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T-type calcium channels, but not Cav3.2, in the peripheral sensory afferents are involved in acute itch in mice

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ABSTRACT

T-type calcium channels are prominently expressed in primary nociceptive fibers and well characterized in pain processes. Although itch and pain share many similarities including primary sensory fibers, the function of T-type calcium channels on acute itch has not been explored. We investigated whether T-type calcium channels expressed within primary sensory fibers of mouse skin, especially $Ca_v3.2$ subtype, involve in chloroquine-, endothelin-1- and histamine-evoked acute itch using pharmacological, neuronal imaging and behavioral analyses. We found that pre-locally blocking three subtypes of T-type calcium channels in the peripheral afferents of skins, yielded an inhibition in acute itch or pain behaviors, while selectively blocking the $Ca_v3.2$ channel in the skin peripheral afferents only inhibited acute pain but not acute itch. These results suggest that T-type $Ca_v3.1$ or $Ca_v3.3$, but not $Ca_v3.2$ channel, have an important role in acute itch processing, and their distinctive roles in modulating acute itch are worthy of further investigation.

1. Introduction

Low voltage-activated calcium channels, known as T-type calcium channels including $Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$ subtypes, are widely distributed in the peripheral and the central nervous systems, in which they tune neuronal excitability, pacemaker activity and neurotransmitter release [1,2]. Notably, they are predominantly expressed in pain pathways [3–5]. In the peripheral nervous system, T-type Ca^{2+} channels are prominent in primary sensory fibers of dorsal root ganglion (DRG) neurons, including nociceptive neurons [3–6], thus their roles in the peripheral pain processes were well recognized [6,7], including inflammatory [8,9], neuropathic [10–13] and visceral pain [12,14]. Among three subtypes, $Ca_v3.2$ channel is well investigated and mainly expressed in the primary sensory fibers and their neurons [3,4]. In addition, $Ca_v3.2$ channel contributes hydrogen sulfide (H_2S), now known as the third gasotransmitter involved in pain processing [15]. Very interestingly, a recent study identified H_2S as a new pruritogen [16]. It is very reasonable, as itch used to be regarded as a sub-modality of pain because they share many similarities [17]. First of all, itch-mediating fibers are small unmyelinated C fibers and lightly myelinated A fibers, responding not only to itch but also to pain stimuli [17]. The study suggested H_2S might be as a new type itch because H_2S-evoked itch is possibly mediated by A fibers [16], based on that itch is used to be categorized two distinct types: histamine-dependent (histaminergic) and histamine-independent (nonhistaminergic) itch [18], which both are mediated by unmyelinated C fibers and lightly myelinated Aδ fibers [17]. In contrast, a series of studies have demonstrated that $Ca_v3.2$ is mainly expressed in small- sized mouse DRG neurons (with C fibers and Aδ fibers) in the peripheral nervous system [3,4,19,20]. Anyway, the function of T-type calcium channels on typical histamine-dependent or histamine-independent itch has not been explored.

Here, we aim to investigate whether T-type calcium channels expressed within primary sensory fibers of mouse skin, especially $Ca_v3.2$ subtype, involve in two classical types of itch: chloroquine (CQ)- and endothelin (ET)-1-induced the histamine-independent itch and histamine-induced the histamine-dependent itch by pharmacological, neuronal imaging and behavioral analyses. Pharmacological blocking agents, mibefradil as a pan-T-type calcium channel blocker and Zn^{2+} as a special $Ca_v3.2$ channel blocker,

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were pre-treated at the local sites of skin in which the pruritogens were intradermally injected. We found that T-type $Ca_v3.2$ channel did not involve in the peripheral processing of CQ-, ET-1- or histamine-evoked itch.

2. Materials and methods

2.1. Animals

All experimental procedures were carried out in accordance with the guidelines of the International Association for the Study of Pain, and approved by the Institutional Animal Care and Use Committee at Xuzhou Medical University. Adult male C57BL/6 mice (≥8 weeks) were provided by Laboratory Animal Center of Xuzhou Medical University (Xuzhou, China), and housed by 2–5 mice/cage under a standard 12 h light/dark cycle at 22 ± 1 °C, with free access to water and chow.

2.2. Reagents

Histamine dihydrochloride (Lot number: WXBC3017V), Chloroquine disphosphate salt (Lot number: 085M4098V) and paraformaldehyde (Lot number: MKBR1837V) were purchased from Sigma (St. Louis., MO, USA). Zinc chloride (Lot number: B527BA0028) was purchased from Sangon Biotech (Shanghai, China). ET-1 (Lot number: 1160) and mibefradil dihydrochloride (Lot number: 116666-83-8) was purchased from Tocris Bioscience (Bristol, UK).

2.3. Acute itch behavior test

Neck model of itch was performed as described [16,21]. Briefly, mice were handled by experimenters for at least one week for habituating the procedure and shaved at the nape of the neck at least two days before the experiment. Then mice were individually placed in a plastic chambers (10 × 10 × 15 cm) on an elevated metal mesh for habituation at least 20 min. Mice were treated with intradermal injection of mibefradil (15 μg in 20 μl saline) or Zinc chloride (10 μM in 20 μl saline) or saline (20 μl) as vehicle control with 26 G needle into the nape of the neck. After 15 min, mice were elicited itch behavior with intradermal injection of histamine (500 μg in 50 μl saline), ET-1 (25 ng in 50 μl saline) or chloroquine (200 μg in 50 μl saline) into the same site of the nape and immediately returned their chambers for video recording for 45 min. Finally, the itch behavior from the videos was quantified by counting the scratch numbers in a blind manner.

2.4. Formalin test

Mice were given intraplantar injection of 20 μl of diluted formalin (2.5% in saline) to induce acute inflammatory pain, after pharmacological blocking agents were injected for 15 min. The spontaneous pain behavior was video-recorded and the time of licking and flinching was measured in a blinded manner in 5 min bins for 45 min after the formalin injection [22,23].

2.5. Immunohistochemistry

Immunofluorescence procedure was carried out as described previously [3,4,22]. Cervical (C)5~thoracic (T)2 or lumbar (L)3~L5 segmental spinal cords sample sections were blocked and then incubated overnight at 4 °C with rabbit antibodies against c-fos (catalog number: 226003, 1:1000, Synaptic System, Goettingen, Germany) and guinea pig against NeuN (catalog number: ABN90, 1:1000, Merck Millipore, Darmstadt, Germany) [3,4,24]. Sections were then incubated for 30 min at 37 °C with AF488- and Cy3-conjugated secondary antibodies (donkey, 1:500, Jackson Immuno-Research, West Grove, PA, USA). Fluorescence images were captured with a FV1000 confocal microscope (Olympus, Tokyo, Japan). For quantification, a total of c-Fos^+ neurons in the spinal dorsal horns were counted with Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA) [23].

2.6. Statistical analysis

All data are presented as means ± S.E.M. and were statistically analyzed with student's *t*-test for two-group, one-way ANOVA for multiple-group, or two-way ANOVA for time course comparison followed by a post hoc Bonferroni multiple comparison test with GraphPad Prism 5.0 (La Jolla, CA, USA) [22]. Data with $P < 0.05$ were considered as statistically significant differences.

3. Results

3.1. Blocking pan-T-type calcium channels in peripheral afferents reduces acute itch-evoked scratching

We firstly observed the effects of intradermal injection (i.d.) of mibefradil, a blocker of pan-T-type calcium channels, on acute histamine-dependent and histamine-independent itch. Local pretreatment with mibefradil into the nape of neck for 15 min significantly reduces i.d. of histamine (90.0 ± 12.7 *v.s.* 137.4 ± 12.3), CQ (129.3 ± 9.9 *v.s.* 220.3 ± 19.7) and ET-1 (124.8 ± 19.7 *v.s.* 281.6 ± 23.0)-evoked hind paw scratching-like behavior in mice, compared with vehicle control (Fig. 1A–C), suggesting that pan-blocking T-type calcium channels in peripheral afferents alleviates acute itch and T-type calcium channels in skin nerves play an important role in histamine-dependent and histamine-independent itch.

3.2. Selectively blocking $Ca_v3.2$ channel in peripheral afferents does not reduce acute itch-evoked scratching behavior

Next, we especially investigated whether $Ca_v3.2$ channel in skin nerves contributes to histamine-dependent and histamine-independent itch. The low concentration of Zn^{2+} (5–20 μM) serves as a special blocker of $Ca_v3.2$ channel. Fifteen minutes after $ZnCl_2$ (10 μM) was locally injected at the nape of neck, injection of histamine, CQ and ET-1 into the same sites induced robust itch-like behaviors (Fig. 2A–C). Unexpectedly, pre-treatment with Zn^{2+} did not reduce any itch behavior induced by histamine (137.1 ± 17.1 *v.s.* 111.7 ± 8.4), CQ (257.6 ± 24.6 *v.s.* 206.7 ± 8.4) or ET-1 (342.4 ± 64.6 *v.s.* 301.7 ± 32.2), compared with vehicle control (Fig. 2A–C), even led to a little increase of scratching (although there were not significant differences), indicating T-type $Ca_v3.2$ channel in skin nerves does not involve in histamine-induced histamine-dependent and ET-1- or CQ-induced histamine-independent itch.

3.3. Selectively blocking $Ca_v3.2$ channel in peripheral afferents relieves formalin-evoked pain behavior

We wanted to know whether the low concentration of Zn^{2+} by blocking $Ca_v3.2$ channel could reduce formalin-induced acute spontaneous pain in mice. So $ZnCl_2$ was locally injected into left hindpaws of mice for 15 min, and then 2.5% formalin was injected at the same sites. Surprisingly, local Zn^{2+} pretreatment alleviated not only the first phase (97.9 ± 10.1 *v.s.* 179.5 ± 19.5) but the second phase (143.7 ± 22.7 *v.s.* 351.9 ± 47.4) pain responses, compared with vehicle control (Fig. 3A and B). We further investigated the effect of mibefradil on formalin-evoked pain behavior and found that local

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