

EFFECTS OF BEE VENOM PEPTIDERGIC COMPONENTS ON RAT PAIN-RELATED BEHAVIORS AND INFLAMMATION

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Abstract—To identify the active components of honeybee venom in production of inflammation and pain-related behaviors, five major peptidergic subfractions were separated, purified and identified from the whole honeybee venom. Among them, four active peptidergic components were characterized as apamin, mast-cell degranulating peptide (MCDP), phospholipase A₂ (PLA₂)-related peptide and melittin, respectively. All five subfractions were effective in production of local inflammatory responses (paw edema) in rats although the efficacies were different. Among the five identified subfractions, only MCDP, PLA₂-related peptide and melittin were able to produce ongoing pain-related behaviors shown as paw flinches, while only apamin and melittin were potent to produce both thermal and mechanical hypersensitivity. As shown in our previous report, melittin was the most potent polypeptide in production of local inflammation as well as ongoing pain and hypersensitivity. To further explore the peripheral mechanisms underlying melittin-induced nociception and hypersensitivity, a single dose of capsazepine, a blocker of thermal nociceptor transient receptor potential vanilloid receptor 1, was treated s.c. prior to or after melittin administration. The results showed that both pre- and post-treatment of capsazepine could significantly prevent and suppress the melittin-induced ongoing nociceptive responses and thermal hypersensitivity, but were without influencing mechanical hypersensitivity. The present results suggest that the naturally occurring peptidergic substances of the whole honeybee venom have various pharmacological potencies to produce local inflammation, nociception and

pain hypersensitivity in mammals, and among the five identified reverse-phase high pressure liquid chromatography subfractions (four polypeptides), melittin, a polypeptide occupying over 50% of the whole honeybee venom, plays a central role in production of local inflammation, nociception and hyperalgesia or allodynia following the experimental honeybee's sting. Peripheral transient receptor potential vanilloid receptor 1 is likely to be involved in melittin-produced ongoing pain and heat hyperalgesia, but not mechanical hyperalgesia, in rats. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: peptidergic biotoxins, local inflammation, ongoing pain, thermal and mechanical hypersensitivity, TRPV1.

It is well known that honeybee's sting on human skin can induce ongoing pain, hyperalgesia (and allodynia) and inflammation. However, the mechanisms underlying the above events are poorly studied. In the past several years, we have reported that following s.c. injection of a solution containing the whole bee venom (BV, *Apis mellifera*), into one hind paw of a rat, the experimentally-produced honeybee's sting, the animal showed unique expressions of persistent spontaneous nociception (PSN) and inflammatory pain hypersensitivity relevant to pathological pain state (Lariviere and Melzack, 1996; Chen et al., 1999b; Chen and Chen, 2000; Lariviere et al., 2002; Chen, 2003). Capsaicin-sensitive primary afferents (CSPA) have been demonstrated to mediate the BV-induced PSN, which lasts for more than 1 h, as well as thermal or mechanical hypersensitivity, which lasts for 3–4 days (Chen and Chen, 2001). These afferents also mediate BV-induced long-term ongoing spike discharges and enhanced heat/mechanical responsiveness of wide dynamic range neurons in the spinal dorsal horn (Chen, 2003). Collectively, these data suggest a contribution of spinal neuronal plasticity to the behavioral PSN and heat and mechanical hypersensitivity in the BV test (Chen et al., 1998, 1999a; You and Chen, 1999; You et al., 2002; Zheng et al., 2002; Chen, 2003). However, BV has also been believed to produce analgesia when applied into an acupoint or painful sites in both animal and clinical studies (Lee et al., 2001; Kwon et al., 2001a,b, 2002).

The whole BV is composed of many chemical agents: polypeptides [melittin, apamin, mast cell degranulating peptide (MCDP), etc.], enzymes [hyaluronidase, phospholipase A₂ (PLA₂), etc.], amines (histamine, dopamine, etc.) and others (Tu, 1977). To study the peripheral mechanisms of pain and inflammation in the BV test, an understanding of the pharmacological roles of each peptidergic component is of particular importance. Recently our laboratory and other laboratories showed that s.c. injection of melittin, which comprises over 50% of the whole BV, can

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Abbreviations: BV, bee venom; CPZ, capsazepine; CSPA, capsaicin-sensitive primary afferent; FMMU, Fourth Military Medical University; F1, major fraction 1; GC, gel chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MCDP, mast cell degranulating peptide; PLA₂, phospholipase A₂; PSN, persistent spontaneous nociception; PWMT, paw withdrawal mechanical threshold; PWTL, paw withdrawal thermal latency; RP-HPLC, reverse-phase high pressure liquid chromatography; TRPV1, transient receptor potential vanilloid receptor 1.

induce persistent spontaneous nociceptive response, and heat and mechanical hypersensitivity in both behavioral and electrophysiological studies (Li and Chen, 2004; Shin and Kim, 2004), partly explaining the pain-related responses observed in the BV test. In a relatively comparative study of s.c. injection of melittin and whole BV, a difference in the intensity of spontaneous nociceptive response, heat and mechanical hypersensitivity was of statistical significance. Based on the results of our previous studies, we propose that there should be other algogenic and pro-inflammatory components in crude BV being responsible for the BV-induced inflammatory pain response. In this report, gel chromatography (GC) and reverse-phase high pressure liquid chromatography (RP-HPLC) were used to successfully isolate and purify four active polypeptides (five subfractions) from the whole BV. The active polypeptides were identified by both matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and amino acid sequence analysis. Then inflammation and pain-related behaviors were observed and quantified in rats receiving s.c. administration of each peptide.

EXPERIMENTAL PROCEDURES

Separation and purification of BV peptidergic components

The crude venom of honeybee was obtained from Georgian gray bees (kindly provided by Floret Ltd. and its partner company New Techniques Laboratory Ltd., Tbilisi, Georgia) and separated by GC followed by RP-HPLC. The methods were based upon the well-established protocols used for separation and purification of scorpion venomous peptides in Dr. Ji's laboratory (for details please refer to Ji et al., 1996 and Ye et al., 2000). Approximately 200 mg crude BV was dissolved in 0.01 M acetic acid solution, and then centrifuged at 10,000 r.p.m. for 5 min at 4 °C. The precipitate was removed and the supernatant was loaded onto a Sephadex G-10 (Pharmacia) column (60×1.5 cm) pre-equilibrated with 0.05 M ammonium acetate buffer. The column was then eluted at a flow rate of 0.5 ml/min. The elution was collected 5 ml/tube and monitored at 280 nm by UV spectrophotometry (Model DU 650, Beckman). The wave length was determined by the fact that the highest ultraviolet absorption peak of protein or polypeptide is at 280 nm. The purification of fractions was performed by C18 RP-HPLC column (Develosil, ODS-UG-5, 25×0.5 cm, Nomura Chemical, Japan) equipped on a HPLC system (Dionex, USA). The eluents employed were 0.01% trifluoroacetic acid as solvent A and 60% acetonitrile as solvent B. Elution was performed with linear gradients of solvent B in solvent A, and monitored at 215 nm at a flow-rate 1.0 ml/min. Since collection of the subfractions was started at the very beginning of elution, salt peaks (peaks 1–6 in this experiment) were also collected but excluded for further analysis. In RP-HPLC, ultraviolet absorption spectroscopy is used to determine the concentration of aromatic amino acid residues so that clearly, this method fails for molecules in which no such residues are present.

Structural identification of BV peptidergic components

The MALDI-TOF MS (Kratos Analytical Co., Shimadzu Group, Manchester) and ABI Procise 491 Protein Sequencer using standard Edman degradation chemistry were used to identify the molecular weight and N-terminal 8 amino acid sequence of each RP-HPLC subfraction. The latter system was equipped with a 140C microgradient apparatus and a 785A UV detector for the

identification of phenylthiohydantoin-amino acids. Generally 0.5–1.0 nmol of sample was used for each sequence run.

Functional identification of BV peptidergic components: behavioral testing

Animals. The experiments were performed on male Sprague–Dawley albino rats weighing 180–250 g. The animals were provided by the Laboratory Animal Center of the Fourth Military Medical University (FMMU) and use of the animals was reviewed and approved by the FMMU Animal Care and Use Committee. The present study was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) revised 1996 or the UK Animals (Science Procedures) Act 1986 and associated guidelines, or the European Communities Council Directive of 24 November 1986 (86/609/EEC). The IASP's guidelines for pain research in conscious animals were also followed (Zimmermann, 1983). The number of animals used and their suffering have been greatly minimized. The animals were housed in plastic boxes with food and water available *ad libitum* in the colony room at 22–26 °C. A 12-h light dark cycle with lights on at 08:00 h was maintained and testing was done between 09:00 and 18:30 h. The rats were acclimatized to the laboratory and habituated to the test boxes and slight restraint for at least 30 min each day for 5 days before testing.

Quantitative analysis of spontaneous pain-related behaviors. A 30×30×30 cm transparent plastic test box with a transparent glass floor was placed on a supporting frame of 30 cm high above the experimental table. The rat was placed in the test box for at least 30 min before administration of the chemical agents. After the acclimation period, s.c. injection of chemical agents was made into the center of the plantar surface of one hind paw with slight restraint. The rat was then returned to the test box, and pain-related responses were recorded for 1 h. The spontaneous nociceptive behavior was determined by counting the number of paw flinches occurring during 5 min intervals following injection (for details see Chen et al., 1999b).

Quantitative measurement of thermal hyperalgesia. Approximately 2 h after s.c. administration of each BV component, spontaneous nociceptive behavior gradually receded, after which the injected paw of the rat was in contact with the floor and showed normal weight bearing. The rat was placed on the surface of a 2 mm thick glass plate covered with a plastic chamber (20×20×25 cm) to measure the sensitivity to heat stimuli with a TC-1 radiant heat stimulator (new generation of RTY-3 made in Xi'an Bobang Technologies of Chemical Industry Co. Ltd., PR China). The distance between the projector lamp bulb (150 W) and the lower surface of the glass floor was adjusted to produce a light spot on the floor surface with 5 mm diameter. Twenty percent of light intensity was adopted as output because for most of the rats tested the paw withdrawal thermal latency (PWTL, s) was around 12 s (Chen et al., 1999b). The heat stimulus was directed onto the injection site or a corresponding area in the contralateral hindpaw. Four stimuli were repeated to the same site and the latter three values were averaged as mean PWTL. The inter-stimulus interval was more than 10 min at the same region, 5 min at the different region. The thermal latency was determined as the duration from the beginning of heat stimulus to the occurrence of a hind paw withdrawal reflex. The stimulus was stopped if the latency exceeded 30 s so as to avoid excessive tissue injury and the region was considered to be completely unresponsive (for details see Chen et al., 1999b). To examine thermal hyperalgesia, sensitivity to radiant heat stimuli applied to the injection area on the hind paw of the rat was examined at 2, 4, 8, 24 and 48 h after s.c. administration of each BV component which was preceded by baseline PWTL measurement prior to s.c. administration of vehicle and drugs.

Quantitative measurement of mechanical hyperalgesia. To observe whether mechanical hyperalgesia and allodynia occurred after the injection of chemical agents, mechanical stimuli were applied by using ascending graded individual von Frey monofilaments with bending forces of 3.5, 4.5, 5.5, 7.8, 11, 15, 20, 25, 30, 40, 50, 60 g. The rat was placed on a metal mesh floor covered with the same plastic box and von Frey filaments were applied from underneath the metal mesh floor to the testing site of the bilateral hind paws. A von Frey filament was applied 10 times (once every several seconds) to the injection site of the hind paw. The bending force value of the von Frey filament that caused a 50% occurrence of paw withdrawal was expressed as the paw withdrawal mechanical threshold (PWMT, g) (for details see Chen et al., 1999b). The time course for measurement of mechanical sensitivity was in accord with that of the thermal sensitivity as described above.

Quantitative measurement of local inflammation. A plethysmometer (Ugo, Basile, Italy) was used to measure the volume of rat hind paw prior to and 1 h after s.c. administration of each BV component. The increase in volume of rat injected hind paw was an index to the inflammatory intensity. The injected hind paw was immersed into water cell full of 0.04–0.05% NaCl solution according to the guide book of the maker, and the volume of spillage was the volume of hind paw.

Drugs

The s.c. dosage of the peptidergic components tested was determined to be 0.1 mg in 50 μ l saline with reference to the dosage of melittin. The reasons are as follows: (1) the dose-response curve with dosage ranging from 0.001–0.2 mg in the BV test of rats showed that 0.2 mg BV was the optimally potent dose producing maximal tonic nociceptive response (Lariviere and Melzack, 1996; Lariviere et al., 2005); (2) the natural amount of melittin is equivalent to about 50% of the whole BV; (3) dose-response observation showed that 0.1 mg melittin (half amount of the whole BV) produced almost the same maximal effect on tonic nociceptive response as 0.2 mg whole BV in both behavioral and electrophysiological recordings (Li and Chen, 2004). Capsazepine (CPZ, 0.3 mg/50 μ l), a blocker of thermal nociceptor TRPV1 (previously termed as vanilloid receptor 1 or capsaicin receptor), was administered s.c. pre- or post-treatment of melittin. The dosage of CPZ was determined on the basis of a previous report showing that intradermal injection of 0.3 mg CPZ could reduce carrageenan-induced inflammatory pain hypersensitivity and spinal c-Fos expression (Kwak et al., 1998).

Statistical analysis

All results were expressed as mean $\pm$ S.E.M. The PWTL and PWMT values prior to and at each time point after s.c. administration of each BV peptidergic component were compared by using Kruskal-Wallis nonparametric ANOVA with post hoc tests. The time course data of spontaneous pain-related behaviors among five subfractions or between control and drug were compared by a non-parametric Mann-Whitney *U* test. *P*-value < 0.05 was considered to be statistically significant.

RESULTS

Separation, purification and identification of BV peptidergic components

The supernatant of BV solution was separated by GC into four fractions through Sephadex G-10 column chromatography (Fig. 1A). After ultrafiltration (with the molecular weight < 3000 Da) of each fraction, molecular weight was measured by MALDI-TOF MS. Typically, the accuracy of

mass determined by MALDI-TOF MS was around $\pm 0.1\%$ for proteins with molecular masses between 1 and 40 kDa (Zaluzec et al., 1995). In our study, due to factors associated with technique, apparatus and the existence of isotope, the error of molecular weights measured by MALDI-TOF MS was around ± 10 for polypeptides. The molecular weight of the major fraction 1 (F1) was 2847.30 (Fig. 1A), which generally corresponded to the molecular weight of melittin (Fischer and Neumann, 1961; Habermann, 1972), while the minor fractions 2, 3 and 4 (F2, F3 and F4) generally corresponded to apamin based upon their molecular weights (2027.29 Da, 2027.29 Da and 2027.23 Da) on the mass spectra (Habermann and Reiz, 1965) (Fig. 1A).

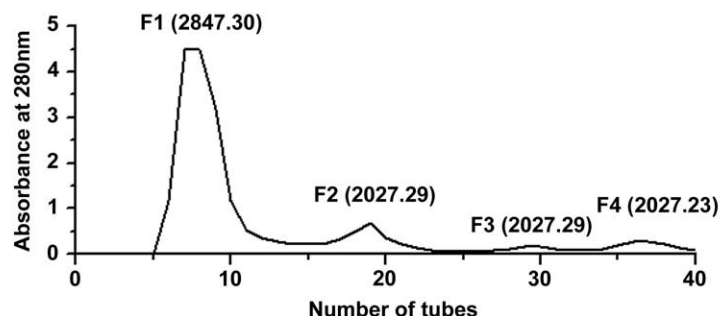
To further identify how many peptidergic components there are in the major GC F1, RP-HPLC was performed and at least 13 subfractions were obtained (Fig. 1B). Because of the existence of salt peaks, the first six subfractions were excluded. Among the remaining seven obtained subfractions (F107–F113), five were identified by both MALDI-TOF MS and amino acid sequence analysis (Fig. 2A–F), while the other two (F107 and F111) were not further analyzed due to their impurity. Among the five identified RP-HPLC subfractions, F108 was characterized as apamin, both F109 and F110 as MCDP, and F113 as melittin, respectively based upon the N-terminal 8 amino acids sequences (Fig. 2F). Because the N-terminal 8 amino acid residues of F112 were homologous to those of PLA₂, it was thus referred to as a PLA₂-related peptide (Fig. 2F).

Pharmacological effects of BV peptidergic components on inflammation and pain-related behaviors in rats

Local inflammatory responses. Following s.c. administration of a single highest dose (0.1 mg/50 μ l), all of the five individual subfractions (F108, F109, F110, F112, and F113) produced striking redness and edema immediately after removal of the needle. The redness radially extended and gradually covered the whole plantar surface of the injected hind paw. The duration of redness and edema lasted for 8–24 h (time course data not shown). Compared with the percent increase in paw volume [(polypeptide–baseline)/baseline $\times$ 100%], the order of relative edema intensity produced was as: F110 (74%) $\approx$ F109 (61%) $\approx$ F113 (53%) $\approx$ F112 (51%) > F108 (28%) (Fig. 3), suggesting that MCDP, melittin, and the PLA₂-related peptide should be major peptidergic components, while apamin should be a minor peptidergic component of BV being responsible for the production of local inflammation.

Ongoing pain-related response. To observe whether some of the five identified subfractions (namely four peptidergic components) of BV can cause ongoing pain-related behaviors, a volume of 50 μ l saline containing 0.1 mg of each lyophilized subfraction was injected s.c. into the posterior surface of one hind paw of rats. Among five subfractions tested, only four (F109, F110, F112 and F113) were able to produce significant ongoing pain-related behaviors shown as paw flinches, however F113

A Gel chromatography



B RP-HPLC

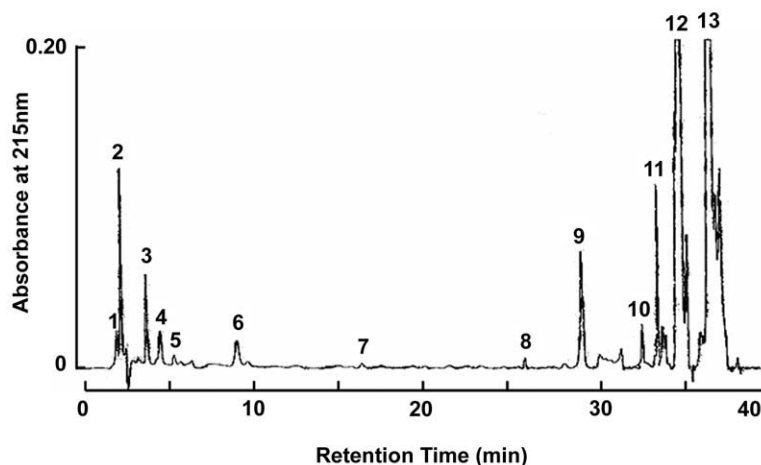


Fig. 1. Separation and purification of honeybee venom peptidergic components by GC and RP-HPLC. (A) Showing one major and three minor fractions obtained by GC (Sephadex G-10 column); B, showing the 13 RP-HPLC subfractions obtained from the GC-derived F1 (peaks 1–6 are the salt peaks, peaks 7–13 are the peptidergic components).

(melittin) produced the longest and the most intensively nociceptive responses in terms of both time course and nociceptive intensity (Fig. 4A and 4B). The results of melittin were consistent with our and other more recent reports (Li and Chen, 2004; Shin and Kim, 2004).

Thermal and mechanical hypersensitivity. Sensitivity of the injection site to heat or mechanical stimuli was investigated 2 h, 4 h, 8 h, 24 h and 48 h after s.c. injection of each subfraction at 0.1 mg/50 μ l. As shown in Fig. 4C, PWTL was only significantly decreased at 2 and 4 h after injection of F113 ($P=0.046$, $n=6$) and F108 ($P=0.042$, $n=5$), respectively. S.c. injection of F109 ($n=5$), F110 ($n=6$) and F112 ($n=5$) did not cause any change in PWTL (Fig. 4C), suggesting that besides melittin (F113), apamin (F108) also results in thermal hypersensitivity following s.c. administration.

Of the five identified subfractions, F108 (apamin) and F113 (melittin) were effective to decrease the PWMT (F108: $P=0.046$, $n=6$; F113: $P=0.042$, $n=5$) at 2 h after s.c. administration (Fig. 4D). Moreover, F113 was the most effective in production of mechanical hypersensitivity 2–48 h after s.c. administration (Fig. 4D). The PWMT baseline

value was not changed by F109, F110 and F112, indicating lack of peripheral actions of these three peptides on mechanical sensitivity (Fig. 4D).

Effects of peripheral CPZ on melittin-induced ongoing pain and hypersensitivity. To further explore the underlying mechanisms of melittin-produced ongoing nociception and hypersensitivity, a single dose of CPZ (0.3 mg/50 μ l), a blocker of thermal nociceptor TRPV1, was administered locally into the paw pad prior to or after melittin injection. Compared with saline control group ($n=6$ for each group), both pre- or post-treatment of CPZ could significantly prevent or suppress the number of the melittin-produced persistent paw flinches observed in 1 h time course (Fig. 5A and 5B, $P<0.05$, $n=10$ for each group). As shown in Fig. 5C, following vehicle administration, s.c. melittin injection produced primary heat hypersensitivity with more than 55–70% reduction in PWTL in the injection site (2 h after melittin). However, both peripherally local pre- and post-treatment with the same dose of CPZ produced a 56% blocking and 58% reversing effect on the development and maintenance of primary heat hypersensitivity (left two panels in Fig. 5C, $P<0.001$, $n=10$ for each group). In sharp contrast, neither pre- nor post-treatment of

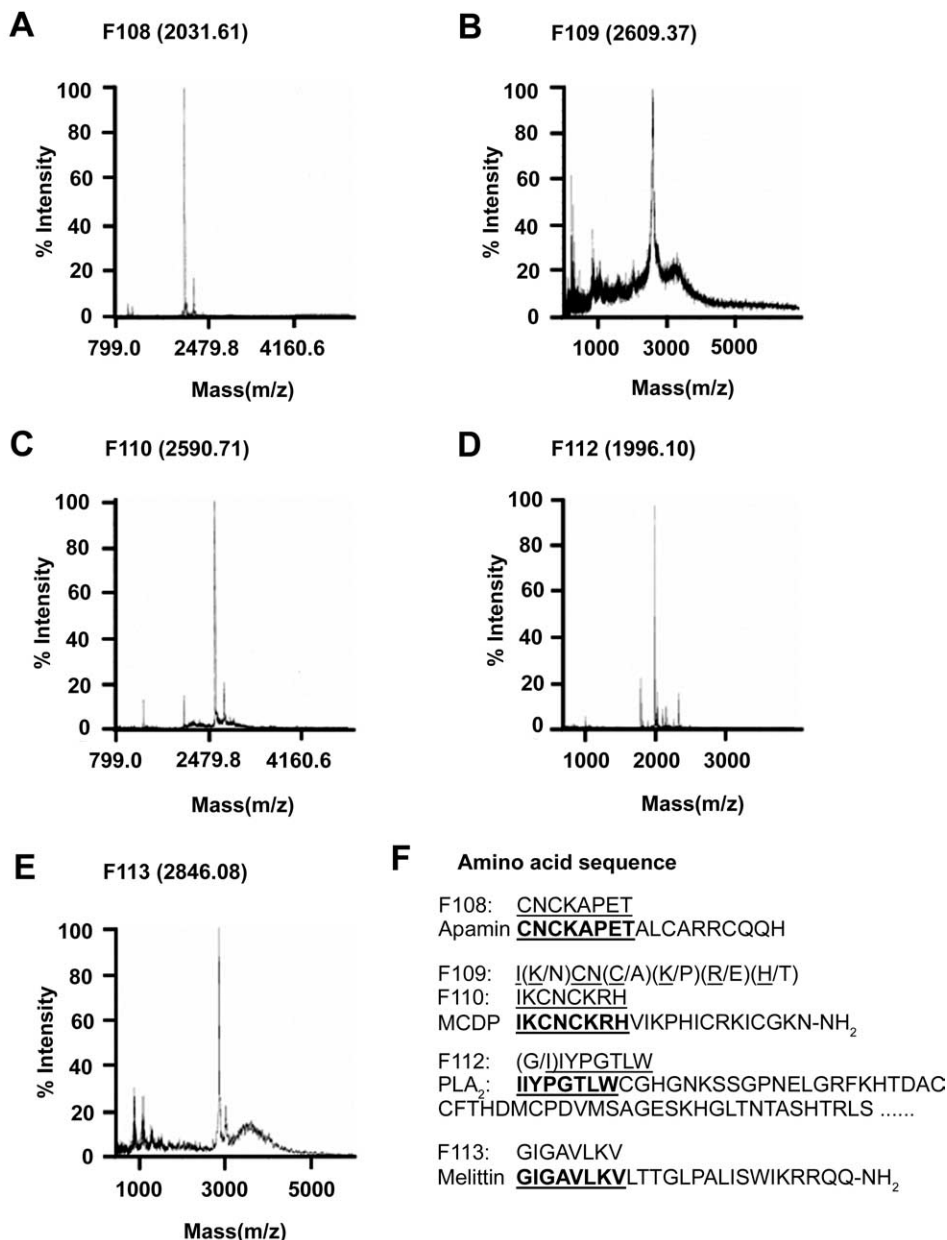


Fig. 2. Structural identification of the honeybee venom peptidergic components by both MALDI-TOF MS and amino acid sequence analysis. (A–E) MALDI-TOF mass spectra of the five RP-HPLC subfractions (F108, F109, F110, F112, F113) which were primarily obtained from GC-derived F1 (as shown in Fig. 1A and B). (F) N-terminal 8 amino acid sequence analysis showing homology of F108 to apamin, of F109 and F110 to MCDP, and of F113 to melittin. Because F112 was an unknown polypeptide with N-terminal 8 amino acid sequence homologous to PLA₂ it was termed as a PLA₂-related polypeptide.

CPZ could block or reverse melittin-produced primary mechanical hypersensitivity (with 65–78% reduction in PWMT) (left two panels in Fig. 5D). The lack of any effect of ipsilaterally local CPZ treatment on the PWTL and PWMT values of contralateral hindpaw compared with both baseline and the melittin-treated state could rule out the systemic effect of CPZ (right two panels in Fig. 5C and 5D).

Table 1 summarized the biological contribution of the five subfractions (four BV peptidergic components) to the production of local inflammation, nociception and hyperalgesia in rats.

DISCUSSION

Structural and functional identification of bioactive components of BV polypeptides and their roles in production of peripheral inflammation, nociception and hyperalgesia in rats

Based upon the molecular weights and N-terminal 8 amino acid sequence analysis of the five RP-HPLC subfractions, three BV peptidergic components were identified as the known polypeptides such as apamin (F108), MCDP (F109,

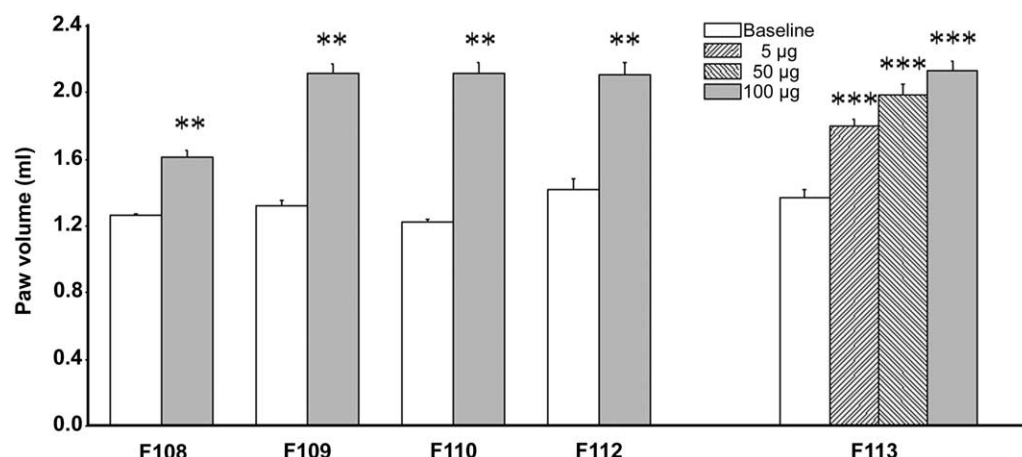


Fig. 3. Evaluation of the pharmacological effects of five identified bee venom peptidergic subfractions on local skin inflammatory responses in conscious rats. Paw volume was measured by plethysmometer prior to and 1 h after s.c. injection of each polypeptide into the left hind paw of rats. Single high dose (0.1 mg in 50 μ l saline) was used for F108, F109, F110, and F112, while three doses (0.005 mg, 0.05 mg, 0.1 mg in 50 μ l saline) were used for study of dose-response effects of F113. Error bars: $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, treatment vs. baseline paw volume.

F110) and melittin (F113) (Habermann and Reiz, 1965; Habermann and Jentsch, 1966; Breithaupt and Habermann, 1968; Gaudie et al., 1978), while one subfraction (F112) was identified as a PLA₂-related peptide due to its 100% homology of the N-terminal 8 amino acids to PLA₂ (Valentin et al., 2000). Regarding the peptidergic components contained in the whole BV, the exact amount of peptidergic components might vary from bee to bee (Minton, 1974) and this might be the reason of discrepancy observed between our present and some previous studies (Habermann and Reiz, 1965; Habermann and Jentsch, 1966; Breithaupt and Habermann, 1968; Gaudie et al., 1978).

For the biological actions of the four naturally occurring polypeptides on mammals, a single dose effect was observed in production of local inflammation, nociception and hyperalgesia. It was interesting to note that all of the four BV peptidergic components (five subfractions) were effective in production of peripherally local inflammatory response (shown as paw edema). However, their roles in production of nociception and hyperalgesia to thermal and mechanical stimuli were various. By comparing the efficacy of an equivalent dose of the four polypeptides, melittin (F113), a 26 amino acid polypeptide, was the only and the most potent one in production of all inflammatory pain symptom and signs including local inflammation, PSN, and thermal or mechanical hyperalgesia in terms of both 1 h long-lasting time course and intensity (or extent). This was highly consistent with our previous results in rats (Li and Chen, 2004) and other results in human subjects (Koyama et al., 2000; Sumikura et al., 2003). For the other three polypeptides, however, the biological functions were not consistent. For instance, MCDP (F109 and F110) and the PLA₂-related peptide were as potent as melittin in production of local inflammation, however, they produced fewer paw nociceptive flinches with short-lasting time course (within 10–15 min) and no hyperalgesia to either heat or mechanical stimuli applied to the inflamed area of the rat hind paw. Moreover, apamin (F108) produced thermal and

mechanical hyperalgesia as well as inflammation, but without any nociceptive effect on paw flinches. These results implicated that: (1) the identified polypeptides might have different contributions to the production of local inflammation, PSN, and thermal or mechanical hyperalgesia observed in the BV test; (2) melittin was likely to be the major algogenic and inflammatory chemical substance of the whole BV.

Biological significance of identification of the BV bioactive polypeptides in study of pain and inflammation

The very much basic scientific question for the study of inflammatory pain and hypersensitivity is to make clear the relationship between pain and hypersensitivity or between pain/hypersensitivity and inflammation. However, so far the question has not been well answered due to lack of pharmacological tools and models which are able to differentiate the processes of pain, hypersensitivity and inflammation. The discovery of different roles of the BV bioactive polypeptides in the production of local inflammation, PSN, and thermal or mechanical hyperalgesia makes it possible to unravel the molecular and cellular mechanisms of either inflammation or pain and hyperalgesia.

Similar to the effect of BV solution, s.c. administration of melittin could also produce a dose-dependent increase in spike discharges of spinal dorsal horn nociceptive neurons which was demonstrated to be mediated by CSPA fibers (Chen and Chen, 2001; Shin and Kim, 2004). This result suggested that the nociceptive and hyperalgesic effects of melittin might be mediated by activation of peripheral capsaicin receptor TRPV1, a noxious heat sensor (Julius and Basbaum, 2001). Thus in the present study we performed local administration of CPZ, a selective blocker of thermal nociceptor TRPV1, in periphery together with melittin and resulted in a blockade of both development and maintenance of PSN and heat hyperalgesia, but without affecting the mechanical hyperalgesia, suggesting in-

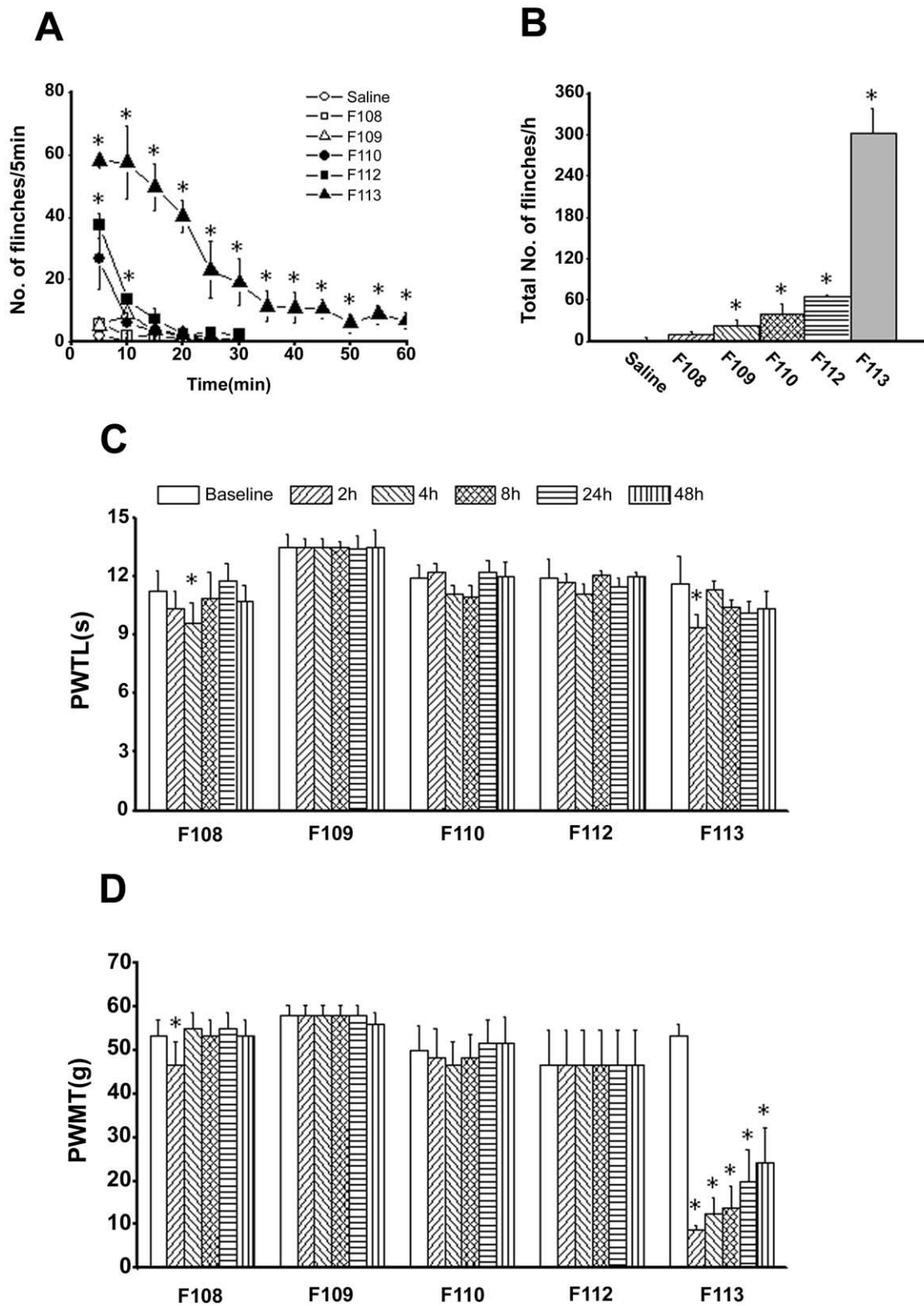


Fig. 4. Evaluation of the pharmacological effects of the five identified bee venom peptidergic subfractions (0.1 mg in 50 μ l saline) on ongoing pain-related behaviors and thermal and mechanical sensitivity in conscious rats. (A) Time-course recording of the rat nociceptive paw flinches following s.c. injection of the same dose of the five bee venom peptidergic subfractions. (B) The mean total number of paw flinches induced by each of the five active subfractions. (C, D) Time-course recording of the change in PWT (s) and mechanical threshold (PWMT, g) prior to and 2, 4, 8, 24 and 48 h after s.c. injection of each subfractions. Error bars: $\pm$ S.E.M. * $P < 0.05$.

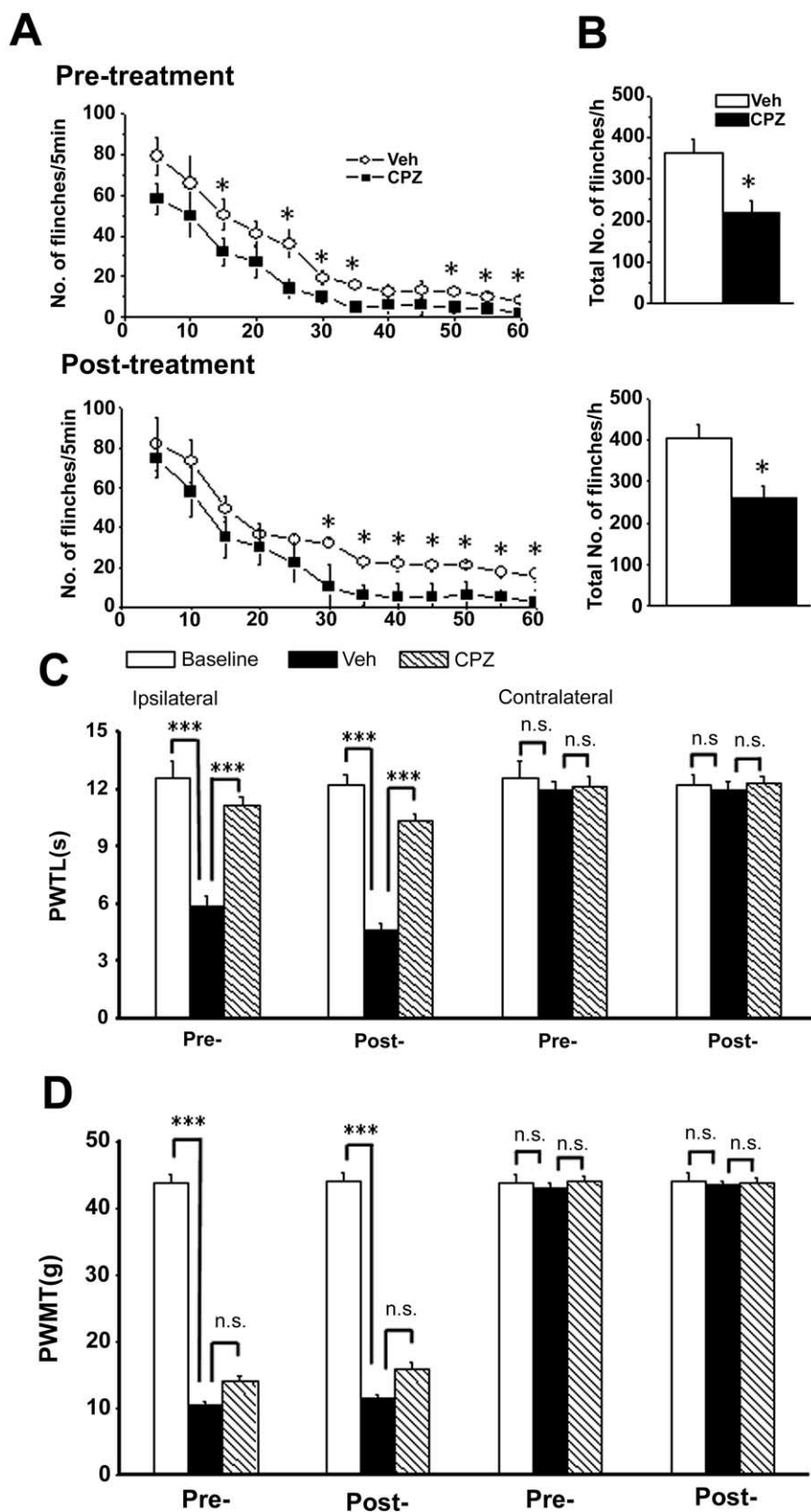


Fig. 5. Evaluation of peripherally local effects of CPZ (0.3 mg/50 μ l), a selective blocker of thermal nociceptor TRPV1, on the melittin-induced PSN and heat and mechanical hyperalgesia. (A, B) Time-course recording and the mean total number of paw flinches induced by melittin with pre- or post-treatment of CPZ. (C, D) Effect of pre- or post-treatment of CPZ on the development and maintenance of thermal and mechanical hyperalgesia. Thermal sensitivity was quantified by measurement of PWTL (s) to radiant heat and mechanical sensitivity by PWMT (g) to a series of von Frey filaments. Ipsilateral and contralateral: hind paw ipsilateral or contralateral to the melittin or CPZ injection. Error bars: $\pm$ S.E.M. * $P < 0.05$, *** $P < 0.001$, n.s., nonsignificant.

Table 1. Biological contribution of the five bee venom peptidergic subfractions to the production of local inflammation, nociception and hyperalgesia in rats

RP-HPLC subfractions (100 μ g/50 μ l)	N-terminal 8 amino acid sequence	Corresponding polypeptide	Increase in paw volume	Ongoing pain	Mechanical hyperalgesia	Thermal hyperalgesia
F108	CNCKAPET	Apamin	+	–	+	+
F109	I(K/N)CN(C/A)(K/P)(R/E)(H/T)	MCDP	+++	+	–	–
F110	IKCNCKRH	MCDP	+++	+	–	–
F112	(I/G)IYPGTLW	PLA ₂ -related peptide	+++	+	–	–
F113	GIGAVLKV	Melittin	+++	+++	+++	+

Notes: +++, strong response; +, minor response; –, no response.

involvement of peripheral thermal nociceptor activation in production of melittin-induced nociception and heat hypersensitivity. Our more recent study further revealed that melittin could activate the small- to medium-sized dorsal root ganglion cells by opening the non-selective cation ion channel TRPV1 through selective activation of PLA₂–lipoxygenase pathway in which protein kinases C and A were necessary, but not PLA₂–cyclooxygenase pathway (Chen et al., 2005). This suggests that melittin in both *in vivo* and *in vitro* experimental models might be a useful pharmacological tool to dissociate the biochemical signaling processing between thermal and mechanical hyperalgesia. Furthermore, it is still intriguing to explore for what the biochemical signaling processes along the pain pathway are associated with mediation of melittin-produced mechanical hypersensitivity. Since the structures and functions of the mechanical sensors and nociceptors in the peripheral primary afferents have not been well identified at the molecular level (Julius and Basbaum, 2001; Julius and McCleskey, 2006), it is thus difficult to discover signaling pathways of the melittin-produced mechanical hypersensitivity at the moment.

Unlike melittin, s.c. administration of MCDP, a well-known powerful histamine-releasing polypeptide (Breithaupt and Habermann, 1968; Gushchin et al., 1977), could only induce local inflammation and short-lasting ongoing pain-related behaviors but with the thermal and mechanical sensitivity remained unchanged. Moreover, s.c. administration of apamin, a well-known blocker of calcium-dependent potassium channel (Hugues et al., 1982), produced both local inflammation and thermal/mechanical hypersensitivity, but with less effect to produce ongoing pain-related behaviors. In sharp contrast, although s.c. administration of the newly identified PLA₂-related polypeptide could produce local inflammation and nociception, no thermal and mechanical hypersensitivity were induced at the highest dose tested. Taken together, during the symphony of the BV-produced inflammatory pain and hypersensitivity, different BV peptidergic components play different roles in the processing, however, melittin is likely to play a central role in production of pain, hyperalgesia or allodynia, and inflammatory processing following honeybee's sting. And opening of the cation channel, namely thermal nociceptor TRPV1 (also referred to as capsaicin receptor), by melittin, might be the prelude of the symphonic processing of BV-induced inflammatory pain and hypersensitivity.

CONCLUSION

In summary, the naturally occurring peptidergic substances of the whole BV have various pharmacological potencies to produce local inflammation, nociception and pain hypersensitivity in mammals, and among the five identified RP-HPLC subfractions (four polypeptides), melittin, a polypeptide occupying over 50% of the whole BV, plays a central role in production of local inflammation, nociception and hyperalgesia or allodynia following the experimental honeybee's sting. Activation of TRPV1, a thermal nociceptor, is likely to be involved in the melittin-produced ongoing pain and heat hyperalgesia, but not mechanical hyperalgesia.

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