Effects of SKF-96365, a TRPC inhibitor, on melittin-induced inward current and intracellular Ca^{2+} rise in primary sensory cells

Jing DING¹, Yong XIAO², Dan LU¹, Yi-Ru DU¹, Xiu-Yu CUI¹, Jun CHEN^{1,2}

¹*Institute for Biomedical Sciences of Pain, Capital Medical University, Beijing 100069, China*

²*Institute for Biomedical Sciences of Pain and Institute for Functional Brain Disorders, Tangdu Hospital, the Fourth Military Medical University, Xi'an 710038, China*

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Abstract: Objective Melittin (MEL) is a major component of bee venom and can produce both persistent spontaneous nociception and pain hypersensitivity when injected subcutaneously in the periphery. The present study aimed to examine the roles of transient receptor potential canonical (TRPC) channels in mediation of MEL-induced activation of primary nociceptive cells. **Methods** Whole-cell patch-clamp and laser scanning confocal calcium detection were used to evaluate the effects of SKF-96365, a TRPC inhibitor, applied on the acutely isolated dorsal root ganglion (DRG) cells of rat, on MEL-induced increase in intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) and inward current. **Results** Under voltage-clamp mode, 43.9% (40/91) DRG cells were evoked to give rise to the inward current by 2 $\mu\text{mol/L}$ MEL, which could be significantly suppressed by 3 doses of SKF-96365 (1, 5 and 10 $\mu\text{mol/L}$) in a dose-dependent manner. Of the other 210 cells, 67.6% responded to MEL with an intracellular Ca^{2+} rise, as revealed by confocal calcium imaging. Of these MEL-sensitive cells, 46.5% (66/142) were suppressed by the highest dose of SKF-96365. **Conclusion** MEL-induced activation of small to medium-sized DRG cells can be suppressed by SKF-96365, suggesting the involvement of TRPC channels in the mediation of MEL-induced activation of primary nociceptive cells.

Keywords: melittin; transient receptor potential canonical channel; dorsal root ganglion; patch-clamp technique; calcium imaging

1 Introduction

Bee venom (BV) is a complex mixture of polypeptides and other bioactive substances. Melittin (MEL) is a major polypeptide ingredient of BV, which accounts for 50% of the lyophilized preparation^[1-3]. Recently, MEL is demonstrated to activate 55%–65% dorsal root ganglion (DRG) cells, partially through activation of transient receptor po-

tential vanilloid receptor 1 (TRPV1) by phospholipase A2 (PLA2)-arachidonic acid-cyclooxygenase (COX)/lipoxygenase (LOX) metabolites^[4]. A series of studies have reported that s.c. injection of MEL could produce most types of pain-related or nocifensive behaviors like those evoked by BV, including long-term spontaneous nociception, and strong mechanical and thermal hyperalgesia in rats^[5-7]. In humans, intradermal injection of MEL also induces spontaneous pain and primary hyperalgesia to mechanical and heat stimuli as well as an increase in skin temperature surrounding the injection site^[8].

As the primary sensory neurons, DRG cells play im-

Corresponding author: Jun CHEN
Tel: +86-29-84777942; Fax: +86-29-84777945
E-mail: junchen@fimmu.edu.cn; chenjun@ccmu.edu.cn
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portant roles in nociceptive transmission from peripheral to the central nervous system (CNS). The soma of DRG neurons are commonly used as models for their own peripheral terminals^[9] which are inaccessible for adequate electrophysiological studies. For this reason, acutely dissociated DRG neurons were used to examine the effects of acute topical application of MEL. MEL-induced behavioral changes and responses of spinal dorsal horn neurons^[5] appear to be partially mediated by the transient receptor TRPV1^[6] and P2X3 receptor^[10].

Transient receptor potential (TRP) channels are a superfamily of non-selective cation-permeable channels^[11]. Based on sequence homology and functional similarities, the TRP canonical (TRPC) subfamily members can be grouped into 4 subfamilies: TRPC1, TRPC2, TRPC3/6/7, and TRPC4/5^[12]. It has been shown that the TRPC channels, which are abundantly expressed in the CNS, are Ca²⁺-permeable nonselective cation channels^[13]. All but TRPC2 mRNA have been detected in human brain tissues^[14] and rat lumbar DRG^[15], with TRPC1, TRPC3 and TRPC6 being the most abundantly expressed in these tissues.

To reveal whether TRPC has a role in mediation of MEL-induced nociception and pain hypersensitivity, effects of 3 doses (1, 5 and 10 $\mu\text{mol/L}$) of SKF-96365, a TRPC inhibitor, on MEL-induced activation of primary nociceptive cells were evaluated in the acutely dissociated DRG cells.

2 Materials and methods

2.1 Animals Male Sprague-Dawley (SD) albino rats weighing 80–100 g were purchased from the Laboratory Animal Center of Capital Medical University (Beijing, China) and The Fourth Military Medical University (Xi'an, China). Animals were housed at room temperature (22–26 °C) under a 12:12 h light/dark cycle, with free access to water and food. All experimental procedures were carried out in accordance with ethical guidelines of the International Association for the Study of Pain for pain research in conscious animals.

2.2 Cell preparation Animals were anesthetized with 7% chloral hydrate (0.7 g/kg, i.p.) and then rapidly decapi-

tated. DRG segments were quickly dissected out from the inner side of each half of vertebrae and cut in half in a Petri dish at 4 °C in oxygenated Dulbecco's Modified Eagle's Medium (DMEM) solution. After removing the attached nerves and surrounding connective tissues, the ganglia were incubated at 37 °C for 40 min in 1 mg/mL collagenase (type IA) and 0.4 mg/mL trypsin (type I). After 3 times washes in standard external solution, cells were triturated with a fire-polished Pasteur pipette and plated on glass coverslips, incubated in standard external solution at room temperature for 40 min and then stored at 4 °C. Detailed procedures could be obtained from the previous reports^[4,16].

2.3 Patch-clamp recordings Whole-cell patch-clamp recordings were conducted at room temperature (20–22 °C) with an EPC10 amplifier and Pulse software (HEKA, Electronik, Germany). Data were analyzed by Igor software (Configuration Metrics, Inc., OR, USA). Patch electrodes fabricated with PB-7 Puller (Narishige, Tokyo, Japan) had resistances of 2–5 M Ω . The internal solution contained (in mmol/L) 150 KCl, 1 MgCl₂, 10 HEPES, and 4 Mg-ATP, adjusted to pH 7.4 with KOH. After cancelling capacitance transient, series of resistance was compensated (> 80%), and leak current was subtracted digitally. The liquid junction potential (approximately -10 mV) between the pipette solution and the bath solution was corrected. Data were low-pass filtered at 10 kHz and obtained only from small- and medium-sized (10–40 μm in diameter) neurons with resting membrane potentials < -50 mV and AP overshoot above 0 mV. Details of the procedures could be obtained from the previous reports^[4,16].

To study the effects of a given drug on the MEL-evoked inward current, an experimental model with 3 applications of MEL (MEL1, MEL2+Drug, MEL3) (or paraformaldehyde) was set, according to the previous reports^[4,16]. The protocol of repeatedly topical applications of an algogen on a DRG cell *in vitro* had been demonstrated to be stable and effective in searching for MEL-sensitive DRG cells and testing the effects of a drug on a cell with MEL activity. Briefly, MEL was first applied to the freshly dissociated DRG cells, followed by MEL plus each of the 3 doses of SKF-96365 (1, 5, 10 $\mu\text{mol/L}$). After washing

out MEL and SKF-96365, the third application of MEL was given to the same cell. The inward current amplitude by different doses of SKF-96365 was calculated as the percentage to that of the first MEL application.

2.4 Measurement of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) Calcium imaging was performed using an upright Olympus IX81 microscope with Olympus FluoView ver.1.7a software (Olympus, Tokyo, Japan). $[\text{Ca}^{2+}]_i$ was represented by the Fluo-3 fluorescence intensity. The acutely isolated DRG neurons were incubated for 40 min at 20–22 °C in the Extracellular Solution (ES) containing Fluo3-AM (3 $\mu\text{mol/L}$; Sigma, St. Louis, MO, USA), followed by 3 times washout with ES. The xyt scan mode was selected to record the changes in fluorescence intensity (temporal resolution of 5 s per scan). Images were collected at 2 Hz with excitation at 488 nm and emission at 530 nm. Data were presented as Fluo-3 fluorescence intensity increase ratio: $R = \Delta F/F_0$, where $\Delta F = F - F_0$. F and F_0 were the maximum and the minimum fluorescence values, respectively. Details of the procedures could be obtained from the previous reports^[4, 16].

To study the effects of a given drug on the MEL-evoked $[\text{Ca}^{2+}]_i$ rise, an experimental model with 2 applications of MEL (MEL1, MEL2+Drug) (or paraformaldehyde) was set, according to the previous reports^[4, 16]. The protocol had also been demonstrated to be stable and effective in searching for MEL-sensitive DRG cells and testing the effects of a drug on a cell with MEL activity using Ca^{2+} imaging test.

2.5 Drug administration MEL stock solution was 2 mmol/L in water. Stock solutions were stored at -20 °C and diluted to final concentrations prior to use in standard external solution, which contained (in mmol/L): 150 NaCl, 5 KCl, 1 MgCl_2 , 2.5 CaCl_2 , 10 HEPES and 10 glucose (pH 7.4). SKF-96365, (1- $\{\beta$ -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenyl}-1*H*-imidazole hydrochloride) (Sigma, St. Louis, MO, USA) was dissolved in 0.9% sterile saline. Test solutions were topically applied to DRG neurons using the DAD-VC Voltage Command Valve Control System (ALA Science, NY, USA), which can quickly change the local solution around individual cells.

2.6 Data analysis All results were expressed as mean $\pm$ SEM. Data were processed by using ANOVA and individual *post hoc* analysis (Fisher's PLSD test) or Student's *t*-test for statistical analysis. $P < 0.05$ was considered as statistically significant.

3 Results

3.1 Suppressive effects of SKF-96365 on the MEL-evoked inward current The voltage clamp recordings at a holding potential of -70 mV revealed that 2 $\mu\text{mol/L}$ MEL could evoke large inward currents in 43.9% (40/91) of the DRG neurons with diameters ranging from 12.5–37.5 μm (Fig. 1A, B). As shown in Fig. 2, co-application of SKF-96365 and MEL reversed MEL-induced inward current in the DRG cells in a dose-related manner. The percentages of inward current amplitude by different doses of SKF-96365 were 66.7% (1 $\mu\text{mol/L}$, $n = 6$, $P < 0.05$), 90% (5 $\mu\text{mol/L}$, $n = 10$, $P < 0.0001$), and 83.3% (10 $\mu\text{mol/L}$, $n = 5$, $P < 0.001$), respectively.

3.2 Suppressive effects of SKF-96365 on the MEL-evoked $[\text{Ca}^{2+}]_i$ rise Using confocal calcium imaging, the effects of SKF-96365 on MEL-induced $[\text{Ca}^{2+}]_i$ increase were also evaluated. Of 210 freshly dissociated DRG cells, 69% were shown to be responsive to MEL with a transient increase in $[\text{Ca}^{2+}]_i$ (Fig. 1C, D). The averaged size of the MEL-sensitive cells with elevated $[\text{Ca}^{2+}]_i$ was larger than that recorded using patch-clamp, ranging mainly between 20–50 μm . Co-application of the highest dose of SKF-96365 (10 $\mu\text{mol/L}$) reversed the MEL-induced $[\text{Ca}^{2+}]_i$ rise of the DRG neurons (Fig. 3C). Among the MEL-sensitive cells ($n = 142$), 46.5% (66/142) were suppressed by SKF-96365 (Fig. 3A).

4 Discussion

4.1 MEL-induced activation of primary nociceptive sensory cells MEL has been demonstrated to be capable of producing both persistent spontaneous nociception and pain hypersensitivity when injected subcutaneously in the periphery. In a more recent report, MEL could activate 55%–65% DRG cells partially through activation of TRPV1 by PLA2-arachidonic acid-COX/LOX metabo-

Fig. 1 Melittin (MEL) induced an inward current and a rise in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in DRG cells. **A:** Whole cell recording of a DRG cell in response to $2 \mu\text{mol/L}$ MEL under voltage-clamp ($V_h = -70 \text{ mV}$). **B:** Distribution histogram of the cell body sizes for MEL-sensitive ($n = 40$) and MEL-insensitive ($n = 91$) DRG neurons by whole-cell patch-clamp. **C:** A subpopulation of acutely isolated rat DRG neurons were able to respond to MEL ($2 \mu\text{mol/L}$) with a $[\text{Ca}^{2+}]_i$ rise (grey vs black). **D:** Distribution histogram of the cell body sizes for MEL-sensitive ($n = 142$) and MEL-insensitive ($n = 210$) DRG neurons. MELs: MEL-sensitive. MELi: MEL-insensitive.

Fig. 2 Topical application of SKF-96365, a TRPC inhibitor, blocked melittin (MEL)-induced inward current in DRG cells. **A:** An example of patch clamp recording of inward current on a MEL-sensitive DRG cell that could be suppressed by co-application of $10 \mu\text{mol/L}$ of SKF-96365 with MEL (2 mol/L) ($V_h = -70 \text{ mV}$). **B:** Statistical analysis of inhibitory effects of 3 doses of SKF-96365 (1, 5 and $10 \mu\text{mol/L}$) on the MEL-induced inward current. * $P < 0.05$, ** $P < 0.01$, MEL + SKF vs MEL (first application).

Fig. 3 Topical application of SKF-96365, a TRPC inhibitor, blocked melittin (MEL)-induced rise in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). **A:** The distribution histogram of the cell body sizes for SKF-96365 (SKF) effectiveness ($n = 66$) and SKF ineffectiveness ($n = 76$) on MEL-sensitive DRG neurons. SKFi: SKF-96365 ineffective. SKFe: SKF-96365 effective. **B:** Repeated application of MEL evoked $[\text{Ca}^{2+}]_i$ rises measured by calcium imaging. **C:** Suppressive effects of SKF-96365 (10 $\mu\text{mol/L}$) on MEL-induced $[\text{Ca}^{2+}]_i$ rise.

lites^[4]. The results suggest that the MEL-sensitive DRG cells are nociceptive in electrophysiological characteristics. Similarly, in the present study, the same treatment of DRG cells with MEL could also evoke $[\text{Ca}^{2+}]_i$ rise in 69% cells and inward current in 43.9% cells tested. The percentage of MEL-evoked $[\text{Ca}^{2+}]_i$ rise was larger than that for the inward current induced in the DRG cells. This can be attributed to the different techniques and experimental protocols used. For Ca^{2+} imaging, MEL was applied onto many DRG neurons simultaneously in a dish each time, while whole-cell patch-clamp recording could only be performed on single DRG cells each time. Thus experimental bias was clearly present in the patch-clamp recordings. This is why 2 techniques were used in the current experiment.

The MEL-sensitive cells are likely to be nociceptive due to the following lines of evidence. First, the MEL-induced persistent spontaneous nociception and primary heat but not mechanical hypersensitivity could be reversed by capsazepine, a TRPV1 antagonist^[6]. Second, the MEL-

induced pain-related behaviors and long-term firing of the dorsal horn pain-related neurons could be suppressed by peripheral administration of inhibitors of ERK, P38 mitogen-activated protein kinase (P38MAPK) and c-Jun N-terminal kinase (JNK)^[17,18]. Third, activation of P2X and P2Y receptors which are mostly co-localized with isolectin B4 (IB4) was shown to be essential for the maintenance of MEL-induced primary thermal and mechanical hyperalgesia. Fourth, the MEL-evoked inward current, $[\text{Ca}^{2+}]_i$ rise and spike firing of the DRG cells could be suppressed by inhibitor of TRPV1, a thermal nociceptor in the periphery^[4]. Accordingly, the MEL-sensitive DRG cells may belong to a subpopulation of nociceptive primary sensory cells.

MEL has long been known to be a pore-forming peptide that may lead to Na^+ and Ca^{2+} influx^[16]. However, this is not likely the case when MEL is applied onto the DRG cells because only 60% of the cells tested can be evoked to produce inward current and $[\text{Ca}^{2+}]_i$ rise by 2 $\mu\text{mol/L}$ concentration, by which both inward current and $[\text{Ca}^{2+}]_i$ rise could be stably

evoked by repetitive applications^[4]. Moreover, as discussed above, co-application of MEL with capsazepine, a competitive TRPV1 blocker in a previous report^[4], or with SKF-96365, an inhibitor of TRPC in the current report, could completely or partially suppress the MEL-evoked inward current and $[Ca^{2+}]_i$ rise. The reasons why complete suppression of the MEL-evoked inward current and/or $[Ca^{2+}]_i$ rise could be produced by SKF-96365 antagonizing against TRPC in the current study or by capsazepine antagonizing against TRPV1 in our previous study^[4] are not clear, but one possibility can be taken into account that TRPV1-expressing DRG cells do not contain TRPC. This is supported by a recent study of Elg *et al.*^[14] which demonstrated that TRPC3 mRNA, one of the most abundant subtype of TRPC family members in the DRG, is almost exclusively present in non-peptidergic IB4-positive small-sized neurons that are largely TRPV1-negative. Since TRPC3/6/7 subfamily has been shown to be directly activated by diacylglycerol (DAG) and its membrane permeable analogue oleylacylglycerol, it is proposed to be G-protein-coupled receptor (GPCR)-operated cation channel regulated by phospholipase C (PLC) metabolites^[19,20]. Current evidence has shown that TRPV1-mediated inward current evoked by MEL is resistant to PLC/PKC inhibitors^[4] and capsaicin-induced calcium transient is resistant to SKF-96365 which acts at TRPC^[19]. In contrast, inhibitors of PLA2-COX/LOX pathways and that of PKA have been shown to be effective in suppression of the MEL-evoked inward current^[4]. These findings demonstrate that DRG cells which are sensitive to both MEL and capsaicin distinguish from those expressing TRPC which are sensitive to MEL. Chen *et al.* have reported that s.c. administration of capsazepine which acts at TRPV1 in the periphery results in suppression of MEL-induced spontaneous persistent nociception and primary heat hyperalgesia but not primary mechanical hyperalgesia^[6]. However, SKF-96365, which acts at TRPC, is effective in suppression of MEL-induced primary mechanical hyperalgesia as well as spontaneous persistent nociception and primary heat hyperalgesia (Ding *et al.*, unpublished data). Taken together, these data suggest that MEL can selectively activate 2 separate subpopula-

tions of primary sensory neurons that may express TRPV1 and TRPC3/6/7, respectively.

4.2 Roles of TRPC channels in mediation of MEL-evoked activation of primary nociceptive cells

One major aim of the present study was to investigate a possible role of TRPC in mediation of the MEL-induced inward current and $[Ca^{2+}]_i$ rise, which are suggestive of activation of primary nociceptive sensory neurons. Similar to TRPV1, TRPC family is also a non-selective cation channel that is highly permeable to Ca^{2+} when being activated. Because TRPC1, TRPC3 and TRPC6 have been demonstrated to be the major subtypes localized in the rat lumbar DRG^[19], and SKF-96365 is believed to potently antagonize TRPC3/6/7^[20], it is suggested that MEL may act directly on TRPC3 and TRPC6, or activate TRPC3/6 via PLC-catalyzed metabolites (DAG-PKC or inositol 1,4,5-triphosphate) as suggested previously^[19,22]. The signaling pathways involved in the activation of TRPC by MEL are of particular interest and need further investigations. It has been recently reported that intraperitoneal injection of GsMTx-4, an inhibitor of TRPC1 and TRPC6, is effective in reducing mechanical hyperalgesia induced by carrageenan or sciatic nerve injury^[23]. Moreover, mRNA expression of TRPCs3, 4 and 5 has been shown to be decreased in the ipsilateral L4 DRG after nerve injury in a rat spared nerve injury model^[24], indicating that TRPC may be also involved in the development of neuropathic pain. More recently, down-regulation of TRPC1 and TRPC6 reverses hyperalgesia to mechanical and hypotonic stimuli^[25]. All these studies indicate that TRPC might be involved in the process of pain. As mentioned above, our behavioral study also revealed that s.c. administration of SKF-96365 reversed the MEL-induced persistent spontaneous nociception as well as primary heat and mechanical hyperalgesia (Ding *et al.*, unpublished data). Taken together, TRPC channels are likely to be involved in mediation of MEL-evoked activation of primary nociceptive cells and may contribute to the development of inflammatory pain and hypersensitivity.

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TRPC 通道阻断剂 SKF-96365 对蜜蜂毒肽诱发初级感觉细胞内向电流和胞内钙增高的作用

丁静¹, 肖勇², 吕丹¹, 杜意如¹, 崔秀玉¹, 陈军^{1,2}

¹首都医科大学疼痛生物医学研究所, 北京 100069

²第四军医大学唐都医院疼痛生物医学研究所, 西安 710038

摘要: **目的** 蜜蜂毒肽是蜜蜂粗毒中的主要物质。外周皮下注射蜜蜂毒肽可导致持续性自发痛和痛觉过敏。本研究旨在研究瞬时受体电势C (transient receptor potential canonical, TRPC)通道在蜜蜂毒肽诱致的初级感觉神经元活化中的介导作用。**方法** 运用全细胞膜片钳和激光共聚焦测钙技术, 检测TRPC通道抑制剂SKF-96365对蜜蜂毒肽诱致的急性分离大鼠背根神经节细胞胞内钙和内向电流升高的影响。**结果** 电压钳记录的91个背根神经节细胞中, 蜜蜂毒肽可诱发43.9% (40/91)的细胞产生内向电流, 而不同浓度的SKF-96365 (1, 5, 10 $\mu\text{mol/L}$) 均明显抑制了背根神经节细胞的内向电流, 且呈剂量相关性。应用激光共聚焦钙成像技术记录的210个背根神经节细胞中, 67.6% 的细胞对蜜蜂毒肽敏感, 产生胞内钙离子浓度的升高, 而SKF-96365能抑制这种胞内钙浓度的升高, 抑制率为46.5%。**结论** SKF-96365能够抑制蜜蜂毒肽引起的背根神经节中小神经元的活化, 提示TRPC通道介导了蜜蜂毒肽对初级感觉神经元的激活作用。

关键词: 蜜蜂毒肽; 瞬时受体电势C通道; 背根神经节; 膜片钳技术; 激光共聚焦测钙