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Multiple 5-Hydroxytryptamine (5-HT) Receptors Are Involved in the Melittin-induced Nociceptive Responses in Rat
I. Role of Peripheral 5-HT Receptor

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Melittin-induced tonic pain model is characterized by local inflammation, edema, spontaneous flinchings, and sustained mechanical hypersensitivity. These nociceptive responses are mediated through selective activation of capsaicin-sensitive primary afferent fibers by melittin. The present study was undertaken to elucidate the role of peripheral 5-hydroxytryptamine (5-HT) receptors in the melittin-induced nociceptive responses. Changes in mechanical threshold, flinching behaviors and paw thickness were measured in rat intraplantarly injected with melittin (40 μg/paw) alone or treated together with melittin and 5-HT receptor antagonists. WAY-100635 (100 μg & 200 μg/paw), isamoltane hemifumarate (100 μg & 200 μg/paw), methysergide maleate (60 μg, 120 μg & 200 μg/paw) and ICS-205,930 (100 μg & 200 μg/paw) were intraplantarly injected 20 min before melittin injection. All 5-HT receptor antagonists tested in this experiment significantly attenuated the ability of melittin to reduce mechanical threshold and to induce flinching behaviors. 5-HT receptor antagonists, except ICS-205,930, had mild inhibitory effect on melittin-induced edema. These experimental findings suggest that multiple peripheral 5-HT receptors are involved in the melittin-induced nociceptive responses.

Key Words: Melittin, Nociceptive responses, Peripheral 5-HT receptors

INTRODUCTION

Intraplantar injection of melittin, a major component of bee venom, causes a dose-dependent and long-lasting decrease in the mechanical threshold and an increase in spontaneous pain with rapid onset which have been reported to be mediated by selective activation of capsaicin-sensitive primary afferent fibers (Li & Chen, 2004; Shin et al, 2004; Shin & Kim, 2004; Chen et al, 2006). Intraplantarly administered melittin also increases the discharge rate of spinal wide dynamic range neurons which receive both A-fiber and C-fiber inputs from the periphery, but wide dynamic range neurons without C-fiber inputs are not activated by melittin injection (Shin et al, 2004; Shin & Kim, 2004). Melittin-induced mechanical hypersensitivity and flinching behaviors are suppressed by intrathecal or intraperitoneal injection of morphine (Shin et al, 2004). These experimental findings suggest that melittin-induced pain model can be very useful for the study of pain mechanism.

Melittin-induced nociceptive responses are significantly suppressed by intrathecal and/or intraplantar administration of ionotropic (Kim & Shin, 2005) and metabotropic (unpublished data) glutamate receptor antagonists. Voltage-gated N-type and L-type, but not P-type, calcium channels and calcium chelators are also implicated in the modulation of melittin-induced nociceptions (Shin & Lee, 2006; Shin et al, 2006). Cyclooxygenase 1 inhibitor, piroxicam, and extracellular signaling-regulated kinase (ERK) inhibitor alleviates melittin-induced spontaneous pain. However, mechanical hyperalgesia was inhibited by piroxicam, but not by ERK inhibitor (Yu & Chen, 2005; Kim et al, 2006).

Many subtypes of 5-hydroxytryptamine (5-HT) receptors have been identified and shown to be involved in the modulation of nociceptive processing in the spinal cord and peripheral site. The distribution of 5-HT receptor subtypes in the spinal cord and sensory neurons is very complex (Millan, 2002), and the same 5-HT receptor subtype has been reported to be involved in both antinociceptive and pronociceptive actions in the spinal cord (Xu et al, 1994; Zhang et al, 2001). However, activation of peripheral 5-HT receptors generally induces nociceptive responses in the inflammatory and neuropathic pain model (Sufka et al, 1991; Doak & Sawynok, 1997; Nitanda et al, 2005; Wei et al, 2005). Intraplantar injection of bee venom containing melittin produces proinflammatory substances such as 5-HT

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ABBREVIATIONS: Ach, acetylcholine; DRG, dorsal root ganglion; 8-OH-DPAT, (±)-8-hydroxy-2-dipropylaminotetralin hydrobromide; ERK, extracellular signaling-regulated kinase; 5-HT, 5-hydroxytryptamine; ICS-205,930, 3-tropanylindole-3-carboxylate methiodide; PG, prostaglandin; PKA, protein kinase A; PWT, paw withdrawal mechanical threshold; TTX-resistant, tetrodotoxin-resistant; WAY-100635 maleate, N-2-[4-(2-methoxyphenyl)-1-piperazinyl]-N-2-pyridinylcyclohexane-carboxamide maleate.

(Calixto et al, 2003) and increases the expression of 5-HT receptor subtypes in the rat dorsal root ganglion (DRG) neurons and spinal cord (Wang et al, 2003; Liu et al, 2005). Intradermal injection of 5-HT also increases c-fos-like immunoreactive neurons in the laminae I-II of spinal dorsal horn which is suppressed by 5-HT_{2A} receptor antagonists, ketanserin (Doi-Saika et al, 1997; Luo et al, 1998). These experimental findings suggest that melittin-induced nociceptive responses can be modulated by the changes in peripheral 5-HT receptor activity. The present study was undertaken to elucidate the role of peripheral 5-HT receptors in the melittin-induced nociceptive responses.

METHODS

Sprague-Dawley male rats (230~300 g) were used. 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). Twenty six grams 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 flinchings. Spontaneous nociception was estimated by counting the total number of flinchings of injected paw for initial 30 min. Flinching behavior is a spontaneous brisk movement of hindpaw without an application of mechanical or any other type of stimulation to hindpaw. Changes in paw thickness were measured by using caliper and expressed as % changes of the control paw thickness measured in the rat before melittin injection. Changes in PWT, flinching behaviors and paw thickness were measured at a given time point after the injection of melittin (40 μg/paw) into mid-plantar area of rat hindpaw. To elucidate the effects of 5-HT receptor antagonists on melittin-induced nociceptive responses, 5-HT receptor antagonists, such as WAY-100635 (100 μg & 200 μg/paw, Sigma), methysergide maleate (60 μg, 120 μg, & 200 μg/paw, Tocris), isamoltane hemifumarate (100 μg & 200 μg/paw, Tocris), and ICS-205,930 (3-tropanylindole-3-carboxylate methiodide, 100 μg & 200 μg/paw, Sigma) were intraplantarly administered 20 min before melittin injection. All drugs were dissolved in 10 μl of saline. 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 injection of melittin (40 μg/paw) induced a sustained decrease of PWTs which were 2.9±0.5 g and 4.7±0.6 g at 10 min and 30 min after melittin injection, respectively (Fig. 1). In general, melittin-induced decrease of PWT did not completely recover to the control level even 6 hr after melittin injection. In the rat pretreated with 5-HT_{1A} receptor antagonist, WAY-100635, the melittin-induced decrease of PWTs was smaller than that of the rat injected with melittin alone, and there was a tendency for rapid recovery of the decreased PWTs compared to the melittin-injected rat. PWTs of the rat pre-injected with 200 μg of WAY-100635 were 7.0±0.8 g and 10.9±1.2 g at 10 min and 30 min after melittin injection, respectively, which were significantly high compared to PWTs of melittin-injected rats ($p<0.01$ or 0.001), and rapidly increased to 21.7±2.7 g 90 min after melittin injection.

Intraplantar pre-treatment of 5-HT_{1B} receptor antagonist dose-dependently attenuated the melittin-induced decrease

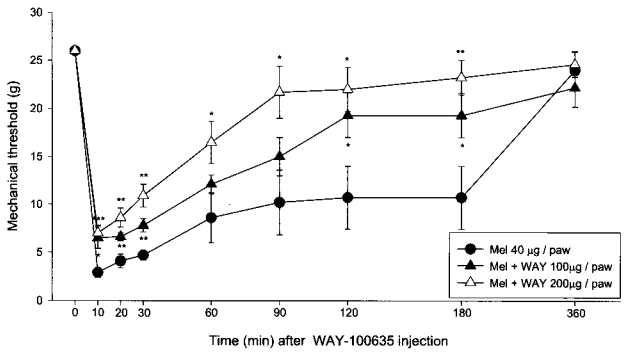

Fig. 1. Modulation of melittin-induced (40 μg/paw, -●-) decrease in mechanical threshold by intraplantar injection of 5-HT_{1A} receptor antagonist, WAY-100635 (100 μg/paw, -▲-). 200 μg/paw, -△-). Data are expressed as mean±S.E. *$p<0.05$, **$p<0.01$, ***$p<0.001$, significant differences from the melittin-induced reduction of mechanical threshold.

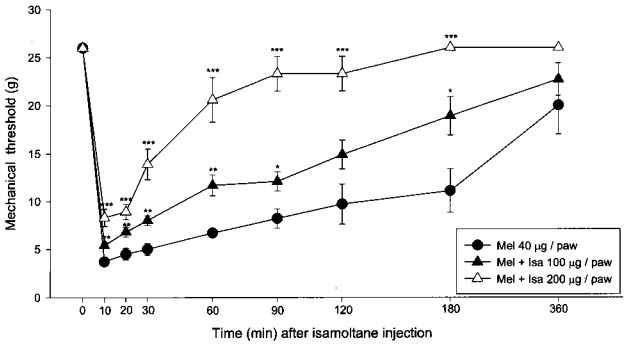

Fig. 2. Intraplantar pretreatment of 5-HT_{1B} receptor antagonist, isamoltane hemifumarate (100 μg/paw, -▲-; 200 μg/paw, -△-) significantly suppressed the decrease in mechanical threshold by intraplantar injection of melittin (40 μg/paw, -●-). Data are expressed as mean±S.E. *$p<0.05$, **$p<0.01$, ***$p<0.001$, significant differences from the melittin-induced reduction of mechanical threshold.

of PWTs (Fig. 2). In the rat pre-injected with 100 μg and 200 μg of isamoltane hemifumarate, PWTs were 5.4±0.3 g and 8.3±0.9 g 10 min after melittin injection, respectively, which were significantly high compared to PWTs of melittin-injected rat (3.7±0.3 g, $p<0.01$ or 0.001). These decreased PWTs increased rapidly to 12.1±1.0 g and 23.3±1.8 g 90 min after melittin injection, respectively, whereas PWT of the rat injected with melittin alone (8.2±1.0 g) was significantly low compared to isamoltane-treated rat at the same time point ($p<0.05$ or 0.001).

5-HT_1 and 5-HT_2 receptor antagonist, methysergide maleate, greatly attenuated the ability of melittin to reduce PWT (Fig. 3). PWT of the rat injected with melittin alone was reduced to 2.7±0.6 g 10 min after melittin injection and remained low until 180 min after melittin injection (5.7±0.6 g). In the rat intraplantarly injected with 60 μg or 200 μg of methysergide maleate, PWTs were reduced to 5.7±1.1 g and 8.8±1.3 g 10 min after melittin injection, respectively, which were significantly high compared to PWT of the melittin-injected rat ($p<0.01$). The increasing rate of decreased PWTs was significantly higher in methysergide-treated rat at all time points than in the rat injected with melittin alone, and PWT of the rat pre-treated with 200 μg of methysergide recovered almost to control PWT 90 min after melitttin injection.

Melittin-induced decrease of PWT was dose-dependently attenuated by intraplantar pre-administration of 5-HT_3 receptor antagonist (Fig. 4). In the rat pre-treated with 100 μg or 200 μg of ICS-205,930, PWTs measured 10 min after melittin injection were 5.0±0.5 g and 10.9±1.3 g, respectively, which were significantly high compared to PWT of the rat injected with melittin alone ($p<0.01$ or 0.001), and increased to 16.8±2.4 g and 21.8±2.1 g 90 min and 180 min after melittin injection in the rat pre-treated with 200 μg of ICS-205,930, respectively.

Flinching behaviors were not observed in the control rat. However, intraplantar injection of melittin caused a great increase in flinching behaviors (Fig. 5). In the rat pre-treated with 100 μg or 200 μg of WAY-100635, melittin-induced flinchings (77.0±5.9/30 min) were significantly reduced to 53.0±6.4/30 min ($p<0.05$) and 23.7±1.8/30 min ($p<0.001$),

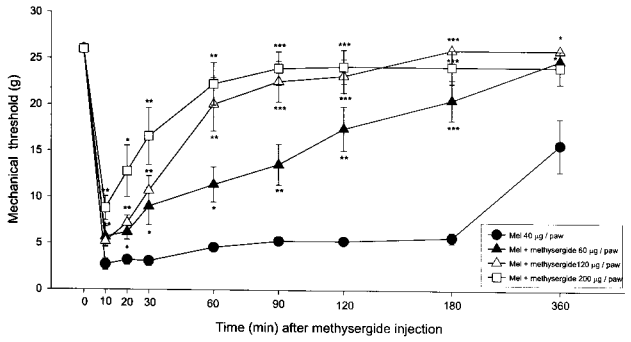

Fig. 3. Intraplantar injection of methysergide maleate (60 μg/paw, -▲-; 120 μg/paw, -△-; 200 μg/paw, -□-) significantly attenuated the ability of melittin (40 μg/paw, -●-) to reduce mechanical threshold. Data are expressed as mean±S.E. *$p<0.05$,**$p<0.01$, ***$p<0.001$, significant differences from the melittin-induced reduction of mechanical threshold.

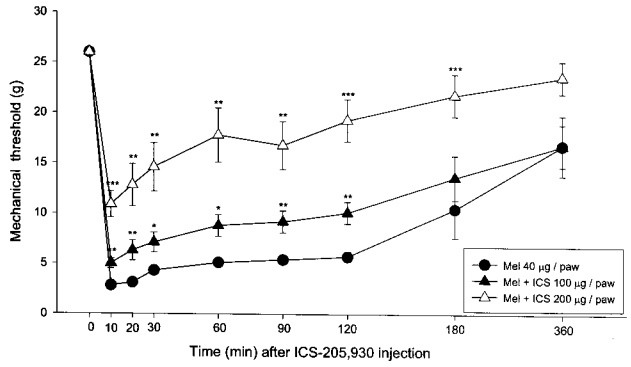

Fig. 4. Melittin-induced decrease in mechanical threshold (40 μg/paw, -●-) was significantly reduced after treatment with 5-HT_3 receptor antagonist, ICS-205,930 (100 μg/paw, -▲-; 200 μg/paw, -△-). Data are expressed as mean±S.E. *$p<0.05$, **$p<0.01$, ***$p<0.001$, significant differences from the melittin-induced reduction of mechanical threshold.

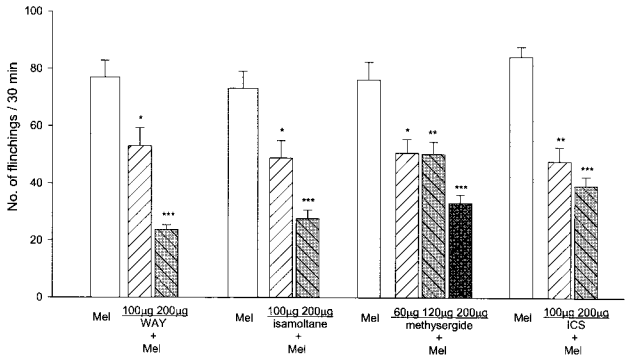

Fig. 5. Melittin-induced increase in flinching behaviors was significantly suppressed following intraplantar injection of WAY-100635 (100 μg & 200 μg), isamoltane hemifumarate (100 μg & 200 μg), methysergide maleate (60 μg, 120 μg & 200 μg), and ICS-205,930 (100 μg & 200 μg). Data are expressed as mean±S.E. *$p<0.05$, **$p<0.01$, ***$p<0.001$, significant differences from the melittin-induced increase in flinching behaviors.

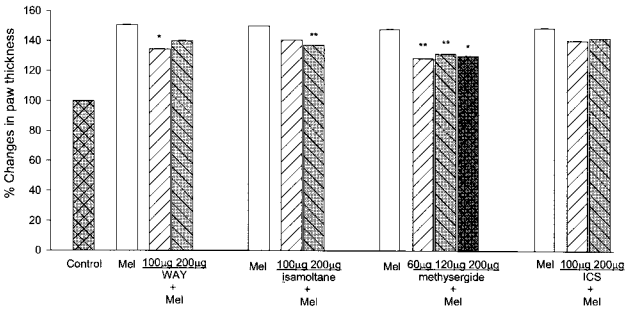

Fig. 6. Intraplantar injection of melittin (40 μg/paw) caused the increase of paw thickness. Intraplantar injection of WAY-100635 (100 μg), isamoltane hemifumarate (100 μg & 200 μg) and methysergide maleate (60 μg, 120 μg & 200 μg), but not ICS-205,930 (100 μg & 200 μg) 20 min before melittin injection had mild inhibitory effects on melittin-induced increase of paw thickness. Data are expressed as mean±S.E. *$p<0.05$, **$p<0.01$, significant differences from the melittin-induced increase in paw thickness.

respectively. Melittin-induced flinching behaviors were also suppressed to 27.7±3.0/30 min, 33.2±2.9/30 min and 39.3± 3.2/30 min after intraplantar pre-injection of 200 μg of isamoltane, methysergide or ICS-205,930, respectively, and their respective flinching numbers induced by melittin alone were 73.1±6.0/30 min, 76.1±6.3/30 min and 84.3± 3.5/30 min respectively. These findings suggest that melittin-induced flinchings were significantly reduced by pre-treatment of rats with isamoltane, methysergide and ICS-205,930.

Paw thickness increased to about 150% of the control paw thickness 30 min after intraplantar injection of melittin (Fig. 6). Melittin-induced increase in paw thickness was weakly suppressed by WAY-100635 and isamoltane, and moderately by methysergide. In the rat pre-treated with 60 μg or 120 μg of methysergide, melittin-induced increase of paw thickness (147.8% of the control) was reduced to 128.4±0.3% and 130.0±0.2% of the control paw thickness ($p<0.01$ or 0.05), and there was no discrete dose-dependence. ICS-205,930 did not have any inhibitory effect on melittin-induced increase of paw thickness.

DISCUSSION

The present study shows that intraplantar injection of 5-HT_{1A}, 5-HT_{1B}, 5-$HT_{1/2}$ and 5-HT_3 receptor antagonists had strong inhibitory effects on the action of melittin to induce spontaneous flinchings and to reduce mechanical threshold. However, melittin-induced edema was mildly inhibited by intraplantar injection of 5-HT receptor antagonists. These experimental findings indicate that melittin-induced nociceptive responses are mediated through the activation of multiple peripheral 5-HT receptors and agree well with the results that peripheral 5-HT receptor activation induces nociceptive responses (Fock & Mense, 1976; Herbert & Schmidt, 1992; Taiwo & Levine, 1992; Tokunaga et al, 1998). Melittin-induced nociceptive responses may result from direct activation of 5-HT receptor by melittin and / or endogenous 5-HT released from bee venom (melittin)-induced inflammatory tissues (Calixto et al, 2003)

Although there is a little difference among the results reported, different subtypes of 5-HT receptors have been shown to be localized in DRG neuron and peripheral afferent fiber (Pierce et al, 1996; Carlton & Coggeshall, 1997; Chen et al, 1998; Nicholson et al, 2003). The expression of 5-HT receptor subtypes is increased in the DRG neuron of the rat inflamed with intraplantar injection of complete Freund's adjuvant and bee venom (Wu et al, 2001; Okamoto et al, 2002). The increased expression of 5-HT_{2A} receptor and thermal hyperalgesia are blocked by oral administration of 5-HT_{2A} receptor antagonist, sarpogrelate (Wu et al, 2001; Okamoto et al, 2002). Intraplantar injection of 5-HT and its agonists also induces pain behaviors and mechanical hyperalgesia (Taiwo & Levine, 1992; Tokunaga et al, 1998), and inflammatory, neuropathic and heat-evoked hyperalgesias are alleviated by peripheral administration of 5-HT receptor antagonists (Doak & Sawynok, 1997; Nitanda et al, 2005; Wei et al, 2005; Sasaki et al, 2006). On the other hand, acute thermal and mechanical pain, formalin-induced licking behavior and the formalin-evoked firing of dorsal horn neurons during the first phase are not different between wild type and mutant mice lacking 5-HT_3 receptor. However, the second phase of dorsal horn neuronal firing and pain behaviors induced by formalin injection are markedly reduced in the mutant mice (Zeitz et al, 2002). These studies indicate that activation of different types of peripheral 5-HT receptor contributes to the induction of nociceptive responses and is consistent with the present results which showed that intraplantar injection of 5-HT_{1A}, 5-HT_{1B}, 5-HT_2, 5-HT_3 receptor antagonists suppressed melittin-induced nociceptions. Other subtypes of 5-HT, except those tested in the present experiment, may contribute to melittin-induced nociceptive responses, because there is no study to elucidate the subtypes of 5-HT which were increased by bee venom or melittin.

Several mechanisms are involved in 5-HT-induced nociceptive responses. 5-HT has direct excitatory and/or sensitizing effect on the peripheral nociceptors. Intra-arterial injection of 5-HT stimulates group III and IV afferent fibers which innervate to muscle and knee joint in cat, but 5-HT did not excite group II and group III fibers with the conduction velocity of more than 16 m/sec (Fock & Mense, 1976; Herbert & Schmidt, 1992). Subcutaneous injection of prostaglandin (PG) E_2 induced paw lifting and licking behaviors, and intra-arterial injection of acetylcholine (Ach) caused vocalization and biting behaviors. These PGE_2- and Ach-induced nociceptive responses were greatly increased after pre-administration of 5-HT and 5-HT_2 agonist, alpha-methyl-5-HT (Nakano & Taira, 1976; Abbott et al, 1997). Rueff and Dray (1992) also reported that bradykinin, capsaicin and heat-evoked depolarization of peripheral afferent nerve were greatly potentiated by 5-HT and 5-HT_2 agonist, alpha-methyl-5-HT, but not by 5-HT_3 agonist, 2-methyl-5-HT and 5-HT_1 agonist, 5-carboxamidotryptamine. 5-HT also depolarizes large number of small or type C DRG neuron (Holziv et al, 1985; Molokanova & Tamarova, 1995). 5-HT-induced depolarization with increased input resistance is antagonized by 5-HT_2 recptor antagonist, and 5-HT-induced depolarization with decreased input resistance is mimicked by 5-HT_3 receptor agonists such as 2-methyl-5-HT and phenylbiguanide (Todorovic & Anderson, 1990a; 1990b).

Cyclic AMP second messenger system has been implicated in 5-HT-induced hyperalgesia. Intradermal injection of 5-HT_{1A} receptor agonist, (±)-8-hydroxy-2-dipropylaminotetralin hydrobromide (8-OH-DPAT), induces dose-dependent mechanical hyperalgesia in rats which is inhibited by protein kinase A (PKA) inhibitor, however, further potentiated by phosphodiesterase inhibitor, rolipram and adenylyl cyclase activator, forskolin (Taiwo et al, 1992).

Changes in Na^+, Ca^{2+} and K^+ currents of sensory afferent fibers may contribute to the development of neuronal hypersensitivity. 5-HT increases tetrodotoxin (TTX)- resistant Na^+ current in capsaicin-sensitive small DRG neuron of rat. 8-bromo-cAMP, adenylyl cyclase activator, forskolin and phosphodiesterase inhibitor, 3-isobutyl-L-methylxanthins also increase TTX-resistant Na^+ current, suggesting that 5-HT increases neuronal excitability via activation of cAMP-coupled TTX-resistant Na^+ channels (Gold et al, 1996; Cardenas et al, 1997; 2001). Although there is no direct result obtained from rat sensory neurons, application of 5-HT to the cell body of *Aplysia* sensory neuron causes prolonged and complete closure of K^+ channel which may result in an increase of action potential duration, Ca^{2+} influx and then neurotransmitter release (Siegelbaum et al, 1982). Fagni et al (1992) also reported that 5-HT-induced inhibition of K^+ current was mediated via cAMP-PKA system in mouse colliculi neurons in culture. In *Aplysia* sensory neuron, bath application of 5-HT and cAMP proloned

the duration of calcium action potential, and the time course of evoked excitatory postsysnaptic potential was consistent with that of Ca^{2+} action potential (Klein & Kandel, 1978). However, Del Mal et al (1994) and Cardenas et al (1995) reported opposite findings that 5-HT and 8-OH-DPAT inhibited high threshold calcium current in capsaicin-sensitive DRG neurons. Taking all these findings together, multiple 5-HT receptor activation by melittin may cause direct excitation and/or sensitization of nociceptive primary afferent fibers via cAMP-coupled increase of Na^+ and Ca^{2+} channel activity and inhibition of K^+ channel which can result in the hyperalgesic state.

We expected that melittin-induced edema could be strongly suppressed by intraplantar injection of 5-HT receptor antagonists, nevertheless, 5-HT receptor antagonists had mild inhibitory effect in the present study. Many proinflammatory substances such as 5-HT, histamine, bradykinin, prostaglandins and neurokinin 1, have been identified in the inflamed tissue after intraplantar injection of bee venom (Calixto et al, 2003). All these inflammatory substances might act together and cause edema. However, only 5-HT receptors were inactivated in the current study. This could be a possible reason of why melittin-induced edema was weakly inhibited after blocking 5-HT receptors.

In summary, intraplantar injection of melittin activates multiple peripheral 5-HT receptors, possibly contributing to the development of mechanical hyperalgesia.

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