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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A B S T R A C T

Subcutaneous melittin injection causes central plasticity at the spinal level in wide-dynamic-range (WDR) neurons, which are hypersensitive to various noxious 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 spinal 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 spinal 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 spinal 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 electrophysiologically 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 neurons 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 \textit{in vitro} activator of secreted phospholipase A\textsubscript{2} (sPLA\textsubscript{2}) with no effect on cytosolic PLA\textsubscript{2} [17,23,37], and has been widely used as a tool to study the roles of sPLA\textsubscript{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.p.l. 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.p.l. 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.p.l. melitin 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.p.l. 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 12/12 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 \textit{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. Laminitectomy was performed from T13 to L1 vertebrae to expose the lumbar enlargement for spinal neuron recording. A parafin 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, Isolated, SS-2023). Nikohn Kohden, Co., Ltd., Japan) at 10Hz 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/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-diacymino-2,3-dicyano-1,4-bis-[oaminophenylmercapto]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 neurons [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 capsaizepine 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.
3. Results

A total of 29 WDR neurons were recorded from the spinal cord dorsal horn in 29 anesthetized rats (11 for persistent spontaneous firing, 13 for thermal and mechanical hypersensitivity, and the remaining 5 for TRPV1). The depth of WDR neurons studied was within the range of 268–1080 μm from the dorsal cord surface, and the mean distance from the dorsal cord surface was 667.63 ± 48.36 μm.

3.1. Effects of local U0126 administration on melittin-induced persistent spontaneous neuronal firing

Subcutaneous injection of melittin caused a tonic increase in firing lasting more than 30 min (Fig. 1A). Compared with DMSO (Fig. 1B), post-treatment with U0126 5 min after melittin resulted in a slow elimination of spike responses, consistent with behavioral results (Fig. 1C; see [22]). Local administration of U0126 produced statistically significant suppression of increased spikes during the time periods of 16–20, 21–25 and 26–30 min with inhibitory rates of 92.57%, 92.45%, and 82.54%, respectively (Fig. 1D).

3.2. Effects of local U0126 administration on melittin-induced hyper-responsiveness

As shown in upper panel of Fig. 2A and left panel of Fig. 2B, U0126 could significantly suppress thermal hyper-responsiveness of a typical WDR neuron to 49 °C hot water. Thermal response was enhanced up to about 3 fold over baseline by melittin. However, the enhanced response was decreased by about 50% 5 min after U0126 administration (Fig. 2B). To determine whether peripheral TRPV1 is involved in thermal hypersensitivity at the spinal cord dorsal horn, another 5 WDR cells were examined for response to local administration of capsazepine. It was demonstrated that melittin-enhanced thermal response could also be reversed by capsazepine (lower panel of Fig. 2A and right panel of Fig. 2B). Short time course observation revealed a time lag for response to U0126 versus capsazepine.

We further investigated the roles of peripheral ERK1/2 in generation of melittin-enhanced mechanical hyper-responsiveness to non-noxious (brush and pressure) and noxious (pinch) mechanical stimuli. A typical WDR neuron demonstrated a progressive increase in spike firing rate (63.96%, 112.27%, and 137.23%) to brush, pressure, and noxious pinch stimuli (Fig. 3). Melittin significantly enhanced mechanical responsiveness of a typical WDR neuron to both non-noxious and noxious stimuli, which were not affected by local U0126 administration (Fig. 3).

4. Discussion

4.1. Activation of ERK1/2 in the primary injury site and its roles in spinal neuronal hypersensitivity

It has been demonstrated that the spinally organized sensorimotor reflex circuitry plays a central role in mediating pain-related nocifensive behaviors, and along the circuitry the spinal cord dorsal horn WDR neurons are believed to act as the ‘central encoder’ of the spinally organized nociceptive reflex [34,35,42]. The BV test and the melittin test have been well established at both behavioral [9,13,16,28] and electrophysiological levels to study the interaction between sensory and motor components along the spinally organized sensorimotor reflex circuitry [28,38–42]. In consequence, i.pl. injection of both BV and melittin resulted in comparable long-lasting increases in ongoing neuronal firing, followed by enhanced responsiveness of both sensory (dorsal horn WDR neurons) and motor (single motor units of related muscles) components, leading to facilitation and sensitization of the spinally organized sensorimotor reflex circuitry [7,8,13,28].

In our previous studies, it was shown that facilitation and sensitization of spinally organized sensorimotor reflex circuitry is
Fig. 2. Effects of localized peripheral administration of U0126 on melittin-enhanced spinal neuronal hyper-responsiveness to noxious thermal stimuli. (A) Following immersion of rat hindpaw into 42, 45 and 49 °C water, the responsiveness of a representative WDR neuron gradually increased. After 90 min of melittin injection, responsiveness to noxious thermal stimulation was clearly enhanced. Local injection of both U0126 (1 μg/10 μl) and capsazepine (0.3 mg/10 μl) significantly inhibited neuronal spikes at different time points after drug administration. (B) Mean time course recording of spike responses. Time 1, 2, and 3 indicates 5, 10, and 15 min after U0126 and 3, 5, and 10 min after capsazepine, respectively. ***P < 0.001 vs. baseline. n.s. not significant, *P < 0.05, **P < 0.01, ***P < 0.001 vs. control. Error bars: ± S.E.M. n = 5 or 6 per group.

triggered and maintained by neuronal firing in primary sensory afferents. Such neuronal firing is mediated by both activation of TRPV1 thermal nociceptors [9,12,13] and involvement of glutamate NMDA and non-NMDA receptors, which may be activated by local release of glutamate through neurogenic inflammatory pain mechanisms [7,8,10,11,14,15,38,40]. Yue et al. [44] found that melittin could increase the spontaneous release of L-glutamate to rat substantia gelatinosa neurons by activating sPLA2 and increasing Ca2+ influx through voltage-gated Ca2+ channels in nerve terminals, providing indirect support for this hypothesis. In the current study, we have demonstrated that ERK1/2 in the primary injury site could also be activated by local melittin injection, and are likely to be involved in the pathophysiological processing associated with spinal hypersensitivity. However, ERK-involved peripheral processing specifically contributed to chemical and thermal hypersensitivity of the spinal dorsal horn WDR cells without demonstrating any observable role in mechanical hypersensitivity, suggesting a dissociation of the signaling pathways between chemical/thermal and mechanical response properties in the periphery. This strongly supports our hypothesis for differential signaling processing of various types of pain at the spinal cord [8]. The differential effects of U0126 on hyperalgesia across thermal and mechanical stimuli in this electrophysiological study were entirely consistent with our previous behavioral study [22], and implicate the existence of a cellular substrate that underlies behavioral expression.

The lack of efficacy of U0126 on melittin-induced mechanical hypersensitivity may be explained by the evidence that reveals the co-localization of ERK1/2-immunoreactivity in a subpopulation of primary sensory neurons that express the thermal nociceptor TRPV1 rather than mechanoreceptors [18]. The blocking effect of capsazepine, a selective TRPV1 blocker, on melittin-induced thermal, but not mechanical, hypersensitivity in both the present electrophysiological and previous behavioral studies highly suggests activation of thermal nociceptors by melittin [12,13]. The result also implies the existence of some receptors other than TRPV1 in the peripheral nerve endings of primary sensory cells, which can be activated by melittin to produce mechanical hypersensitivity. This is particularly intriguing and remains to be further studied in dorsal root ganglion cells by observing the actions of melittin on various ion channels. Nonetheless, several lines of evidence indicate an important role for the Gi/o protein–Ras–ERK1/2 pathway in the mediation of epinephrine- and prostaglandin E2-
induced mechanical hyperalgesia [19], which thereby suggests the existence of separate mechanisms underlying mechanical hypersensitivities induced by different chemicals. Taken together, the ERK1/2 signaling pathway in the primary injury site can be activated by local melitin injection, and such activation is likely to be involved in the peripheral processing of thermal nociceptor (TRPV1) activation, which contributes in turn to the maintenance of a spinal thermal hypersensitive state and behavioral thermal hypersensitivity to peripheral stimuli. Meanwhile, the ERK1/2 signaling pathway is not likely to be associated with melitin-induced dorsal horn hypersensitivity to mechanical stimuli.

4.2. Possible regulation of nociceptors by activation of the ERK1/2 signaling pathway

During tissue injury and inflammation, peripheral nociceptor sensitization may be associated with posttranslational regulation within a few minutes of the initial injury, or with transcriptional regulation that occurs for several hours post-injury (for reviews see [4,24]). Time course observation of UI0126 and capsazepine revealed a fast and short-lived effect on thermal hypersensitivity, indicating that contribution of peripheral ERK1/2 to melitin-induced heat hypersensitivity occurs via posttranslational regulation of thermonociceptors (e.g., TRPV1) [13]. Increases in ERK1/2 phosphorylation in small-sized TRPV1-expressing sensory neurons were localized and blocked by i.pl. administration of UI0126 [18], which further supports this hypothesis. Moreover, activation of the phosphatidylinositol-3-kinase pathway through TRPV1 regulation was reported to be ERK dependent [46]. Additionally, peripheral ERK1/2 are able to modulate the current potentiation of peripheral tetrodotoxin-resistant sodium channels (Nav1.8/1.9) [19]. Therefore, peripheral ERK1/2 activation may regulate multiple targets through posttranslational regulatory mechanisms.

In conclusion, activation of the ERK1/2 signaling pathway at the primary injury site is likely necessary for the maintenance of the spinal sensitized state caused by i.pl. injection of melitin, however, activation of ERK1/2 at the primary injury site contributes predominantly to the processing of chemically and thermally, but not mechanically associated hypersensitivity in the dorsal horn.

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