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Research report

Extracellular signal-regulated kinases mediate melittin-induced hypersensitivity of spinal neurons to chemical and thermal but not mechanical stimuli

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ABSTRACT

Subcutaneous melittin injection causes central plasticity at the spinal level in wide-dynamic-range (WDR) neurons, which are hypersensitive to various nociceptive stimuli. Previous behavioral studies demonstrated that the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase 1/2(ERK1/2), p38 MAPK, and c-Jun N-terminal kinase are involved in both peripheral and spinal processing of melittin-induced nociception and hypersensitivity. Yet the functional roles of the three MAPKs vary among different stimulus modalities, and must be further studied at the cellular level *in vivo*. In this report, extracellular single unit recordings were performed to investigate whether activation of ERK1/2 in the primary injury site of melittin is essential to the establishment of a spinally sensitized state. Localized peripheral administration of a single dose of the MEK inhibitor U0126 (1 μg/10 μl) significantly suppressed neuronal hyper-responsiveness to thermal stimulus and chemical (melittin)-induced tonic firing of WDR neurons after full establishment of a spinally sensitized state. However, U0126 failed to affect mechanical hypersensitivity to both noxious and non-noxious stimuli. Melittin-induced enhancement of thermal hypersensitivity was also greatly inhibited by a single dose of capsazepine, a thermal nociceptor (TRPV1) blocker. These results suggest that activation of the ERK signaling pathway in the periphery is likely necessary for maintenance of a spinally sensitized state; activation of ERK1/2 in the primary injury site may regulate TRPV1, leading to dorsal horn hypersensitivity to thermal and chemical stimuli. ERK signaling pathways are not likely to be associated with melittin-induced dorsal horn hypersensitivity to mechanical stimuli.

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1. Introduction

Intraplantar (i.pl.) injection of whole honeybee venom, an experimental model of honeybee sting, produces long-term behavioral and neuronal changes along the spinal sensorimotor reflex circuitry [7,8]. The bee venom (BV) model is behaviorally characterized by an immediate persistent spontaneous pain-related paw flinching reflex lasting for more than 1 h, followed by 72–96 h of primary heat and mechanical hypersensitivity [7–9,16]. The BV model is also elecrophysiologically characterized by an immediate increase in spontaneous spike discharges lasting more than 1 h, followed by long-term enhancement of stimulus-evoked responsiveness of both sensory neurons in the dorsal horn of the spinal cord and single motor units involved in the spinal nociceptive withdrawal reflex [7,8,14,15,39,42]. BV-induced long-term behavioral and sensorimotor neuronal changes were demonstrated to be primarily dependent upon increased ongoing firing activity at the primary injury site of the periphery [7,8,15,29,31,38,40]. Moreover, activation or sensitization of the peripheral thermal nociceptor, transient receptor potential vanilloid receptor 1 (TRPV1) and peripheral *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors are also involved in the nociceptive processing described above, which requires capsaicin-sensitive primary afferent fibers [9,13,14,29,36,40]. We recently found that melittin, a 26-amino acid polypeptide comprising over 50% of the whole BV, plays a central role in the production of acute local inflammation, persistent spontaneous nociception, and hypersensitivity to heat and mechanical

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stimuli in both behavioral and electrophysiological surveys [13,28]. Melittin was a known *in vitro* activator of secreted phospholipase A_2 ($sPLA_2$) with no effect on cytosolic PLA_2 [17,23,37], and has been widely used as a tool to study the roles of $sPLA_2$ in modulating synaptic transmission, particularly glutamatergic transmission in the central nervous system [2,3,33]. Based upon the biological and pharmacological actions of melittin, we hypothesized that activation and sensitization of peripheral nociceptors (including TRPV1) by i.pl. injection of the peptide might involve several known or unknown signaling pathways responsible for induction and maintenance of central changes along the spinal sensorimotor neuronal circuitry, resulting in persistent spontaneous pain-related behaviors and hypersensitivity.

Extracellular signal-regulated kinases (ERKs) are members of the mitogen-activated protein kinases (MAPKs) family, and are activated by an upstream kinase, MAPK/ERK kinase (MEK) [6]. ERKs in the spinal cord dorsal horn have recently been revealed as one of the most important intracellular signaling pathways involved in the production and regulation of nociception and pain hypersensitivity [21,25,26,30,32]. We found that activation of three subfamilies of spinal MAPKs, including ERK1/2, p38 MAPK, and c-Jun N-terminal kinase (JNK), were involved in the processing of ongoing pain-related behaviors and heat hypersensitivity, but not mechanical hypersensitivity, induced by i.pl. injection of melittin and BV; this implicates differential roles of certain spinal MAPKs in various types of modality-associated pain hypersensitivity [5,43]. Moreover, compared with intrathecal administration, localized peripheral administration of ERK1/2, p38, and JNK inhibitors also effectively suppressed ongoing pain-related behaviors and heat hypersensitivity, but did not suppress mechanical hypersensitivity, in rats receiving i.pl. melittin injection. This indicates that peripheral MAPKs have similar mediating roles in processing various 'phenotypes' of BV-induced nociception and pain hypersensitivity in the spinal cord [22]. In support of this hypothesis, immunoreactivity assays demonstrated increased phosphorylation of ERK1/2 in nerve endings in rats receiving i.pl. capsaicin injection [18]. However, the roles of MAPKs in the primary injury site in the mediation of abnormal central neuronal activities remain in need of clarification.

Wide-dynamic-range (WDR) neurons in the deep layers (IV–VI) of the spinal cord dorsal horn are considered a 'central encoder' of spinal nociceptive withdrawal reflex circuitry [7,8,27,34,35,41,42]. Spinal dorsal horn WDR neurons are a major population that is persistently activated by both BV and melittin [7,14,15,28,38–41]. Moreover, the BV-induced long-term tonic firing of WDR neurons can be completely blocked by both peripheral sciatic nerve blockade and local administration of NMDA and non-NMDA receptor antagonists, as well as propofol [14,15,38,40]. In the present study, extracellular single unit recordings were performed in rats to determine whether activation of ERK1/2 in the primary injury site of melittin is involved in maintenance of the spinally processed hypersensitive state associated with chemical, thermal, and mechanical modalities.

2. Materials and methods

2.1. Animals

The described experiments were performed on male Sprague–Dawley albino rats (purchased from Laboratory Animal Center of Fourth Military Medical University, Xi'an) weighing 180–220 g. The animals had access to water and food *ad libitum*, and were maintained at room temperature (22–26 °C) with a light/dark cycle of 12 h. The ethical guidelines established for pain research in conscious animals by the International Association for the Study of Pain were followed [47]. The present study was also carried out in accordance with either the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) revised 1996, the UK Animals (Science Procedures) Act 1986 and associated guidelines, or the European Communities Council Directive of 24 November 1986(86/609/EEC).

2.2. Electrophysiological experiments

2.2.1. Animal preparation and surgical procedures

The animal preparation procedures for *in vivo* electrophysiological recordings have been previously described in detail [28,42]. Briefly, the rat was initially anesthetized with sodium pentobarbital (50 mg/kg, i.p.). A tracheal cannula and a left jugular vein catheter were inserted, and adequate anesthesia was confirmed intermittently during the experiment by examining whether the animal made spontaneous movements or had arousal responses to noxious pinch applied to the skin when the muscle relaxant wore off. Laminectomy was performed from T13 to L1 vertebrae to expose the lumbar enlargement for spinal neuron recording. A paraffin pool was made with ambient skin flaps around the exposed incision area of the lumbar spinal cord and filled with warm paraffin oil (37 °C) to prevent drying. Core body temperature was monitored through a thermistor probe inserted into the rectum, and maintained at 37.5 ± 0.5 °C by means of a feedback-controlled heating pad under the ventral surface of the abdomen.

2.2.2. Extracellular single unit recording

Electrophysiological responses of a single spinal WDR neuron with the cutaneous receptive field (cRF) located on the ipsilateral hind paw were recorded extracellularly using glass capillary microelectrodes (10–15 MΩ, filled with 0.5 M sodium acetate). The recording electrode was advanced in 2 μm steps using an electronically controlled microstepping manipulator. Electrical current pulse (Electric Stimulator, SEN-3301, Isolator, SS-202J, Nihon Kohden, Co., Ltd., Japan) at Aβ strength (100 μA, 50 μs, 1 Hz) was applied to the skin of the hind paw ipsilateral to the recording site as a search stimulus to identify neurons. The original signals were filtered (20 Hz–20 kHz) by AC/DC Differential Amplifier (A-M Systems, Inc. USA) and simultaneously sampled with PowerLab/8SP (AD Instruments, Australia) at 10 kHz. Chart 4.0 software (AD Instruments, Australia) was applied for recording and off-line analysis.

2.2.3. Characteristics of WDR neurons in response to thermal and mechanical stimuli

WDR neurons in response to heat and mechanical stimuli were identified based on our previous studies [15,28]. WDR neuronal responses are characterized by an intensity-dependent increase in response to both thermal and mechanical stimuli [15,28]. The responsiveness of a typical WDR neuron gradually increases with increased skin temperature (from 35 °C baseline up to 42, 45, 47 and 49 °C). Neuronal responses to mechanical stimuli were assessed by: (1) brush, performed by stroking on the center of the cRF at a frequency of 1–2 times/s with a hairy paint brush; (2) pressure, performed by picking up a skin fold with a flattened alligator clip to produce a consistent strength that was not painful when tested on the experimenter's skin; and (3) noxious pinch, performed by pinching a fold of skin with a small serrated clip to produce a consistent strength that was obviously painful when tested on the experimenter's skin. The forces of pressure and pinch stimuli applied to the cRF were uniform, although the precise strength of the stimulus was not available (for details see [42]).

2.3. Drug administration

A volume of 50 μl melittin solution (50 μg/50 μl, Sigma, St. Louis, MO, dissolved in 0.9% sterile saline) was used throughout the experiment [13,28]. A single dose (1 μg/10 μl) of the MEK inhibitor 1,4-diamino-2,3-dicyano-1,4-bis-[*o*-aminophenylmercapto] butadiene (U0126, Sigma, St. Louis, MO) was used in the current study, because it could selectively inhibit MEK1 and MEK2 [20], produce anti-nociception in behavioral tests [22], and prevent capsaicin-induced increases in pERK labeling in peripheral nerve terminals and fibers [18]. To investigate the effects of local U0126 administration on maintenance of either persistent spontaneous firing or hypersensitivity, U0126 or vehicle (30% dimethyl sulfoxide, DMSO) was subcutaneously injected into the center of cRF 5 min or 2–3 h after melittin injection. Capsazepine (0.3 mg/10 μl) or vehicle (DMSO) was also administered locally in order to investigate the possible roles of thermal nociceptor TRPV1 in melittin-enhanced responsiveness to thermal stimulation [13]. The systemic effects of capsazepine were ruled out by the negative effectiveness of U0126 on melittin-induced pain behaviors following local administration of the same dose into the contralateral hind paw [22]. Details of the experimental protocol can be referred to our previous report [45].

2.4. Data analysis

All results were expressed as mean ± S.E.M. The data between drug- and vehicle-treated groups were compared by ANOVA and individual *post hoc* analysis (Fisher's PLSD test). P value < 0.05 was considered to be statistically significant.

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