

Calcium Ions are Involved in Modulation of Melittin-induced Nociception in Rat: II. Effect of Calcium Chelator

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Melittin, a major component of bee venom, produces a sustained decrease in mechanical threshold, and an increase in spontaneous flinchings and paw thickness, which are characteristics similar to those induced by whole bee venom. Melittin-induced nociception has been known to be modulated by the changes in the activity of excitatory amino acid receptors, voltage-dependent calcium channels, cyclooxygenase and serotonin receptors. The present study was undertaken to investigate the role of calcium chelators (TMB-8 & Quin 2) in melittin-induced nociceptive responses. Changes of mechanical threshold and spontaneous flinching behaviors were measured at a given time point following intraplantar injection of melittin (30 µg/paw). Intrathecal or intraplantar pre-administration and intrathecal post-treatment of TMB-8 and Quin 2 significantly prevented the melittin-induced reduction of mechanical threshold, and intraplantar or intrathecal pre-treatment of TMB-8 and Quin 2 suppressed melittin-induced flinching behaviors. These results indicate that calcium ion in the spinal dorsal horn neurons and peripheral nerves plays an important role in the production and maintenance of mechanical allodynia and spontaneous pain by melittin.

Key Words: Melittin, Nociceptive response, TMB-8, Quin 2

INTRODUCTION

Lariviere and Melzack (1996) introduced the bee venom (BV) model which has been suggested to be more useful for the study of pain than the formalin test (Chen et al, 1999). BV-induced edema, hyperalgesia, spontaneous pain and an increase of discharge of wide dynamic range (WDR) neurons have been reported to be caused mainly by the action of melittin, a major component of BV (Li & Chen, 2004; Shin et al, 2004). In the rat whose sciatic nerves were pre-treated with capsaicin, melittin-induced mechanical hyperalgesia and spontaneous flinchings were almost completely blocked (Shin & Kim, 2004), and intraplantar (i.pl.) injection of melittin increased only the discharge rate of WDR neurons with, but not without, C-fiber inputs from the peripheral receptive field (Shin et al, 2004; Shin & Kim, 2004). Topical application of lidocaine onto sciatic nerve completely blocked the firing of WDR neuron induced by i.pl. injection of melittin (Shin et al, 2004; Shin & Kim, 2004). These findings indicate that melittin activates selectively capsaicin sensitive primary afferent fibers, which transmit noxious inputs to nociceptive dorsal horn neurons.

Recently Yu and Chen (2005) showed that intrathecal (i.t.) administration of mitogen-activated protein kinase inhibitor suppresses melittin-induced production and main-

tenance of spontaneous flinchings and thermal hypersensitivity, however with no effect on the maintenance of mechanical hyperalgesia, suggesting that activation of spinal extracellular signaling-regulated kinase contributes to melittin-induced nociceptive responses. Spinal and peripheral N-methyl-D-aspartate (NMDA) receptors, cyclooxygenase and serotonin receptors are also implicated in melittin-induced decrease of mechanical threshold and spontaneous flinchings which are sensitive to i.t. and intraperitoneal (i.p.) administration of morphine (Kim & Shin, 2005; Lee et al, 2005; Kim et al, 2006).

There is a growing evidence for the essential role of calcium ions in triggering the nociceptive response in the spinal dorsal horn and peripheral site. Formalin-induced phase 2 nociceptive response, neuropathic mechanical allodynia and thermal hyperalgesia are greatly reduced in mice lacking $\alpha 1 \beta$ subunit of N-type calcium channel (Hatakeyama et al, 2001; Saegusa et al, 2001). On the other hand, peak calcium current recorded from small dorsal root ganglion neuron and the amplitude and duration of dorsal horn neuron to innocuous and noxious stimulations are significantly increased in mice overexpressed with $\alpha 2 \delta 1$

ABBREVIATIONS: BV, bee venom; EGTA, 3,12-bis(carboxymethyl)-6,9-dioxo-3,12-diazatetradecanedioic acid; i.p., intraperitoneal; IP₃, phosphoinositol 1,4,5-trisphosphate; i.pl., intraplantar; i.t., intrathecal; NMDA, N-methyl-D-aspartate; PWT, paw withdrawal mechanical threshold; Quin 2, 2-([2-Bis(carboxymethyl)amino-5-methylphenoxy]methyl)-6-methoxy-8-bis(carboxymethyl)aminoquinoline; TMB-8, 8-(N,N-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride; WDR, wide dynamic range.

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subunit of voltage-gated calcium channel. Furthermore, in the behavioral test, mechanical allodynia and thermal hyperalgesia have also been observed (Li et al, 2006). N-type, L-type and other calcium channel antagonists have been known to induce antinociceptive actions in acute, tonic and chronic model of pain, and analgesic effects of calcium channel blockers have also been reported in both the behavioral test and the responses of nociceptive dorsal horn neurons (Miranda et al, 1992; Chaplan et al, 1994; Neugebauer et al, 1996; Diaz & Dickenson, 1997; Shin et al, 1997; Todorovic et al, 2004). Calcium chelator and calcium channel antagonist have antinociceptive action, also. Spinal application of TMB-8 and EGTA suppress the response of WDR neurons to C-fiber stimulation, NMDA and thermal stimuli (Shin et al, 1999; Koo et al, 2002). Another calcium chelator, Quin 2, induces antinociceptive action on formalin-induced tonic pain behaviors and neuropathic hyperalgesia (Coderre & Melzack, 1992). However, there has been no report on the effect of calcium chelator on the melittin-induced nociceptive responses. The present study, therefore, was undertaken to investigate the role of calcium chelator in the production and maintenance of nociceptive responses after i.pl. injection of melittin.

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 algometric 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 able to evoke a brisk paw withdrawal in more than 50% of 6 trials was expressed as the paw withdrawal mechanical threshold (PWT, g). Twenty six grams of bending force of the von Frey filament was selected as the upper limit for testing, since stiffer filaments with a bending force of more than 10% of body weight tended to passively raise the entire limb rather than to cause an active brisk withdrawal (Chaplan et al, 1994). When von Frey filaments with weak bending force less than 26 g were applied, rats that sharply withdrew their paws were not used in this 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 melittin (30 µg/paw, Sigma, St. Louis, Missouri, USA) into the mid-plantar area of the right hindpaw. This dose of melittin has been shown to be proper for the production of sustained nociception without any excessive tissue damage (Shin et al, 2004). The total number of flinchings was measured for the first 30 min, because more than 95% of flinchings were observed within the first 30 min after i.pl. injection of melittin. To observe the effects of 8-(Diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8, Sigma, USA) and Quin 2 (Sigma, USA) on the melittin-induced nociception, TMB-8 (0.5 µg & 1 µg for i.t. administration, 50 µg & 100 µg for i.pl. administration) and Quin 2 (5 µg & 10 µg for i.t. administration, 50 µg & 100 µg for i.pl.

administration, Sigma) were administered intrathecally or intraplantarly 20 min before or intrathecally 60 min after the melittin injection. In the post-treatment groups, calcium chelators were administered 60 min after the melittin injection, because melittin-induced nociceptions were shown to be in the established phase 60 min after the melittin injection (Shin et al, 2004). Intrathecal administration of each calcium chelator was followed by an additional injection of 10 µl saline to ensure complete flush of each chelator into intrathecal space. For i.t. administration of TMB-8 and Quin 2, 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 TMB-8 (100 µg) and Quin 2 (100 µg), which were the highest dose intraplantarly administered, did not induce any changes in PWT and spontaneous flinchings. These results suggest that intrathecally or intraplantarly administered TMB-8 and Quin 2 did not have any systemic effects. 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 the experiments were completed, rats were euthanized by an overdose of pentobarbital sodium.

RESULTS

Intraplantar injection of melittin (30 µg/paw) caused sustained decrease of PWTs; 3.4 ± 0.3 g, 5.1 ± 0.6 g, 7.2 ± 0.8 g and 9.1 ± 1.4 g 10 min, 30 min, 60 min and 180 min after the melittin-injection, respectively. Many of the melittin-induced decreases of mechanical threshold were recovered to the control level 24 h after the melittin injection (*n*=13,

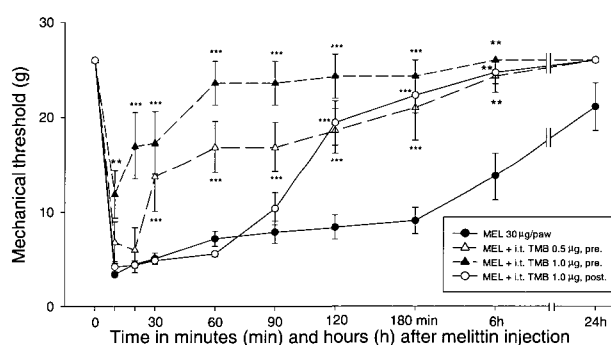


Fig. 1. Melittin-induced reduction of mechanical threshold (30 µg/paw, —●—) was significantly attenuated by the intrathecal administration of TMB-8 (0.5 µg, —△—; 1 µg, —▲—) 20 min before intraplantar injection of melittin. Intrathecal post-treatment of TMB-8 (1 µg, —□—) 60 min after melittin injection also accelerated the recovery of the mechanical threshold reduced by melittin injection. Data are expressed as mean ± S.E. ***p* < 0.01, ****p* < 0.001, significant differences from the melittin-induced reduction of mechanical threshold.

Fig. 1). The i.t. pre-administration of TMB-8 ($0.5 \mu\text{g}$, $n=8$ & $1 \mu\text{g}$, $n=9$) 20 min before the melittin injection significantly attenuated the decrease of mechanical threshold at all time points, and the decreased thresholds were almost completely recovered to the control level 6 h after the melittin injection ($p < 0.01$ or 0.001). However, a low dose of TMB-8 ($0.5 \mu\text{g}$, i.t.) did not suppress significantly the decrease of mechanical threshold during the first 20 min after the melittin injection. After i.t. post-administration of TMB-8 ($1 \mu\text{g}$, $n=12$), the decreased mechanical threshold increased significantly to 19.4 ± 2.3 g and 22.3 ± 1.9 g 120 min and 180 min after the melittin injection, respectively ($p < 0.001$).

Although i.t. or i.pl. administered dosages of TMB-8 and Quin 2 were different, i.pl. injection of TMB-8 (Fig. 2) and

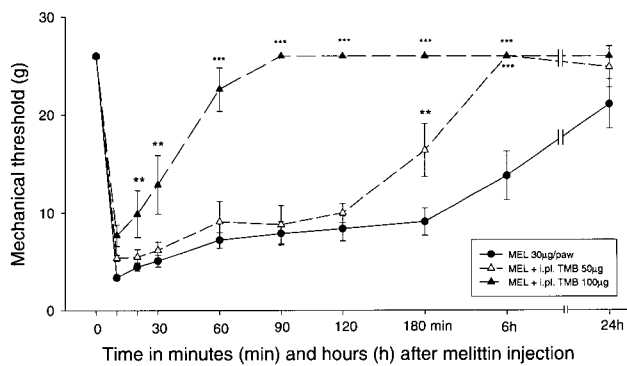


Fig. 2. Intraplantar injection of melittin ($30 \mu\text{g}/\text{paw}$, \bullet) induced sustained decrease of mechanical thresholds which were strongly attenuated by intraplantar administration of $100 \mu\text{g}$ TMB-8 (\blacktriangle) 20 min before melittin injection. However, intraplantar pre-treatment of $50 \mu\text{g}$ TMB-8 significantly prevented the reduction of mechanical threshold only at 180 min and 6h after melittin injection (\triangle). Data are expressed as mean \pm S.E. $**p < 0.01$, $***p < 0.001$, significant differences from the melittin-induced reduction of mechanical threshold.

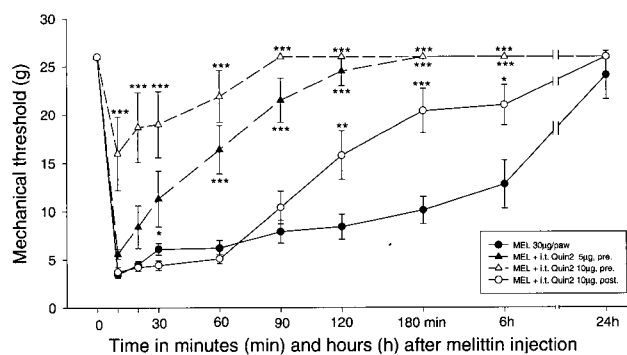


Fig. 3. Intrathecal pretreatment of Ca^{2+} chelator, Quin 2 ($5 \mu\text{g}$, \blacktriangle ; $10 \mu\text{g}$, \triangle), significantly attenuated the effect of melittin to reduce the mechanical threshold ($30 \mu\text{g}/\text{paw}$, \bullet). Melittin-induced decreases of mechanical thresholds rapidly increased following intrathecal administration of Quin 2 ($10 \mu\text{g}$, \circ) 60 min after intraplantar injection of melittin. Data are expressed as mean \pm S.E. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, significant differences from the melittin-induced reduction of mechanical threshold.

Quin 2 (Fig. 4) produced similar inhibitory effects on the melittin-induced decrease of mechanical threshold, and the inhibitory effect was dose-dependent. Fifty μg of TMB-8 ($n=10$) did not have any significant inhibitory action on PWT until 120 min after the melittin-injection, however, PWTs at 180 min and 6h after the melittin-injection were significantly higher than those of the rat injected with melittin alone (Fig. 2, $p < 0.01$ or 0.001). In the rat i.pl. administered with $100 \mu\text{g}$ of TMB-8 ($n=8$), PWTs were decreased to 9.9 ± 2.4 g and 22.6 ± 2.2 g 20 min and 60 min after the melittin injection, which were significantly higher than those of melittin-injected group ($p < 0.01$ and $p < 0.001$, respectively), and the decreased threshold was almost completely recovered to the control level 90 min after the melittin injection.

Quin 2 also produced a suppressive effect on the melittin-induced decrease of PWT similar to those induced by TMB-8 (Fig. 3). After i.t. pre-administration of $10 \mu\text{g}$ Quin 2 ($n=9$), the ability of melittin to reduce PWT was greatly suppressed, and the decreased PWTs were completely recovered to the control level 90 min after the melittin injection ($p < 0.001$). Five μg of intrathecally administered Quin 2 also reduced the melittin-induced decrease of PWT, but the suppressive effect of $5 \mu\text{g}$ Quin 2 was not significant during the first 20 min ($n=10$). The decreased PWT (5.1 ± 0.5 g) 60 min after the melittin injection was significantly recovered to 15.8 ± 2.5 g and 20.4 ± 2.3 g 60 min and 120 min after i.t. post-administration of Quin 2 ($10 \mu\text{g}$, $n=13$), which were significantly higher than the PWTs of the melittin-injected group ($p < 0.01$ or 0.001 , respectively).

Intraplantar injection of Quin 2 also inhibited the melittin-induced decrease of PWT, but low dose of Quin 2 ($50 \mu\text{g}/\text{paw}$, $n=8$) did not have any significant effect at all time-points except 180 min (Fig. 4). However, higher dose of Quin 2 ($100 \mu\text{g}/\text{paw}$, $n=9$) attenuated the effect of melittin to reduce PWT at all time-points except the initial 30 min after the melittin injection. In the rat pre-treated with $100 \mu\text{g}$ of Quin 2, PWTs 60 min and 180 min after the melittin injection were 16.4 ± 3.0 g and 21.9 ± 2.7 g, which were significantly higher than the PWTs of melittin-injected

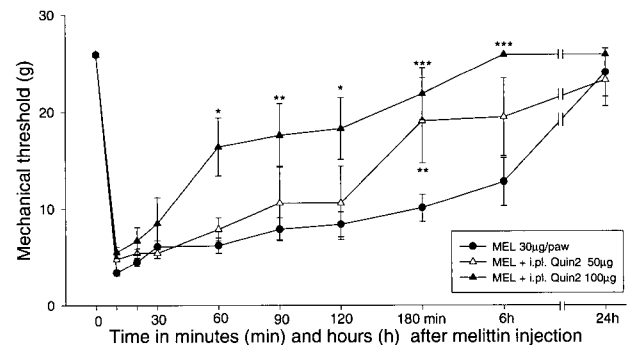


Fig. 4. Melittin-induced reductions of mechanical threshold for the first 30 min were not affected by intraplantar administration of Quin 2. However, the mechanical thresholds of rats intraplantar administered with $100 \mu\text{g}$ Quin 2 were higher than those of rats injected with melittin alone at all time-points 60 min after melittin injection. Fifty μg of Quin 2 did not have any significant effect on melittin-induced reduction of mechanical threshold at all time-points except 180 min after melittin injection. Data are expressed as mean \pm S.E. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, significant differences from the melittin-induced reduction of mechanical threshold.

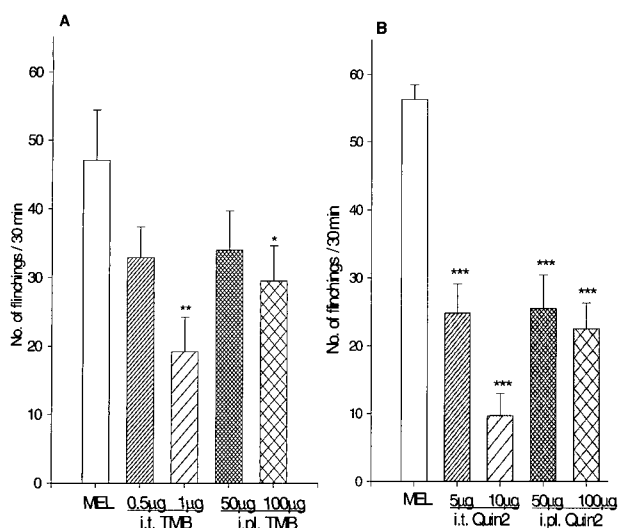


Fig. 5. Intrathecal (i.t.) and intraplantar (i.pl.) pre-administration of TMB-8 (A) and Quin 2 (B) reduced flinching behaviors induced by i.pl. injection of melittin (MEL, 30 µg/paw). Data are expressed as mean ± S.E. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significant differences from the melittin-induced reduction of mechanical threshold.

group ($p < 0.05$ and $p < 0.001$, respectively), and recovered to the control level 6 h after melittin injection ($p < 0.001$).

Melittin-induced flinchings were suppressed by i.pl. or i.t. administration of TMB-8 and Quin 2 (Fig. 5). A low dose of i.t. (0.5 µg, $n=8$) or i.pl. (50 µg, $n=10$) TMB-8 caused insignificant inhibition of melittin-induced flinchings, however, melittin-induced flinchings ($47.1 \pm 7.3/30$ min, $n=13$) were greatly reduced to $19.2 \pm 5.0/30$ min ($p < 0.01$) and $29.5 \pm 5.1/30$ min ($p < 0.05$) after i.t. (1 µg, $n=9$) or i.pl. (100 µg, $n=8$) administration of TMB-8, respectively (Fig. 5A). Low and high doses of i.t. or i.pl. Quin 2 strongly suppressed melittin-induced flinchings (Fig. 5B). Melittin-induced flinchings ($56.2 \pm 4.2/30$ min, $n=12$) were significantly suppressed to $9.7 \pm 2.3/30$ min ($p < 0.001$) and $22.5 \pm 3.8/30$ min ($p < 0.001$) after i.t. (10 µg, $n=7$) or i.pl. (100 µg, $n=8$) administration of Quin 2.

DISCUSSION

The present study shows that i.t. and i.pl. injection of TMB-8 and Quin 2 strongly suppressed the melittin-induced nociceptions by chelating calcium ions and reducing calcium influx into nociceptors, suggesting that calcium ions in the spinal dorsal horn neurons and peripheral nociceptive afferent nerves play an important role in the melittin-induced decrease of PWT and increase of flinchings. Although no direct evidence from nociceptive neurons is available, intraplantarly administered melittin seems to directly activate nociceptive afferent fibers. Melittin-induced decrease of PWT and increases of flinchings have very rapid onset, and the discharge rate of spinal WDR neurons with capsaicin-sensitive C fiber inputs from the peripheral receptive field increases greatly immediately after i.pl. injection of melittin (Shin et al, 2004; Shin & Kim, 2004). The increased discharges of spinal WDR neurons are blocked by topical application of lidocaine onto sciatic nerve (Shin et al, 2004), suggesting that melittin-in-

duced discharges are of peripheral origin.

In Swiss 3T3 cells, subtoxic concentration of melittin stimulated Na^+ influx, which could cause membrane depolarization (Rozenfurt et al, 1981), and increased Ca^{2+} influx in rat pheochromocytoma PC12 cells, which was blocked by organic calcium channel antagonists such as verapamil and nifedipine (Choi et al, 1992). Lee et al (2004) reported that Ca^{2+} influx through voltage-dependent calcium channels in the afferent nerves is involved in the melittin-induced decrease of PWT and increase of flinchings (Lee et al, 2004). Melittin has been known to activate phospholipase C which converts phosphoinositide to phosphoinositol 1,4,5-trisphosphate (IP_3) (Rozenfurt et al, 1981). IP_3 induces the release of Ca^{2+} from the intracellular calcium stores. Taken these findings together, melittin-induced increase of intracellular calcium concentration (Choi et al, 1992) could trigger a series of chain reactions responsible for the production of pain, including the activation of protein kinase C and an increase of membrane permeability and neurotransmitter release (Koizumi et al, 1992; Barber & Vasko, 1996; Manseau et al, 1998). In the present study, it is highly possible that, i.pl. administration of TMB-8 and Quin 2 reduced Ca^{2+} availability necessary for calcium influx into nociceptive neurons, thus resulting in the inhibition of a series of pain reactions. At the late phase of melittin-induced nociceptive response, the melittin-produced proinflammatory substances, such as arachidonic acids, tachykinin, histamine, serotonin and prostaglandin E_2 , act together and activate nociceptive peripheral nerves, resulting in the sustained increase of noxious inputs to nociceptive dorsal horn neurons (Mufson et al, 1979; Calixto et al, 2003).

Because noxious stimuli have been shown to increase the release of nociceptive neurotransmitters from the central ending of primary afferent fibers, sustained noxious inputs from the capsaicin-sensitive primary afferent fibers selectively activated by melittin may contribute to the spinal mechanism of melittin-induced nociception. The common feature of the action of nociceptive neurotransmitters is to increase intracellular calcium concentration and production of IP_3 in the dorsal horn neurons (Sladeczek et al, 1985; Mayer et al, 1987; Koizumi et al, 1992), and these neurotransmitters include substance P, NMDA and non-NMDA receptor agonists. The increased intracellular calcium ions by sustained noxious inputs can activate protein kinase C, which has been known to increase NMDA current, neurotransmitter release and calcium channel activity (Kelso et al, 1992; Koizumi et al, 1992; Reeve et al, 1995; Barber & Vasko, 1996), and can increase the production of arachidonic acid by the activation of phospholipase A_2 (Dumuis et al, 1988). The combined action of all these pro-nociceptive factors may produce spinal sensitization and a sustained hyperalgesia. This possibility is further supported by earlier studies showing that melittin- or BV- induced nociceptive responses are suppressed by i.t. administration of nonselective neurokinin receptor antagonist, protein kinase C inhibitor, NMDA and non-NMDA receptor antagonists and calcium channel antagonists (Li et al, 2000; Zheng & Chen 2001; Lee et al, 2004; Kim & Shin, 2005). As in the peripheral afferent nerves, i.t. pre-administration of TMB-8 and Quin 2 in the present study can reduce Ca^{2+} availability needed for triggering of serial pain reactions in the dorsal horn neurons, possibly resulting in the suppression of melittin-induced nociceptive responses. Intrathecal post-treatment of chelators also strongly attenuated the melittin-induced reduction of mechanical threshold in

the established phase, indicating that calcium chelators inhibited maintenance as well as development of melittin-induced nociceptions. It appears in the present study that i.t. or i.p.l. administered TMB-8 and Quin 2 chelated calcium ions mainly in the extracellular space and then reduced Ca^{2+} availability for Ca^{2+} influx into nociceptive neurons. This proposal is further supported by a recent study to show that blockade of voltage-gated calcium channel strongly reduces melittin-induced nociception (Lee et al, 2004). However, the possibility that TMB-8 and Quin 2 reduced intracellular calcium concentration can not be ruled out. TMB-8 has an ability to inhibit caffeine-induced release of calcium ions from sarcoplasmic reticulum of skeletal muscle (Chiou & Malagodi, 1975) and to suppress melittin-induced increase of intracellular calcium ion concentration in cultured human fibroblast (Mix et al, 1984). Malagodi and Chiou (1974) also suggested that TMB-8 might produce its inhibitory effect on guinea pig ileum by blocking the calcium release from the intracellular stores (Malagodi & Chiou, 1974). These limited evidences from smooth and skeletal muscles may be indicative of the possibility that TMB-8 acted as an intracellular calcium antagonist. The results in the present study show that calcium ions mainly in extracellular fluid (Lee et al, 2004) and some Ca^{2+} ions released from intracellular Ca^{2+} stores (Chiou & Malagodi, 1975) are needed for the production and maintenance of nociceptive responses by melittin.

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