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PKM ζ is not required for development of postsurgical pain

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Abstract

Previous studies have shown that protein kinase M zeta (PKM ζ), a brain-specific isoform of protein kinase C, is involved in the central processing of nociception in several pain models by using a synthetic zeta inhibitory peptide. In the present study, we investigated whether PKM ζ contributes to the pathogenesis of postsurgical pain using both conditional and conventional PKM ζ knockout mice. Our results showed that the expression of PKM ζ in anterior cingulate cortex, but not spinal cord, of the conditional PKM ζ knockout mice was inhibited following tamoxifen injection. And the conditional PKM ζ knockout mice displayed similar plantar incision-produced postsurgical pain responses as those in wild-type mice. Moreover, the expression of PKM ζ was inhibited in both anterior cingulate cortex and spinal cord of the conventional PKM ζ knockout mice. And there were no significant differences in the development of postsurgical pain among wild-type, heterozygous and homozygous conventional PKM ζ knockout mice. These data suggest that PKM ζ is not required for the development of postsurgical pain after plantar incision.

Keywords

PKM ζ ; postsurgical pain; anterior cingulate cortex; spinal cord; plantar incision; synaptic plasticity

Introduction

It has been shown that protein kinase M zeta (PKM ζ), a brain-specific, autonomously active isozyme of protein kinase C, is necessary and sufficient for maintaining the late-phase of synaptic potentiation in hippocampal slices [1]. Many forms of long-term memory in a wide

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variety of neural circuits were also maintained by the persistent activity of PKM ζ in the central nervous system (CNS) [2–5]. However, most of previous studies supporting the involvement of PKM ζ in long-term potentiation (LTP) and long-term memory rely heavily on pharmacological inhibition of PKM ζ . By using genetic knockout (KO) mouse models, a recent study from Dr. Richard Huganir Laboratory at Johns Hopkins University School of Medicine demonstrates that PKM ζ is not required for hippocampal synaptic plasticity, learning and memory [6]. In that study, both conventional and conditional PKM ζ KO mice show normal synaptic transmission and LTP at hippocampal synapses, and have no deficits in several hippocampal-dependent learning and memory tasks [6]. Another study using PKC ζ /PKM ζ null mice has also shown that absence of PKM ζ does not impair learning and memory in mice [7]

It is widely believed that long-lasting changes (e.g., LTP) in central sensitization of the CNS serve as a neural basis of chronic pain development. Thus, it is interesting to know whether PKM ζ contributes to nociceptive plasticity and central mechanisms underlying pain transmission. Previous studies using pharmacological inhibition of PKM ζ have shown that PKM ζ may play a role in the central processing of nociception [8–15]. These studies employed a synthetic zeta inhibitory peptide (ZIP) to inhibit the activities of PKM ζ in the CNS, but it has been indicated that the effect of ZIP may be independent of PKM ζ , because ZIP still reverses LTP in PKM ζ KO mice [6, 7]. Therefore, it remains to be illuminated whether PKM ζ is involved in pain processing. To further clarify this issue, we used both conventional and conditional PKM ζ KO mice to investigate the effect of PKM ζ deletion on postsurgical pain in the present study.

Materials and Methods

Animals

Both conditional and conventional PKM ζ KO mice [6] with C57BL/6 background were obtained from Dr. Richard Huganir Laboratory at Johns Hopkins University School of Medicine. Male mice were used in this study and all animal procedures were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals, and were approved by Texas A&M University College of Dentistry Institutional Animal Care and Use Committee. All animals weighing 25–30 g were housed up to four per cage on a standard 12-h light/dark cycle, with water and food pellets available *ad libitum*. All efforts were made to minimize pain or discomfort and to reduce the number of animals used.

The conditional PKM ζ KO mice were generated by crossing PKM $\zeta^{fl/fl}$ mice with CaMKII-CreER^{T2} mice [6], which express Cre recombinase that becomes active only upon addition of tamoxifen driven by the CaMKII promoter. Thus, we induced PKM ζ KO by intraperitoneal injection of tamoxifen (Sigma, 2 mg per day for 5 days) dissolved in sunflower seed oil. Control mice were either PKM $\zeta^{fl/fl}$ mice injected with tamoxifen (Tamoxifen control) or PKM $\zeta^{fl/fl}$; CaMKII-CreER^{T2} mice injected with sunflower seed oil (Vehicle control). The conventional PKM ζ KO mice were generated by breeding to a CMV-Cre transgenic mouse line [6]. PCR amplification for genotyping of PKM ζ KO mice was performed as described previously [6].

Plantar incision

A plantar incision was made in the left hind paw according to previous studies [16, 17]. In brief, mice were anesthetized with 1.5–2% isoflurane. After antiseptic preparation of the left hindpaw, a 5-mm longitudinal incision was made through the skin and fascia of the plantar foot. The incision was started 2 mm from the proximal edge of the heel and extended toward the toes. The underlying muscle was elevated with a curved forceps, leaving the muscle origin and insertion intact.

Pain behavior testing

Mechanical and thermal pain hypersensitivities were measured as described in our previous studies [16, 18–20] at 1 day before incision surgery as baseline and 1, 3, 5, 7, 10, 15, 20, and 30 days after surgery. For mechanical pain testing, mice were placed on an elevated wire mesh floor and were covered with a clear Plexiglas chamber. After acclimation for 30 min, paw withdrawal responses to mechanical stimuli were determined using calibrated von Frey filaments (0.08, 0.15, 0.25, 0.41, 0.75, 1.2, and 2.0 g). Each monofilament was applied five times to the plantar side of the hind paws for approximately 1 s with a 10-s interval, starting with a force of 0.08 g and continuing in ascending order. A stimulus-related withdrawal was considered a positive response. The paw withdrawal threshold was calculated as the force at which the positive response occurred in three out of five stimuli. For thermal pain testing, mice were placed in a Plexiglas chamber on a glass plate under which a light box was located. A radiant heat stimulus was applied by aiming a beam of light through a hole in the light box to each hind paw through the glass plate. The light beam was turned off when the animal lifted the paw, allowing the measurement of time between start of the light beam and the paw lift. This time was defined as the paw withdrawal latency. The stimulus intensity was adjusted to give 10–12 s withdrawal latency in the normal mouse (baseline). A cutoff time of 20 s was used to avoid tissue damage to the hind paw. For all mice, both ipsilateral (incision) and contralateral (non-incision) hind paws were tested five times at 5-min intervals to obtain an average response latency for each hind paw. All pain behavioral tests were done by an investigator blinded to animal treatment groups.

Western blotting

The mice were sacrificed, and lumbar spinal cord and anterior cingulate cortex (ACC) tissues were harvested. PKM ζ expression in the spinal cord and ACC was analyzed with quantitative Western blotting. The primary antibody against PKM ζ (1:1000, anti-rabbit, Thermo Fisher Scientific) was used in our experiments. The intensity of Western blots was quantified with densitometry.

Statistical analysis

Data are expressed as mean $\pm$ SEM. Comparisons among groups were performed by one-way analysis of variance followed by the Student–Newman–Keuls method. Differences with $P < 0.05$ were considered statistically significant.

Results

Conditional PKM ζ KO mice showed specifically PKM ζ expression deficiency in the ACC after tamoxifen injection

Western blot analysis was used to assess the expression of PKM ζ protein in the lumbar spinal cord and ACC of different groups of mice. Tissues from control mice were harvested at 2 weeks after sunflower seed oil (Vehicle control) or tamoxifen (Tamoxifen control) injection. As shown in Fig. 1a, PKM ζ protein was expressed in both spinal cord and ACC of control and conditional PKM ζ KO mice. The expression of PKM ζ in the ACC significantly decreased at 2 weeks, but not 3 and 6 weeks, after tamoxifen injection compared to control groups (Fig. 1b, * $P < 0.05$ vs. control). In the spinal cord, no significant difference of PKM ζ protein expression was shown among different groups (Fig. 1c).

PKM ζ in the ACC is not required for pain processing following plantar incision

To investigate if PKM ζ protein in the ACC is involved in postsurgical pain, we prepared a plantar incision-induced postsurgical pain model in conditional PKM ζ KO mice at 2 weeks after tamoxifen injection. We found that plantar incision decreased paw withdrawal threshold and paw withdrawal latency in the ipsilateral hind paws of the conditional PKM ζ KO mice in a similar pattern as those in both tamoxifen and vehicle control mice (Fig. 2a,b). The time course of postsurgical pain in the conditional PKM ζ KO mice was not significantly different from that in the control mice (Fig. 2). In the contralateral hind paws, no mechanical and thermal pain behaviors were observed in all the mice (Data not shown).

Conventional PKM ζ KO mice showed PKM ζ protein deletion in both ACC and spinal cord

To further investigate the possible role of PKM ζ in pain processing, we used conventional PKM ζ KO mice in our study. We observed that PKM ζ protein was deleted completely in both ACC and spinal cord of the conventional PKM ζ KO (Homo) mice (Fig. 3). In the heterozygotes (Het) of the KO mice, PKM ζ was down-regulated in the ACC and spinal cord compared to wild-type (WT) mice (Fig. 3).

PKM ζ in the spinal cord is also not required for plantar incision-induced postsurgical pain

As shown above, PKM ζ protein deletion occurred in both ACC and spinal cord of the conventional PKM ζ KO mice. Our study using conditional PKM ζ KO mice has indicated that PKM ζ in the ACC is not required for pain processing following plantar incision. To further clarify if PKM ζ in the spinal cord is involved in postsurgical pain, we prepared a plantar incision-induced postsurgical pain model in conventional PKM ζ KO mice. We found that plantar incision decreased paw withdrawal threshold and paw withdrawal latency in the ipsilateral hind paws of WT, Het, and Home PKM ζ KO mice in a similar pattern (Fig. 4a,b). The time course of postsurgical pain in these mice was not significantly different (Fig. 4).

Discussion

Although pharmacological studies using PKM ζ inhibitors have shown that PKM ζ may contribute to the maintenance of LTP in the CNS [1–5, 21, 22], recent studies using transgenic mouse models demonstrate that PKM ζ is not required for learning and memory

[6, 7]. Thus, it remains uncertain whether PKM ζ plays a role in synaptic plasticity of the CNS. By using ZIP (a synthetic zeta inhibitory peptide), previous studies have shown that PKM ζ in both spinal cord and brain may be involved in the modulation of neuropathic and inflammatory pain [8–15, 23–25]. However, ZIP is not specific for inhibiting the activities of PKM ζ in the CNS and the inhibitor can still reverse LTP in PKM ζ KO mice [6, 7], indicating that the effect of ZIP is independent of PKM ζ inhibition.

In the present study, we employed the conditional and conventional PKM ζ KO mice [6] from Dr. Richard Huganir Laboratory. We investigated the effect of PKM ζ deficiency in the spinal cord and anterior cingulate cortex (ACC, a pain-related brain area) on plantar incision-induced postsurgical pain. By using conditional PKM ζ KO mice, we showed that the expression of PKM ζ in the ACC, but not spinal cord, is significantly decreased after tamoxifen induction. And the PKM ζ deficiency in the ACC of the conditional KO mice does not affect the development of postsurgical pain. We observed that the expression of PKM ζ in the ACC of the conditional KO mice returns to normal level after 3 weeks following tamoxifen injection. The recovery of PKM ζ expression could be related to the degradation of tamoxifen or potential mosaic recombination in the ACC. Moreover, by using conventional PKM ζ KO mice, we showed that the expression of PKM ζ in both ACC and spinal cord is completely deleted in the Homo mice and markedly decreased in the Het mice compared to that in WT mice. And the PKM ζ deletion in both ACC and spinal cord of the conventional KO mice also does not affect the development of postsurgical pain. It is possible that other PKC subtypes may compensate PKM ζ function in the conventional PKM ζ KO mice. Thus, we used both conditional and conventional PKM ζ KO mice to confirm each other in the present study. Taken together, our results suggest that PKM ζ in both ACC and spinal cord is not required for the pathogenesis of postsurgical pain.

The recent study using the same conditional and conventional PKM ζ KO mice as described in the present study supports our findings by showing that PKM ζ is not required for synaptic plasticity, learning and memory [6], because central sensitization (a type of synaptic plasticity) of the CNS plays a critical role in pain transmission and chronification. Regarding the role of PKM ζ in pain processing, we found that PKM ζ in both ACC and spinal cord is not necessary during the development of postsurgical pain, but previous studies using a synthetic zeta inhibitory peptide ZIP show that PKM ζ in the CNS contributes to pain mechanisms in different animal models [8–15, 23–25]. The controversy between our findings and these previous pain studies using ZIP is because ZIP is not a specific inhibitor for PKM ζ and it may cause non-specific effects on pain transmission, which is independent of PKM ζ activities in the CNS. In our study, we used tamoxifen-induced knockdown and conventional knockout mouse models. These genetic approaches allow us to examine the specific role of PKM ζ in pain processing. Our results indicate that PKM ζ in both ACC and spinal cord is not involved in plantar incision-induced postsurgical pain. We may need to use our transgenic mouse models to clarify if PKM ζ in the CNS contributes to other types of pain in the future.

In conclusion, our findings in the present study demonstrate that PKM ζ is not required for development of postsurgical pain, which is consistent with recent studies on the role of PKM ζ in learning and memory [6, 7]. Those studies show that absence of PKM ζ does not

impair learning and memory in mice, and that ZIP can reverse LTP and erase memory even when PKM ζ is not present [6, 7], suggesting that PKM ζ is not required for synaptic plasticity in the CNS and the effect of ZIP is independent of PKM ζ . Therefore, genetic approach-based transgenic models should be used to investigate the specific role of PKM ζ in different types of pain.

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Figure 1. Decreased PKMζ expression in the ACC of conditional PKMζ KO mice after tamoxifen injection

a Western blot analysis showed that PKMζ protein was expressed in both spinal cord and ACC of control and conditional PKMζ KO mice. The expression of PKMζ in the ACC, but not spinal cord, significantly decreased at 2 weeks after tamoxifen injection compared to control groups. At 3 and 6 weeks after tamoxifen, no significant change in PKMζ expression was observed in the conditional PKMζ KO mice. β-actin served as a loading control. The data shown are representative of three independent experiments. **b** Statistical analysis of the PKMζ expression in the ACC. $n = 3$ for each group; $*P < 0.05$ vs. control groups. **c** Statistical analysis of the PKMζ expression in the spinal cord. $n = 3$ for each group. There was no significant difference among different groups.

Figure 2. Tamoxifen-induced PKM ζ deficiency in the ACC has no effect on postsurgical pain in conditional PKM ζ KO mice

a Mechanical testing: Plantar incision decreased paw withdrawal threshold in the ipsilateral hind paws of the conditional PKM ζ KO mice with tamoxifen injection in a similar pattern as those in both tamoxifen and vehicle control mice. The time course of postsurgical mechanical pain in the conditional PKM ζ KO mice was not significantly different from that in the control mice. **b** Thermal testing: Plantar incision decreased paw withdrawal latency in the ipsilateral hind paws of the conditional PKM ζ KO mice with tamoxifen injection in a similar pattern as those in both tamoxifen and vehicle control mice. The time course of postsurgical thermal pain in the conditional PKM ζ KO mice was not significantly different from that in the control mice.

Figure 3. Conventional PKMζ KO mice display PKMζ protein deletion in both ACC and spinal cord

Note that PKMζ protein was deleted completely in both ACC and spinal cord of the conventional PKMζ KO (Homo) mice. In the heterozygotes (Het) of the KO mice, PKMζ was down-regulated in the ACC and spinal cord compared to wild-type (WT) mice. β-actin served as a loading control. The data shown are representative of three independent experiments.

Figure 4. Conventional PKM ζ KO mice show similar postsurgical pain as that in WT mice
a Mechanical testing: Plantar incision decreased paw withdrawal threshold in the ipsilateral hind paws of WT, Het, and Homo PKM ζ KO mice in a similar pattern. The time course of postsurgical mechanical pain in these mice was not significantly different. **b** Thermal testing: Plantar incision decreased paw withdrawal latency in the ipsilateral hind paws of WT, Het, and Homo PKM ζ KO mice in a similar pattern. The time course of postsurgical thermal pain in these mice was not significantly different.