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Melittin-induced Nociceptive Responses are Alleviated by Cyclooxygenase-1 Inhibitor

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Melittin-induced pain model has been known to be very useful for the study of pain mechanism. Melittin-induced nociceptive responses are reported to be modulated by the changes in the activity of excitatory amino acid receptor, calcium channel, spinal serotonin receptor and extracellular signaling-regulated kinase. The present study was undertaken to investigate the role of cyclooxygenase (COX) in the melittin-induced nociception. Changes in mechanical threshold, flinchings and paw thickness were measured before and after intraplantar injection of melittin in the rat hind paw. Also studied were the effects of intraperitonealy administered diclofenac (25 mg & 50 mg/kg), piroxicam (10 mg & 20 mg/kg) and meloxicam (10 mg & 20 mg/kg) on the melittin-induced nociceptions. Intraplantar injection of melittin caused marked reduction of mechanical threshold that was dose-dependently attenuated by non-selective COX inhibitor (diclofenac) and selective COX-1 inhibitor (piroxicam), but not by COX-2 inhibitor (meloxicam). Melittin-induced flinchings were strongly suppressed by non-selective COX and COX-1 inhibitor, but not by COX-2 inhibitor. None of the COX inhibitors had inhibitory effects on melittin-induced increase of paw thickness (edema). These experimental findings suggest that COX-1 plays an important role in the melittin-induced nociceptive responses.

Key Words: Melittin, Mechanical threshold, Flinching, Cyclooxygenase inhibitors

INTRODUCTION

Bee venom (BV) has two opposite effects such as antinociception and pronociception. BV injection into acupoint (apipuncture) has been known to be effective for the treatment of inflammatory pain in human (Kwon et al, 2001a) and animal pain models (Kwon et al, 2001b; Kim et al, 2003). Apipuncture also has been reported to have stronger antinociceptive effect on pain than those induced by needle acupuncture and BV injection into non-acupoints (Kwon et al, 2001b; Kim et al, 2003). BV contains many ingredients such as melittin, apamin, phospholipase A_2, adolapin, and mast cell degranulating peptide. Of these ingredients, ethylacetate soluble fraction dose not have any antinociceptive action. Active ingredients of BV responsible for antinociception are soluble in water, resistant to heat (100°C) and has molecular weight less than 10 kDa. Injections of water soluble fraction into acupoint inhibit Fos expression in the spinal cord, development of edema, production of interleukin-1 β, thermal and mechanical hyperalgesia (Kwon et al, 2002; Kwon et al, 2005).

Morderate or low dose of BV injection into acupoint induced antinociception even in resiniferatoxin-treated mice (Roh et al, 2004a) and BV-induced antinociception was significantly attenuated by the intrathecal administration of idazoxan and methysergide (Roh et al, 2004b; Kim et al, 2005). Injection of BV into zusanli acupoint increased Fos expression in arcuate nucleus, dorsal raphe, locus coeruleus and other catecholaminergic nuclei (Kwon et al, 2004). On the basis of these experimental findings, Kim et al (2005) and Roh et al (2004a) suggested that apipuncture-induced antinociception is mediated via α_2-adrenergic and serotonergic components of descending pain inhibitory system activated by input signals from capsaicin-insensitive primary afferent fibers.

On the other hand, Lariviere and Melzack (1996) introduced tonic pain model, in which subcutaneous injection of BV induced Fos expression in the spinal dorsal horn, edema, spontaneous flinchings, referred mirror hyperalgesia, thermal and mechanical hyperalgesia in the behavioral test (Luo et al, 1998; Chen et al, 1999b, 2000) and increased the discharge rate of wide dynamic range (WDR) dorsal horn cell in electrophysiological studies (Chen et al, 1998). This BV-induced nociceptive responses are modulated by intrathecal and/or peripheral administration of antagonists of N-methyl-D-aspartate (NMDA) receptor, non-NMDA receptor, neurokinin 1/2 receptor, protein kinase A, protein kinase C and P_{2X} purinoceptor (Chen et al, 1999a; Chen & Chen, 2000; Li et al, 2000; Zheng & Chen, 2000, 2001; You et al, 2002; Li & Chen, 2003). Changes in the discharge rate of WDR cell and spontaneous flinchings have similar time courses (Chen et al, 1998). BV-induced nociception has been known to be mediated by selective activation of

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ABBREVIATIONS: BV, bee venom; CGRP, calcitonin gene-related peptide; COX, cyclooxygenase; PG, prostaglandin; PWT, paw withdrawal threshold; SP, substance P; WDR, wide dynamic range.

capsaicin-sensitive primary afferent fibers (Chen et al, 1998; Chen & Chen, 2001). Bilateral lesions of the rostral medial medulla with ibotenic acid inhibited BV-induced spontaneous flinchings and referred mirror hyperalgesia (Chen et al, 2003), suggesting that descending facilitatory pathway contributes to spontaneous pain and contralateral heat hyperalgesia. About 50% of dry BV is melittin which induces dose-dependent and sustained nociceptive responses. Melittin-induced nociceptive responses have almost all the characteristics of nociception induced by subcutaneous injection of BV, and there are no differences in the maximum decrease in paw withdrawal threshold, flinching behaviors and changes in time courses induced by subcutaneous injection of either whole BV or melittin at one half of the whole BV dosage (Li & Chen, 2004; Shin et al, 2004a). Intrathecal administration of melittin decreases mechanical threshold and increases flinching behaviors (Shin et al, 2004a).

Topical application of 1% capsaicin onto the sciatic nerve almost completely blocked the increase of flinching behaviors, the enhanced discharge rate of WDR cell, and the decrease of mechanical threshold induced by melittin injection, suggesting that melittin selectively activated capsaicin-sensitive afferent fibers (Shin & Kim, 2004). Melittin-induced spontaneous pain and mechanical hyperalgesia have been reported to be modulated by the changes in the activity of excitatory amino acid receptor, calcium channel and spinal serotonin receptor (Lee et al, 2004, 2005; Shin et al, 2004b; Kim & Shin, 2005). In the recent study, extracellular signaling-regulated kinase has been known to be implicated in melittin-induced spontaneous pain and thermal hyperalgesia (Yu & Chen, 2005).

In mouse fibrobastic cell, melittin has been known to increase the activity of phospholipase A_2 which catalyzes the conversion of phosphatidylcholine to arachidonic acid (Shier, 1979; Choi et al, 1992). Arachidonic acid can be further converted to prostaglandins by cyclooxygenase (COX). Noxious stimulations such as inflammation and nerve injury have been reported to increase the activity of COX and the release of prostaglandins, which are well known pronociceptive substances (Hay et al, 1997; Maihöfner et al, 2000; Zhao et al, 2000). The present study was undertaken to investigate the role of prostaglandin in the melittin-induced nociceptive responses.

METHODS

Seventy five Sprague-Dawley male rats (200−250 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 in a transparent plastic compartment 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 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. The number of flinchings was measured for 30 min after melittin injection, because flinching behaviors almost completely disappeared 30 min after melittin injection. 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 melittin (30 μg) into mid-plantar area of the hindpaw of normal rats.

The effects of cyclooxygenase (COX) inhibitors on melittin-induced changes in mechanical threshold, flinching behaviors and paw thickness were studied by intraperitoneally administering diclofenac (25 mg & 50 mg/kg), piroxicam (10 mg & 20 mg/kg), and meloxicam (10 mg & 20 mg/kg) 30 min before melittin injection. Melittin and diclofenac were dissolved in saline, and piroxicam and meloxicam were dissolved in dimethylsulfoxide. In preliminary experiments, it was observed that mechanical threshold and flinching behaviors were not affected after intraperitoneal administration of saline and dimethylsulfoxide. The data are expressed as mean±S.E. and analyzed using ANOVA followed by the Newman-Keuls test. P values less than 0.05 were considered statistically significant. When experiments were completed, rats were euthanized by an overdose of pentobarbital sodium.

RESULTS

PWT of a normal rat was approximately 26 g. Intraplantar injection of melittin (30 μg) dramatically reduced mechanical threshold, which was 3.5±0.4 g at 10 min after melittin injection (n=7). The decreased PWT recovered very slowly to 7.4±0.9 g and 9.9±1.2 g 180 min and 360 min after the injection of melittin, respectively (Fig. 1). Melittin-induced decrease in mechanical threshold was very sustained and stable, suggesting that melittin model could be very useful for the study of nociceptive mechanisms. The ability of melittin to reduce PWT was dose-denpendently attenuated in the rat pretreated with diclofenac (Fig. 1). Although there was a tendency that melittin-induced decrease of PWT was smaller in the rat pretreated with low dose of declofenac (25 mg/kg, n=8) than in the rat injected with melittin alone, this reduced decrease of PWT was

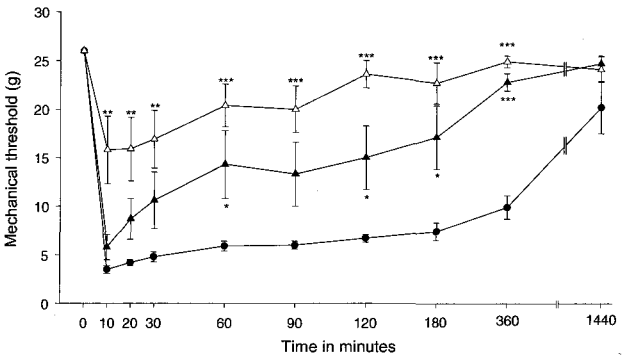

Fig. 1. Non-selective cyclooxygenase inhibitor (diclofenac) dose-dependently prevented melittin-induced reduction of mechanical threshold. Diclofenac (25 mg; ▲, n=8 & 50 mg/kg; △, n=10) was injected into abdominal cavity 30 min before intraplantar injection of melittin. $*P<0.05$, $**P<0.005$, $***P<0.001$, statistically significant differences from melittin-induced decrease in mechanical threshold (30 μg/paw; ●, n=7).

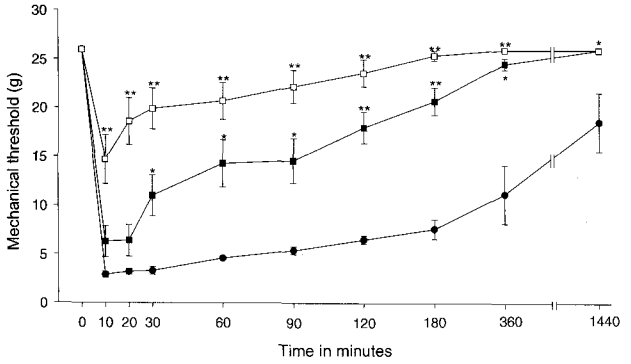

Fig. 2. Effect of cyclooxygenase-1 inhibitor on the melittin-induced changes in mechanical threshold. Melittin (30 μg/paw; ●, n=9) strongly lowered mechanical threshold, and piroxicam (10 mg; ■, n=11 & 20 mg/kg; □, n=12) significantly suppressed melittin-induced decrease in mechanical threshold. *$P<0.05$, **$P<0.001$, statistically significant differences from the melittin-treated group.

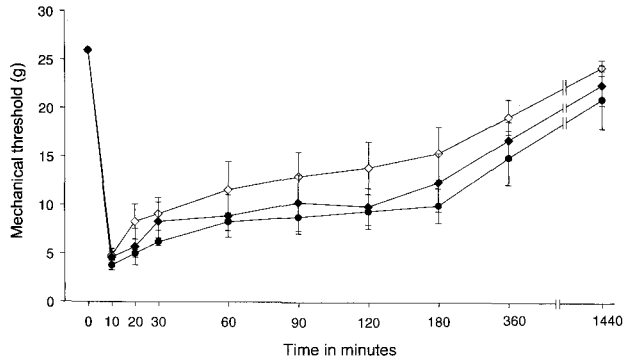

Fig. 3. Melittin-induced decrease in mechanical threshold (●) was not affected by intraperitoneal injection of COX-2 inhibitor, meloxicam (10 mg/kg; ◆, n=8 & 20 mg/kg; ◇, n=9) 30 min before melittin injection (30 μg/paw, n=8).

significantly different from PWT of the rat injected with melittin alone only at time points 60 min after melittin injection ($P<0.05$ or 0.001). In the rat pretreated with high dose of diclofenac (50 mg/kg, n=10), PWT decreared to 15.8± 3.5 g 10 min after melittin injection, which was significantly higher than that of the rat injected with melittin alone (3.5±0.4 g, $P<0.005$), and the decreased PWT recovered already to 23.6±1.4 g at 120 min after melittin injection, which was not different from PWT of normal rat (26 g) without any treatment.

To investigate which type of COX was involved in the diclofenac-induced suppression of a decrease in PWT induced by melittin, COX-1 and COX-2 selective inhibitors were intraperitoneally administered into mid-plantar area of hindpaw 30 min before melittin injection. COX-1 selective inhibitor (piroxicam) dose-dependently reduced melittin-induced decrease in PWT (Fig. 2). In the rat pretreated with 20 mg/kg of piroxicam (n=12), PWT was decreased to 14.7± 2.5 g 10 min after subcutaneous injection of melittin, which was significantly higher than that of the rat injected with melittin alone ($P<0.001$).

The decreased PWT rapidly recovered to 25.4±0.5 g 180

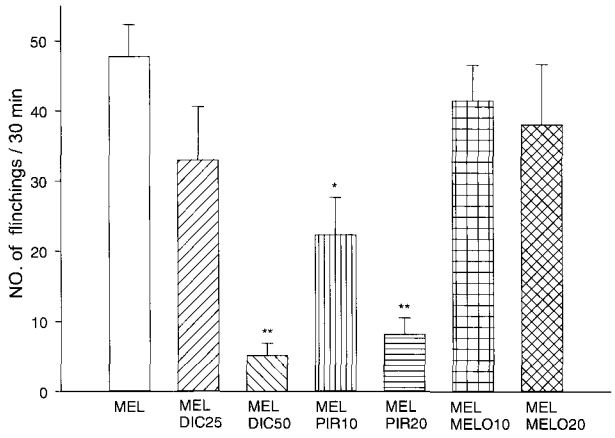

Fig. 4. Changes in melittin (MEL)-induced flinchings measured for the first 30 min in the rat intraperitoneally administered with cyclooxygenase inhibitor. MEL-induced flinchings (n=11) were dose-dependently suppressed by diclofenac (DIC, 25 mg, n=8 & 50 mg/kg, n=8, i.p) and piroxicam (PIR, 10 mg, n=11 & 20 mg/kg, n=10, i.p), but not affected by meloxicam (MELO, 10 mg, n=8 & 20 mg/kg, n=9, i.p). *$P<0.05$, **$P<0.001$, statistically significant differences from the melittin-induced flinchings.

min after melittin injection ($P<0.001$) which was almost identical to that of normal rat. Low dose of piroxicam (10 mg/kg, n=11) also significantly attenuated the ability of melittin to reduce PWT at all time points, except the initial 20 min after melittin injection. In the rat pretreated with 10 mg/kg of piroxicam, PWTs were 11.0±2.1 g and 24.6± 0.6 g 30 min and 360 min after melittin injection, respectively, whereas PWTs of the rat injected with melittin alone were 5.7±0.5 g and 15.8±2.5 g at the respective time points ($P<0.05$).

Meloxicam, selective COX-2 inhibitor did not have any significant suppressive effect on melittin-induced decrease in mechanical threshold (Fig. 3, n=17). PWTs of the rat pretreated with 20 mg/kg of meloxicam together with melittin, and injected with melittin alone were 4.8±0.4 g and 4.5±0.9 g 10 min after melittin injection, respectively. There was a tendency that 20 mg/kg of meloxicam attenuated the melittin-induced decrease in PWT, but this suppressive effect of meloxicam was not statistically significant.

Flinching behaviors were not observed in normal rat. However, flinching behaviors increased to 47.8±4.5 for the initial 30 min after the injection of melittin alone (Fig. 4, n=11). Diclofenac and piroxicam dose-dependently suppressed melittin-induced flinching behaviors. The melittin-induced flinching behaviors decreased to 5.2±1.7/30 min and 8.2±2.3/30 min, when pretreated with 50 mg/kg of diclofenac (n=8) and 20 mg/kg of piroxicam (n=10), respectively ($P<0.001$). Intraperitoneally administered meloxicam (n=8 & 9) had weak suppressive effect on melittin-induced flinching behaviors (38.1±8.6/30 min), but this suppressive effect was not significant.

Subcutaneous injection of melittin (n=10) caused an increase in paw thickness which reached the maximal level (152.9±2.6%, $P<0.01$) approximately 30~60 min after melittin injection (Fig. 5). Diclofenac (n=8 & 8) had very weak inhibitory effect on melittin-induced increase in paw thickness, however none of the COX inhibitors had any significant inhibitory effect.

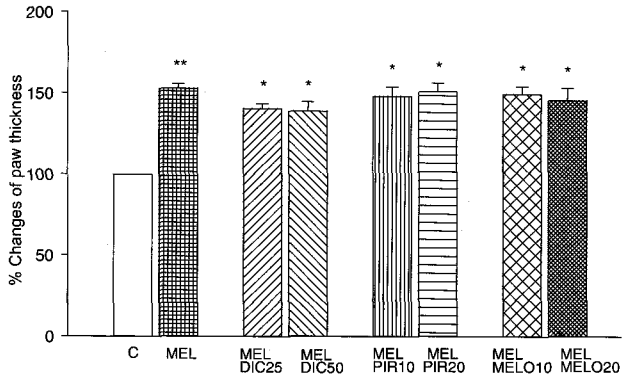

Fig. 5. Melittin (MEL) caused the increase in paw thickness. The increased paw thickness was not affected by any of cyclooxygenase inhibitors including diclofenac (DIC, 25 mg, n=8 & 50 mg/kg, n=8, i.p), piroxicam (PIR, 10 mg, n=10 & 20 mg/kg, n=11, i.p) and meloxicam (MELO, 10 mg, n=8 & 20 mg/kg, n=9, i.p). All data are expressed as percent changes in the control state (C). $*P<0.05$, $**P<0.01$, statistically significant differences from the control paw thickness.

DISCUSSION

Two types of COX isozymes, constitutive COX-1 and inducible COX-2, have been identified in many tissues. There is growing evidence in experimental studies that COX-2 plays an important role in the production of nociceptive responses. Noxious stimulations such as inflammation and nerve injury cause an increase in COX-2 mRNA expression and the number of COX-2 positive neurons in the superficial layer of spinal cord, whereas the number of COX-1 positive neurons remains unchanged by noxious inputs (Beiche et al, 1998; Maihöfner et al, 2000; Zhao et al, 2000). High density of prostanoid receptor immunoreactivity is also localized in the superficial layer of dorsal horn (Kawamura et al, 1997; Beiche et al, 1998). When inflammation is induced by carrageenan and complete Freund's adjuvant, the expression of COX-2 mRNA and concentration of prostaglandin (PG) E_2 in tissue and cerebrospinal fluid increase simultaneously, and their time courses are almost identical. Inflammation-induced increase in COX-2 mRNA expression, PGE_2 concentration and nociceptions are significantly suppressed by selective COX-2, but not COX-1 inhibitors (Masferrer et al, 1994; Hay et al, 1997; Zhang et al, 1997; Maihöfner et al, 2000). Interleukin-1β has been known to increase SP-like immunoreactivity in rat dorsal root ganglion neurons in culture and to enhance PGE_2 synthesis in cultured mouse astrocytes. These effects of interleukin-1β are significantly suppressed by selective COX-2 inhibitor, NS-398 (Inoue et al, 1999; O'Banion et al, 1996). Intrathecal administrations of SP and N-methyl-D-aspartate induce thermal hyperalgesia and increase PGE_2 release into spinal cord, which are strongly inhibited by the administration of selective COX-2 inhibitor, SC-58125, but not by selective COX-1 inhibitor (Yamamoto & Sakashita, 1998; Yaksh et al, 2001). All these experimental findings indicate that inflammation- and nerve injury-induced nociceptive responses are mediated mainly by the activation of COX-2 and are in sharp contrast with the results of present study in which selective COX-2 inhibitor did not have any significant inhibitory effect on melittin-induced nociceptions.

In the present study, the effect of melittin to dramatically decrease mechanical threshold and to increase flinching behaviors was dose-dependently suppressed by intraperitoneal administration of nonselective COX inhibitor (diclofenac) and selective COX-1 inhibitor (piroxicam), but not by selective COX-2 inhibitor (meloxicam). There are a number of reports indicating that COX-1 rather than COX-2 is a major factor to mediate the development of nociceptive responses. In the rat with cutaneous inflammation, peritonitis and type II collagen-induced arthritis, increased production of PGE_2 and hyperalgesia are significantly inhibited by selective COX-1 inhibitors such as piroxicam, ketorolac and FR122047 (Engelhardt et al, 1996; Zhang et al, 1997; Ochi & Goto, 2002). In air pouch model of mice, carrageenan-induced increase in PGE_2 production was suppressed by aspirin that had relatively higher affinity to COX-1 than COX-2, but increased PGE_2 production was not affected by selective COX-2 inhibitor, NS-398 (Gilroy et al, 1998). Capsaicin increases the release of substance P (SP) and calcitonin gene-related peptide (CGRP) from spinal cord slice of rat with carrageenan-induced hyperalgesia and this capsaicin-induced increase of SP and CGRP was significantly blocked by intrathecal administration of ketorolac and ibuprofen (Southall et al, 1998).

Neuropathic tactile allodynia as well as inflammatory hyperalgesia was also significantly suppressed by intrathecal administration of ketorolac (Lashbrook et al, 1999; Ma et al, 2002), and sustained ectopic activity of dorsal root ganglionic and dorsal horn neurons with peripheral nerve injury was also inhibited by subcutaneous administration of indomethacin, but not by selective COX-2 inhibitors such as celecoxib and NS-398 (Omana-Zapata & Bley, 2001). A 1 cm longitudinal incision of plantar aspect of rat hindpaw caused an increase in COX-1 immunoreactivity in the spinal cord and mechanical hyperalgesia that were dose-dependently suppressed by intrathecal administration of COX-1 preferring inhibitor, ketorolac and SC-560, but not by COX-2 inhibitor, NS-398 (Zhu et al, 2003). Ballou et al (2000) reported that, COX-1-deficient mice showed weaker nociception than wild-type controls in hot plate test and acetic acid writhing test. In immunocytochemical and morphometric study, COX-1 immunolabelling was found to be almost exclusively restricted to small diameter dorsal root ganglionic neurons in which the sensory neuron-specific (SNS) Na^+ channels were expressed, and COX-1 labelling was colocalized with CGRP and isolectin B4. However, COX-2 labelling was absent in dorsal root ganglionic neurons. On the basis of these findings, Chopra et al (2000) suggested that COX-1 is a marker for a subpopulation of putative nociceptive neurons in rat dorsal root ganglion. All these experimental findings indicate that PG synthesized by COX-1 may be important for nociception, in good agreement with the present results that COX-1 inhibitor suppressed melittin-induced nociceptive responses.

There are differences also in the dosages of COX inhibitors which were administered intraperitoneally. For example, Motta et al (2003) administered 0.1~0.5 mg/kg of diclofenac which induced dose-dependent antinociception in carrageenan-induced arthritic rat, whereas formalin-induced flinchings were inhibited by intraperitoneal administration of 0.3~27 mg/kg of diclofenac (Euchenhofer et al, 1998). Different doses of intraperitoneally administered meloxicam (single dose of 0.2~2 mg/kg or 0.1~4 mg/kg/day for 5 days) had antinociceptive action on thymulin- and carrageenan-induced joint hyperalgesia, respectively (Laird et

al, 1997; Safieh-Garabedian et al, 2000). The doses of COX inhibitors administered in these studies were lower than doses used in the present experiments. The difference in the dosage appears to be due to the type of pain models and rats raised under different conditions. Different types of pain model induce different severity of pain, and antinociceptive action of drugs is inversely proportional to the severity of pain (Luttinger, 1985). In our experience, rats of the same strain, when raised under different conditions, showed greatly different sensitivities to drug administration (unpublished data).

Melittin has been reported to selectively activate capsaicin-sensitive primary afferent fibers (Shin & Kim, 2004) and melittin-induced nociceptions are modulated also by the changes in the activity of extracellular signaling-regulated kinase, calcium channel, excitatory amino acid receptor and spinal serotonergic receptors (Lee et al, 2004; Shin et al, 2004b; Kim & Shin, 2005; Lee et al, 2005; Yu & Chen, 2005). Melittin has an ability to activate phospholipase A_2 which catalyzes the conversion of phosphatidylcholine to arachidonic acid and PG release (Shier, 1979; Choi et al, 1992). In recent *in vitro* study, melittin was shown to induce excitatory postsynaptic current in spinal substantia gelatinosa neurons which was reduced by phospholipase A_2 inhibitor (Yue et al, 2005). Melittin and phospholipase A_2 activating proteins increase also the synthesis of interleukin-1 and tumor necrosis factors which stimulate PG synthesis (Burch et al, 1988; Bomalaski et al, 1995). Because COX inhibitors were intraperitoneally administered in the present study, COX inhibitors are most likely to act on both spinal and peripheral sites. In summary, the activation of phospholipase and/or COX-1 by melittin may increase the release of arachidonic acid and PG which may play an important role in melittin-induced nociceptions.

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