Phospholipase C-related but catalytically inactive protein modulates pain behavior in a neuropathic pain model in mice

Tomoya Kitayama1, Katsuya Morita1, Rizia Sultana1, Nami Kikushige1, Keisuke Mgit2, Shinya Ueno2, Masato Hirata3 and Takashi Kanematsu1*

Abstract

Background: An inositol 1,4,5-trisphosphate binding protein, comprising 2 isoforms termed PRIP-1 and PRIP-2, was identified as a novel modulator for GABA_A receptor trafficking. It has been reported that naive PRIP-1 knockout mice have hyperalgesic responses.

Findings: To determine the involvement of PRIP in pain sensation, a hind paw withdrawal test was performed before and after partial sciatic nerve ligation (PSNL) in PRIP-1 and PRIP-2 double knockout (DKO) mice. We found that naive DKO mice exhibited normal pain sensitivity. However, DKO mice that underwent PSNL surgery showed increased ipsilateral paw withdrawal threshold. To further investigate the inverse phenotype in PRIP-1 KO and DKO mice, we produced mice with specific siRNA-mediated knockdown of PRIPs in the spinal cord. Consistent with the phenotypes of KO mice, PRIP-1 knockdown mice showed allodynia, while PRIP double knockdown (DKD) mice with PSNL showed decreased pain-related behavior. This indicates that reduced expression of both PRIPs in the spinal cord induces resistance towards a painful sensation. GABA_A receptor subunit expression pattern was similar between PRIP-1 KO and DKO spinal cord, while expression of K^+-Cl^-cotransporter-2 (KCC2), which controls the balance of neuronal excitation and inhibition, was significantly upregulated in DKO mice. Furthermore, in the DKD PSNL model, an inhibitor-induced KCC2 inhibition exhibited an altered phenotype from painless to painful sensations.

Conclusions: Suppressed expression of PRIPs induces an elevated expression of KCC2 in the spinal cord, resulting in inhibition of nociception and amelioration of neuropathic pain in DKO mice.

Keywords: KCC2, GABA_A Receptor, Neuropathic pain, Partial sciatic nerve ligation, PRIP

Findings

Background
We identified a D-myo-inositol 1,4,5-trisphosphate-binding protein with a domain organization similar to phospholipase C-δ, but with no enzymatic activity. We therefore termed this protein phospholipase C-related but catalytically inactive protein (PRIP) [1-3]. PRIP exists in 2 subtypes and can bind to GABA_A receptor β subunits [4], GABA_A receptor associated protein [5], and protein phosphatase 1 and 2A [6,7].

Naive PRIP-1 knockout (KO) mice demonstrate a marked decrease in the withdrawal threshold in the von Frey hair test because of altered expression of GABA_A receptor subunit in their central nervous system [8]. In the present study, we investigated the role of PRIP-1 and PRIP-2 in pain sensation using PRIP-1 and PRIP-2 double knockout (DKO) mice, and PRIP-1 and/or PRIP-2 knockdown (KD) mice.

Materials and methods

Animals
Ten- to fourteen-week-old male PRIP-1 KO [5,8] and DKO [7,9] mice, in a C57BL/6) mouse background, and ddY mice were used. All procedures and handling of...
animals were performed with permission according to the guidelines of Hiroshima University.

**Seltzer model and paw withdrawal threshold test**
Partial sciatic nerve ligation (PSNL) was performed according to the procedure described by Seltzer et al. [10]. A paw withdrawal threshold in response to probing with von Frey hair (gram weight to buckling) was measured.

**Generation of PRIP knockdown mice by intrathecal injection with siRNA**
Three siRNA target sequences for each PRIP-1 and PRIP-2 gene were designed using a manufacturer-provided software (see Table 1). Synthetic siRNAs (0.45 pmol [0.15 pmol for each]/5 μl/animal, purchased from iGENE, Therapeutics Inc., Tokyo, Japan) were injected into the subarachnoid space between L5 and L6 vertebrae of mice using hemagglutinating virus of Japan envelope (HVJ-E) vector system (GenomeONE; Ishihara Sangyo Kaisha, Ltd., Osaka, Japan) [11].

**Immunoblot analysis**
The region of L5 and L6 vertebrae, into which siRNAs were injected, was homogenized at 3 days postinjection with a homogenization buffer, and whole-cell fractions (for PRIPs, K⁺-Cl⁻ cotransporter-2 [KCC2], Na⁺-K⁺-Cl⁻ cotransporter-1 [NKCC1], glycine receptor [GlyR] α1, and tubulin) or cell membrane fractions (for GABAA receptor subunits) were obtained [12]. The homogenates were subjected to SDS-PAGE followed by immunoblotting using specific primary antibodies of interest. Antibodies used are as follows: anti-PRIP-1 antibody [5], anti-PRIP-2 antibody [13], anti-β tubulin (Thermo Scientific, CA), anti-GABAA receptor α1 subunit, anti-NKCC1 (Alpha Diagnostic International, TX), anti-GABAA receptor α2 subunit (Aviva Systems Biology, CA), anti-GABAA receptor α5 subunit (R&B Systems, MN), anti-GABAA receptor α6 subunit (Imgenex, CA), anti-GABA A receptor α4, β2/3, and γ2 subunit, anti-GlyRa1 (Merck Millipore, MA), anti-KCC2 (Santa Cruz Biotechnology, CA), and anti-phosphoserine (Acris antibodies, CA) antibodies. An enhanced chemiluminescence western detection system (Nacalai Tesque Inc., Kyoto, Japan) was used for development (ImageQuant™ LAS 4000 mini detection system; GE Healthcare Japan).

**Statistical analyses**
The density of each band was analyzed using NIH ImageJ software, and the densitometric units were corrected for tubulin. The data were expressed as the mean ± S.E.M. Statistical analyses are described in the figure legends.

**Results and discussion**
To examine pain-related behavior in DKO mice, PSNL was performed, and the withdrawal threshold of the hind paw was measured by applying von Frey filaments. Naïve DKO mice had normal sensation levels in terms of withdrawal threshold (Figure 1A). This differed greatly from the significant reduction in the withdrawal threshold observed in PRIP-1 KO mice [8]. After PSNL, the withdrawal threshold in the contralateral hind paw of DKO mice was not significantly different from presurgical baselines.

![Figure 1 Pain-related behavior in WT and DKO mice.](image)
PRIP is a modulator for GABA_A receptor intracellular trafficking [7,9,17]. The β 2/3 subunit is upregulated, and the γ2 subunit is downregulated in the spinal cord of PRIP-1 KO or DKO mice [8,18]. Therefore, we examined the expression levels of GABA_A receptor subunits by immunoblotting using commercially available subunit-specific antibodies. The examined expressions were similar between the genotypes, with the exception of α5 expression, which was increased in DKO mice (Figure 3A). Knabel et al. reported that α2 and α3 contribute to diazepam-induced antihyperalgesia actions, but that α1 and α5 subunits do not [19], suggesting less involvement of spinal α5 subunit-containing GABA_A receptors in nociception [16]. Therefore, the different pain sensation between PRIP-1 KO and DKO mice is probably not due to the alteration of GABA_A receptor expression in the spinal cord.

Inhibitory signaling is regulated by the intracellular chloride ion concentration, which is established in part via KCC2. Therefore, a high level of KCC2 expression drives chloride extrusion from neurons and maintains a low intracellular chloride ion concentration, i.e., GABAergic input may even acquire a net cell inhibitory response [20].

Next, we observed the influence of suppression of the PRIP gene on pain sensation by using a PSNL model. PSNL was performed on ddY mice 10 days before intrathecal siRNA injection, after which an allodynia score of contralateral and ipsilateral sides was analyzed during the 8 days after the injection of PRIP siRNA. In the contralateral paw, PRIP-1 KD mice showed an allodynia in accordance with PRIP-1 fluctuation 2–5 days after siRNA injection (initial score, 1.17 ± 0.09 at day 0; peak score, 0.46 ± 0.117 at day 2; and recovered score, 1.22 ± 0.08 at day 8) (Figure 2D). The PRIP-1 protein expression was analyzed by immunoblotting (data not shown). The allodynia observed in PRIP-1 KD mice was not seen in PRIP-2 KD, DKD, and other control mice (Figure 2D). However, the withdrawal threshold for the ipsilateral paw was dramatically increased in DKD mice (initial score, 0.104 ± 0.02 at day 0 and peak score, 0.628 ± 0.068 at day 3), but not other experimental mice, including PRIP-1 KD and PRIP-2 KD mice; the relief gradually reverted to painful levels within 7 days (Figure 2E). This suggested that suppression of both PRIP genes, but not either, induces resistance for pain sensation associated with allodynia.

Neuropathic pain in a model animal induces an altered expression of GABA_A receptors, including the down-regulation of γ2 subunit-containing receptors [15,16]. PRIP is a modulator for GABA_A receptor intracellular trafficking [7,9,17]. The β 2/3 subunit is upregulated, and the γ2 subunit is downregulated in the spinal cord of PRIP-1 KO or DKO mice [8,18]. Therefore, we examined the expression levels of GABA_A receptor subunits by immunoblotting using commercially available subunit-specific antibodies. The examined expressions were similar between the genotypes, with the exception of α5 expression, which was increased in DKO mice (Figure 3A). Knabel et al. reported that α2 and α3 contribute to diazepam-induced antihyperalgesia actions, but that α1 and α5 subunits do not [19], suggesting less involvement of spinal α5 subunit-containing GABA_A receptors in nociception [16]. Therefore, the different pain sensation between PRIP-1 KO and DKO mice is probably not due to the alteration of GABA_A receptor expression in the spinal cord.
resulting in a high concentration of intracellular chloride ion and reduced nociceptive threshold [23]. In addition, upregulation of KCC2 induced inhibitory postsynaptic potentials [24], suggesting that high expression level of KCC2 observed with DKO spinal cord enhances inhibitory synaptic transmission. We then tested if inactivation of KCC2 by R-\(+\)-(dihydroindenyl)oxy] alkanolic acid (R-DIOA), an inhibitor of KCC2, affects pain sensitivity. The paw withdrawal threshold was decreased dose-dependently by intrathecal administration of R-DIOA (Figure 3D), indicating the importance of KCC2 activity. Therefore, similar KCC2 expression in the spinal cord of naive WT and DKO PSNL mice (Figure 3C) may cause the allodynia-resistant phenotype observed in the DKO PSNL model (Figure 1B).

**Figure 2** Involvement of PRIPs in the pain-related behavior. (A, B) Expression of PRIP-1 and PRIP-2 in the spinal cord of PRIP-1 KD, PRIP-2 KD and DKD mice 3 days after intrathecal siRNA injection. Immunoblot analyses were conducted using anti-PRIP-1 (A) and anti-PRIP-2 (B) antibodies. HVJ-envelope (HVJ-E) or scrambled siRNA (SC) was used as a negative control. For each, a representative image is shown in the upper panel. The level of immunoreactivity was normalized to β-tubulin and represented as % induction compared with the values of untreated mice (control) (means ± S.E.M., n = 4). *P < 0.05, †P < 0.005, ‡P < 0.05, §P < 0.05 and ¶P < 0.05 compared with the corresponding values in untreated, SC, HVJ-E, PRIP-1 KD, and PRIP-2 KD mice, respectively (Tukey-Kramer test). (C) Influence on pain sensitivity by the suppression of the PRIP gene using intrathecal siRNA injection in mice. Paw withdrawal threshold was measured the day before (open column) and 3 days after (closed column) injection. Values represent withdrawal threshold (mean ± S.E.M., n = 5–10). **P < 0.01 compared with the corresponding values from before injection (Student’s t-test). (D, E) Influence on pain sensitivity by the suppression of the PRIP gene in PSNL-operated mice. Paw withdrawal threshold of both contralateral (D) and ipsilateral (E) sides were measured each day after the intrathecal siRNA injection (day 0). The values before the PSNL surgery represent as “naive.” Lines used in graphs are as follows: green, violet, blue, black, and red are for PRIP-1 KD, PRIP-2 KD, DKD, HVJ-envelope, and scrambled siRNA-injected mice, respectively. Values represent withdrawal threshold (mean ± S.E.M., n = 7). *P < 0.05 compared with the corresponding values of untreated mice (day 0) (Dunnet test).
To further confirm the involvement of KCC2 in neuropathic pain regulated by PRIP, we performed a hind paw withdrawal test by using WT, PRIP-1 and PRIP-2 KD, and DKD mice with R-DIOA. Withdrawal thresholds were not changed in PSNL-operated WT and a single gene KD mice, and R-DIOA administration did not affect the pain threshold (Figure 3E). However, relief from pain (as indicated by the increase of the threshold) in the DKD PSNL model was significantly inhibited by the administration of R-DIOA 3 days postinjection (Figure 3E).

Conclusions
We demonstrated that the regular expression of KCC2 in DKO mice even after PSNL surgery induces the

Figure 3 Alteration of expression of GABAA receptor subunits and KCC2 in KO or KD mice. (A) Expression of GABA_A receptor subunits in PRIP-1 KO (open column) and DKO (close column) mice. Expression levels of PRIP-1 KO and DKO are based on the corresponding WT (represented as 100%, n=5). *P < 0.05, for values in DKO vs PRIP-1 KO mice (Student’s t-test). (B) Expression of KCC2 in PRIP-1 KO (open column) and DKO (close column) mice. Expression levels represent each corresponding WT value as 100%. *P < 0.05, for values in DKO vs PRIP-1 KO mice (Student’s t-test). (C) Expression of GlyRα1 subunit, NKCC1 and KCC2 in WT and DKO 1 day after PSNL or sham operation (sham). The level of immunoreactivity was normalized to β-tubulin (mean ± S.E.M., n=5–7). *P < 0.05, compared with values in WT sham-operated mice (Dunnet test). (D) R-DIOA administration induces pain relation behavior. Intrathecal administration of R-DIOA dose-dependently induced a decreased pain withdrawal threshold in WT mice. (E) Influence of KCC2 activity on pain sensitivity in PSNL mice. Each siRNA injection was performed 10 days after PSNL surgery. After 3 days of siRNA injection, R-DIOA (3 μg/mouse) was administrated intrathecally, and a paw withdrawal test was carried out 30 min after the injection. PSNL-operated WT mice were also treated with R-DIOA at 8 days after the surgery (representing as 8 in graph) and performed a paw withdrawal test. Naive represents prior to the surgery. Lines used in graph are as follows: black, green, violet, and blue are for WT, PRIP-1 KD, PRIP-2 KD, and DKD mice, respectively. The graph shows the withdrawal threshold value (mean ± S.E.M., n=8). *P < 0.05, for values in 30 min after injection vs before injection (Student’s t-test).
inhibition of nociceptive transmission and ameliorates PSNL-mediated neuropathic pain, even though the alteration of GABA_\text{A} receptor subunits in PRIP-1 KO mice causes allodynia [8]. The current findings led us to hypothesize that regulation of KCC2 expression is a critical modulator of pain sensation.

Abbreviations
DID: [dihydropyridine/loxo] alkaloidic acid; DKD: PRIP-1 and 2 gene double knockdown; DKO: PRIP-1 and 2 gene double homologous knockout; GABA: \gamma-aminobutyric acid; GlyR: Glycine receptor; KCC2: K^+Cl^-cotransporter-2; NKCC1: Na^+K^+Cl^-cotransporter-1; PSNL: Partial sciatic nerve ligation; PRIP: Phosphophilic C-related but catalytically inactive protein; PRIP-1 KO: PRIP-1 gene homologous knockout; WT: Wild type.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
TK carried out the paw withdrawal threshold test, immunoblotting, and data analyses. KM performed PSNL surgery and intrathecal siRNA injection. SR, NM, and KM participated in the data analyses. SJ and MH provided the knockout mice and participated in the design of the study. TK conceived of the study, participated in its design and coordination of the experiments, and wrote the manuscript. All authors read and approved the final manuscript.

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