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Melittin activates TRPV1 receptors in primary nociceptive sensory neurons via the phospholipase A2 cascade pathways

Yi-Ru Du [a,c,1], Yong Xiao [b,1], Zhuo-Min Lu [b], Jing Ding [a], Fang Xie [b], Han Fu [b], Yan Wang [b], Judith A. Strong [c], Jun-Ming Zhang [c,*], Jun Chen [a,b,*]

[a] Institute for Biomedical Sciences of Pain, Capital Medical University, Beijing 100069, PR China
[b] Institute for Biomedical Sciences of Pain and Institute for Functional Brain Disorders, Tangdu Hospital, The Fourth Military Medical University, Xi'an 710038, PR China
[c] Pain Research Center, Department of Anesthesiology, 231 Albert Sabin Way, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0531, USA

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ABSTRACT

Previous studies demonstrated that melittin, the main peptide in bee venom, could cause persistent spontaneous pain, primary heat and mechanical hyperalgesia, and enhance the excitability of spinal nociceptive neurons. However, the underlying mechanism of melittin-induced cutaneous hypersensitivity is unknown. Effects of melittin applied topically to acutely dissociated rat dorsal root ganglion neurons were studied using whole-cell patch clamp and calcium imaging techniques. Melittin induced intracellular calcium increases in 60% of small (<25 μm) and medium (<40 μm) diameter sensory neurons. In current clamp, topical application of melittin evoked long-lasting firing in 55% of small and medium-sized neurons tested. In voltage clamp, melittin evoked inward currents in sensory neurons in a concentration-dependent manner. Repeated application of melittin caused increased amplitude of the inward currents. Most melittin-sensitive neurons were capsaicin-sensitive, and 65% were isolectin B4 positive. Capsazepine, the TRPV1 receptor inhibitor, completely abolished the melittin-induced inward currents and intracellular calcium transients. Inhibitions of signaling pathways showed that phospholipase A_2, but not phospholipase C, was involved in producing the melittin-induced inward currents. Inhibitors of cyclooxygenases (COX) and lipoxygenases (LOX), two key components of the arachidonic acid metabolism pathway, each partially suppressed the inward current evoked by melittin. Inhibitors of protein kinase A (PKA), but not of PKC, also abolished the melittin-induced inward currents. These results indicate that melittin can directly excite small and medium-sized sensory neurons at least in part by activating TRPV1 receptors via PLA2-COXs/LOXs cascade pathways.

1. Introduction

The bee venom test, as a tonic pain test in rats, was introduced in 1996 by Lariviere and Melzack [1]. Our group has demonstrated that subcutaneous injection of bee venom induced primary hyperalgesia to mechanical and heat stimuli, and secondary heat hyperalgesia, but not secondary mechanical hyperalgesia [2]. Bee venom is a complex mixture of polypeptides, enzymes, amines, lipids, and amino acids. with various biological, pharmacological, and toxicological activities [2]. Melittin is the principal polypeptide in bee venom [2]. Previously [3,4] we found that melittin played a central role in the production of nociceptive responses and cutaneous hypersensitivity after whole bee venom injection. In humans, intradermal injection of melittin induced spontaneous pain and increased skin temperature around the injection site, and subcutaneous injection of melittin induced primary hyperalgesia to mechanical and heat stimuli together with neurogenic inflammation [5,6].

Dorsal root ganglion (DRG) primary sensory neurons responding to noxious stimuli are generally characterized by broader action potentials (AP) with an inflection on the falling phase, larger overshoot, longer duration, and larger amplitude of afterhyperpolarization (AHP) [7]. Melittin-induced behavioral changes

Abbreviations: AP, action potential; COX, cyclooxygenase; DRG, dorsal root ganglion; IB4, isolectin B4; LOX, lipoxygenase; PKA, protein kinase A; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; TRPV1, transient receptor potential vanilloid receptor 1.

* Corresponding authors at: Pain Research Center, Department of Anesthesiology, University of Cincinnati College of Medicine, 231 Albert Sabin Way, P.O. Box 670531, Cincinnati, OH 45267-0531, USA. Fax: +513 558 0995 (J.-M. Zhang), Institute for Biomedical Sciences of Pain, Tangdu Hospital, The Fourth Military Medical University, #1 Xinsi Rd, Baqiao District, Xi'an 710038, China. Fax: +86 29 8477 7945 (J. Chen).

E-mail addresses: Jun-Ming.Zhang@uc.edu (J.-M. Zhang), junchen@fmmu.edu.cn (J. Chen).

[1] Equal contributors.

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and responses of spinal dorsal horn neurons [3,8] appeared to be mediated by capsaicin-sensitive primary afferent Aδ and C fibers [9–11] via mechanisms involving the transient receptor potential vanilloid receptor 1 (TRPV1) [4]. However, it is currently unknown if melittin can alter the excitability of sensory neurons.

The soma of DRG neurons are commonly used as models for their own peripheral terminals [12] which are inaccessible for adequate electrophysiological studies. Here we examined effects of acute topical application of melittin on the excitability of acutely dissociated DRG neurons. Since melittin is known as an in vitro activator of secreted phospholipase A_2 ($sPLA_2$) [13,14], we further examined the role of $sPLA_2$ in modulating melittin-induced neuronal responses.

2. Material and methods

2.1. Animals

The study protocol was approved by the institutional animal care and use committees of the University of Cincinnati, Capital Medical University (CCMU), and The Fourth Military Medical University (FMMU). The ethical guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) were followed. Experiments were performed on acutely dissociated DRG neurons from male Sprague–Dawley rats (Laboratory Animal Centers of CCMU, Beijing and FMMU, Xi'an, China or Harlan, Indianapolis, IN, USA) weighing 80–100 g. Animals had free access to water and food and were maintained at room temperature (22–24 °C) with a 12 h light/dark cycle.

2.2. Cell preparation

Animals were anesthetized with pentobarbital sodium and decapitated. L4–L6 DRG were dissected out and cut in half in a Petri dish at 4 °C in oxygenated DMEM solution. The ganglia were then incubated at 37 °C for 40 min in 1 mg/ml collagenase (type IA) and 0.4 mg/ml trypsin (type I). After three washes in standard external solution (see below), cells were triturated with a fire-polished Pasteur pipette and plated on glass cover slips coated with poly-D-lysine, incubated in standard external solution at room temperature for 0.5–1 h then stored at 4 °C. When used within 8 h, cells retained a healthy appearance, negative resting potentials and overshooting APs.

2.3. Electrophysiological recordings and calcium imaging

Recordings were made at room temperature (20–22 °C) with an EPC10 amplifier and Pulse software (HEKA, Electronik, Germany). Patch electrodes fabricated with PB-7 Puller (Narishige, Tokyo, Japan) had resistances of 2–4 MΩ. The internal solution contained (in mM): 150 KCl, 1 $MgCl_2$, 10 HEPES, and 4 Mg-ATP, adjusted to pH 7.4 with KOH. The capacitance transient was cancelled, series resistance was compensated (>80%), and leak current was subtracted digitally. The liquid junction potential (approximately −10 mV) was corrected. Data were low-pass filtered at 10 kHz and obtained only from small and medium-sized neurons (10–40 μm in diameter) with resting membrane potentials <−50 mV and AP overshoot above 0 mV. In some patch clamp experiments, isolectin B4 (IB4) conjugated to fluorescein isothiocyante (FITC) (1 μg/μl) was added to the culture medium for 30–60 min prior to recording to identify IB4-positive and IB4-negative neurons. Calcium imaging was performed using an upright Olympus IX81microscope with Olympus Fluociew ver.1.7a software (Olympus, Tokyo, Japan). Intracellular Ca^{2+} level ($[Ca^{2+}]i$) was represented by the Fluo-3 fluorescence intensity as described previously [15]. Briefly, cells were preloaded with media containing 5 μM Fluo-3/AM for 30 min at room temperature. Images were collected at 2 Hz with excitation at 488 nm and emission at 530 nm. Data are presented as Fluo-3 fluorescence intensity increase ratio: $R = \Delta F/F_0$, where $\Delta F = F - F_0$, and F *and* F_0 are the maximum and minimum fluorescence values, respectively.

2.4. Chemicals and solutions

Melittin was isolated and purified from whole bee venom using gel chromatography and reverse-phase high pressure liquid chromatography. Melittin stock solution was 2 mM in water. Capsaicin (8-methyl-N-vanillyl-6-nonenamide, Sigma) stock solution was10 mM in ethanol. Capsazepine, Antiflammin (Anti), U73122, Indomethacin (Indo), NDGA, H-89, and Bisindolylmaleimide (BIM) stock solutions (DMSO) were 5, 5, 2, 10, 10, 1, and 1 mM, respectively. All chemicals were purchased from Sigma–Aldrich, Inc., St. Louis, MO, USA. Stock solutions were stored at −20 °C and diluted to final concentrations prior to use in standard external solution, which contained (in mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES and 10 glucose (pH 7.4 adjusted with NaOH). Test solutions were topically applied to DRG neurons using the DAD-VC Voltage Command Valve Control System (ALA Science, New York, USA), which can quickly change the local solution around individual cells.

2.5. Statistical analysis

All results were expressed as mean ± S.E.M. Nonparametric statistics were used for data not normally distributed. The specific test used in each case is indicated in the figure legends or text. P value < 0.05 was considered to be statistically significant. For each cell, if the calcium ratio increased more than 0.20 (20%) above baseline after melittin or capsaicin application, it was counted as melittin-sensitive or capsaicin-sensitive, respectively. In patch clamp recording experiments, a cell was defined as melittin-sensitive if melittin-induced firing persisted more than 10 s in current clamp mode.

3. Results

3.1. Acute topical application of melittin excited DRG neurons

We first measured calcium responses in acutely dissociated DRG neurons. In most experiments, a melittin concentration of 2 μM was chosen based on literature review [16,17]. We found that topical application of melittin increased $[Ca^{2+}]i$ by at least 20% in 93 of 134 (69%) cells tested (Fig. 1A). In melittin-sensitive cells, the average response latency was 124 ± 57 (s) and the average $[Ca^{2+}]$ increase ratio was 1.5 ± 0.1.

Examples of DRG cells responding to melittin are given in Fig. 1A in which of nine cells tested, five responded to melittin, and 4 did not show any responses. The melittin-sensitive cells ranged from 20 to 35 μm in diameter, which is the size range for small and medium-sized cells (Fig. 1B).

Whole-cell patch clamp technique was used to measure the electrophysiological responses of DRG neurons after melittin application. In current clamp mode, 55% (56/102) of recorded DRG neurons responded to melittin. A typical response to a 50-s application of melittin (2 μM) is shown in Fig. 1C. The cell responded with a slow depolarization (average value, 18 ± 2 mV) and long lasting discharges. The amplitude of the APs decreased dramatically over time after melittin application, possibly caused by accumulation of inactivated sodium channels. The duration of melittin-induced firing ranged from 70 to 1200 s (Fig. 1D) and was most intense