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Sensitization of TRPV1 by protein kinase C in rats with mono-iodoacetate-induced joint pain

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SUMMARY

Objective: To assess the functional changes of Transient receptor potential vanilloid 1 (TRPV1) receptor and to clarify its mechanism in a rat mono-iodoacetate (MIA)-induced joint pain model (MIA rats), which has joint degeneration with cartilage loss similar to osteoarthritis.
Methods: Sensitization of TRPV1 in MIA rats was assessed by transient spontaneous pain behavior induced by capsaicin injection in knee joints and electrophysiological changes of dorsal root ganglion (DRG) neurons innervating knee joints in response to capsaicin. Mechanisms of TRPV1 sensitization were analyzed by a newly developed sandwich enzyme-linked immunosorbent assay that detects phosphorylated TRPV1, followed by functional and expression analyses of protein kinase C (PKC) *in vivo* and *in vitro*, which involves TRPV1 phosphorylation.
Results: Pain-related behavior induced by intra-articular injection of capsaicin was significantly increased in MIA rats compared with sham rats. In addition, capsaicin sensitivity, evaluated by capsaicin-induced inward currents, was significantly increased in DRG neurons of MIA rats. Protein levels of TRPV1 remained unchanged, but phosphorylated TRPV1 at Ser800 increased in DRG neurons of MIA rats. Phosphorylated-PKCε (p-PKCε) increased and co-localized with TRPV1 in DRG neurons of MIA rats. Capsaicin-induced pain-related behavior in MIA rats was inhibited by intra-articular pretreatment of the PKC inhibitor bisindolylmaleimide I. In addition, intra-articular injection of the PKC activator phorbol 12-myristate 13-acetate increased capsaicin-induced pain-related behavior in normal rats.
Conclusion: TRPV1 was sensitized at the knee joint and at DRG neurons of MIA rats through PKC activation. Thus, TRPV1 sensitization might be involved in chronic pain caused by osteoarthritis.

Introduction

Transient receptor potential vanilloid 1 (TRPV1) is a non-selective cation channel gated by endogenous ligands (noxious heat, protons, lipids) and exogenous ligands (capsaicin)[1,2]. It is highly expressed in unmyelinated C-fibers and activated in response to multiple noxious stimuli. Genetic and pharmacological approaches demonstrated TRPV1 is involved in the development of inflammatory pain[3–8]. However, under chronic pain conditions, the modulation of TRPV1 remains elusive. Osteoarthritis (OA) often develops knee hyperalgesia into chronic pain (OA pain). Recent

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studies have reported that the involvement of TRPV1 in OA pain, but the functional changes of TRPV1 under painful OA of the knee are poorly understood. TRPV1 expression was increased in the synovium of OA patients[9], and the Ile585Val TRPV1 variant was associated with reduced risk of symptomatic knee OA[10]. Furthermore, TRPV1 expression was increased in dorsal root ganglions (DRG) in rat OA-like pain models[11,12]. Thus, TRPV1 modulation might occur in OA pain. We investigated the functional change of TRPV1 in OA pain using a mono-iodoacetate (MIA) induced joint pain model (MIA rats). MIA inhibits glyceraldehyde-3-phosphate dehydrogenase and intra-articular injection of MIA induces chondrocyte death resulting in joint degeneration and cartilage loss with joint pain in rats, which resembles features of OA pain. MIA rats are commonly used to examine the effect of analgesics and mechanisms of OA pain[13–16]. The present study investigated functional changes of TRPV1 *in vivo* and *in vitro* in the context of MIA rats.

Materials and methods

Experimental animals

Experiments used male Sprague Dawley rats (Charles River Labs, Yokohama, Japan), weighing 250–350 g. Overall, 243 rats were used for behavioral and electrophysiological experiments. Sample size was determined from previous studies[17,18] to detect a statistical difference. Rats were housed in groups of three in plastic cages under controlled temperature and humidity, and provided free access to food and water under a 12/12 h reversed light–dark cycle (lighting at 8:00 AM). All procedures were approved by internal animal care and use committee of Shionogi Pharmaceutical Research Center (Osaka, Japan) instructed by Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) guidelines. The results of all studies were reported in accordance with the ARRIVE guidelines for reporting experiments involving animals[19,20].

Induction of MIA rats

MIA rats were established as previously described[21]. Briefly, 2 mg of MIA (Sigma–Aldrich, St. Louis, MO, USA) in 50 μL saline was injected into the articular cavity of the right leg knee joint using a Hamilton gas-tight syringe. Sham-operated rats received a saline injection. Left leg knee joints were untreated in all rats. All rats were anaesthetized with isoflurane during the procedure. Pain development was measured using the grip strength test on days 1, 3, 7, 11, 14, 18, 21, 25 and 28 after MIA or saline injection. Grip strength was measured using the animal grip strength system (San Diego Instruments, San Diego, CA, USA) as previously described[17]. Briefly, each rat was gently restrained and allowed to grasp the wire mesh frame with its hind limbs and was moved in a rostral-to-caudal direction until the grip released. Grip strength was averaged from two readings with a minimal interval of 5 min. Selection criteria for MIA rats were <950 grip strength (g)/body weight (kg) value. Overall, 147/172 rats fulfilled the criteria.

Assessment of capsaicin-induced pain-related behavior

To examine TRPV1 function in rat knee joints, capsaicin-induced pain-related behavior was evaluated on day 14 after MIA or saline injection. Rats were randomly allocated by body weight and grip strength. Behavioral tests were conducted during the light phase. Capsaicin (Sigma–Aldrich) was dissolved into 50 μL 10% ethanol in saline and injected into the articular cavity of the right leg knee joint. Response behavior was measured by duration of flinching and licking behavior for 10 min in a plexiglas cylinder (25 cm diameter, 30 cm high) after capsaicin injection by a blinded investigator. Bisindolylmaleimide I (PKC inhibitor, EMD Millipore, Billerica, MA, USA) and KT5720 (PKA inhibitor, EMD Millipore) were dissolved in 50 μL of 10% DMSO in saline and administered before capsaicin injection. Phorbol 12-myristate 13-acetate (PMA) (50 μL, Invivogen, San Diego, CA, USA) was administered before capsaicin injection. Drugs were administered into the knee joint cavity to examine the effect of those in knee joint. Rats were tested in order of allocated groups.

Isolation of dorsal root ganglia (DRG) neurons

Rats were decapitated under deep anesthesia, right-side L3 and L4 DRGs were isolated and incubated in low-sodium Ringer's solution (212.5 mM sucrose, 3 mM KCl, 1 mM NaH_2PO_4, 25 mM $NaHCO_3$, 11 mM D-glucose, 5 mM $MgCl_2$) containing 2 mg/mL collagenase (Yakult Pharmaceutical Industry Co., Ltd., Tokyo, Japan) for 1 h at 37°C and subsequently treated with 0.05% trypsin-EDTA for 5 min at room temperature. Trituration was gently applied to dissociate neurons from DRGs in culture medium containing Dulbecco's modified Eagle's medium (Sigma–Aldrich) with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 20 mM HEPES (Gibco, Carlsbad, CA, USA), 1% penicillin–streptomycin solution (Nacalai Tesque, Kyoto, Japan), and 4 mM L-glutamine (Nacalai Tesque). Following centrifugation at 1500× *g* for 5 min, DRG neurons were resuspended in culture medium containing 0.5 μg 2.5 S mouse nerve growth factor (Gibco), placed onto glass coverslips precoated with poly-L-lysine and laminin, and incubated at 37°C with 5% CO_2 overnight.

Electrophysiology

DRG neurons were back-labeled by injection of WGA-Alexa 488 into knee joints of rats on day 14 after MIA or saline injection. WGA-Alexa 488 (Invitrogen, W11261) was dissolved in 2% w/v phosphate-buffered saline (PBS) and injected into the knee joint 72 h before decapitation. External recording solutions (145 mM NaCl, 2.5 mM KCl, 2 mM $BaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 11 mM D-glucose) were adjusted to pH 7.4 with NaOH. Patch electrodes were fabricated from borosilicate glass capillaries using an electrode puller (P97; Sutter Instrument Co., Novato, CA, USA). Tip resistances of patch electrodes were 1.8–3.8 MΩ when filled with internal solution (135 mM CsCl, 10 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, 3 mM ATP-Mg, and 0.3 mM GTP-Tris adjusted to pH 7.2 with CsOH). In the whole-cell configuration, capsaicin-induced Ba^{2+} currents were recorded with an EPC-10 amplifier and PatchMaster software (HEKA, Freiburg, Germany), and digitized at 10 kHz with PowerLab and LabChart software (ADInstruments, Colorado Springs, CO, USA).

Capsaicin (0.03–30 μM) were cumulatively applied to DRG neurons until the current reached a plateau, and maximum Ba^{2+} currents were measured at each concentration. EC_{50} values of capsaicin-induced Ba^{2+} currents in each DRG neuron were calculated from the concentration response curve using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA). To evaluate the current density of capsaicin-induced Ba^{2+} currents, a high concentration of capsaicin (30 μM) was applied to DRG neurons, and maximum Ba^{2+} currents were measured. Currents were normalized by the membrane capacitance of each DRG neuron, and the current density of capsaicin-induced Ba^{2+} currents in each DRG neuron was expressed as pA/pF. All recordings were performed at room temperature.

Protein lysate preparations

DRG protein lysates were prepared by homogenizing tissues in T-PER Tissue Protein Extraction Reagent (Thermo Scientific Pierce Protein Biology Products, Rockford, IL, USA, containing a complete mini-EDTA-free tablet and PhosSTOP tablet; Roche, Indianapolis, IN, USA) and centrifuged at 10,000× *g* for 5 min at 4°C, and supernatants were collected. Protein concentrations were determined by BCA Protein Assay-Reducing Agent Compatible kit (Thermo Fisher Scientific, Yokohama, Japan).

Western blotting

Protein levels of TRPV1, phospho-PKCε, or PKCε in DRGs were measured by western blot analysis. Protein lysates were separated by 4–12% SDS-PAGE, transferred onto PVDF membranes that were then incubated in blocking buffer (0.1% Tween-20 in Tris-buffered saline containing Block Ace, DS Pharma Biomedical, Osaka, Japan) for 1 h, and incubated with primary antibodies (Table I, in blocking buffer) at 4°C overnight. Then they were incubated with peroxidase-labeled anti-rabbit IgG (for TRPV1, 111-035-003, 1:10,000 dilution; Jackson Immuno Research Laboratories, West Grove, PA, USA), peroxidase-labeled anti-mouse IgG (for β-actin, 115-035-174, 1:10,000 dilution; Jackson Immuno Research Laboratories) or peroxidase-labeled anti-goat IgG (for PKCε and p-PKCε, 705-035-003, 1:10,000 dilution; Jackson Immuno Research Laboratories) for 1 h at room temperature. Proteins were detected using ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences, Tokyo, Japan). Signal intensities of each protein band were assessed using Multi Gauge software (Fujifilm, Tokyo, Japan).

Phospho-TRPV1 ELISA

Amounts of phosphorylated TRPV1 in DRGs were measured by enzyme-linked immunosorbent assay (ELISA). Polyclonal antibodies against phosphorylated TRPV1 at Ser502 and Ser800 were raised in rabbits using KLH-conjugated LQRRP(pS)LKSLFVDSC and VPLLRDA(pS) TRDRHATQQEC as antigens, respectively, by Sigma–Aldrich. Briefly, two rabbits were used for each antigen, and antibody titers were measured at days 49 and 77 (1 day before sacrifice) after immunization. Sera from each rabbit were purified by positive affinity purification (using phosphorylated peptide), followed by negative affinity purification (using non-phosphorylated peptide). Antibody specificity was confirmed by titration using phosphorylated or non-phosphorylated peptide-coated plates. A 96-well MaxiSorp plate (Nunc/Thermo Fisher, Rochester, NY, USA) was coated with anti-phospho-TRPV1 antibody (each 5 μg/mL; phospho-Ser502 or phospho-Ser800) overnight at 4°C, then blocked with 1% Block Ace for 2 h at room temperature. After washing three times with PBS containing 0.05% Tween 20 (PBST), 50 μL of protein lysates were incubated overnight at 4°C. After washing with PBST, 50 μL of streptavidin-Poly-HRP (500 ng/mL; Thermo Scientific, Waltham, MA, USA) with biotinylated anti-TRPV1 antibody (500 ng/mL; Alomone Labs, Jerusalem, Israel), prepared with NHS-PEG4-Biotin (Thermo Scientific), was placed into each well for 2 h at room temperature. After washing with PBST, 50 μL of TMB (Dako, Glostrup, Denmark) substrate solution was added. The reaction was stopped by addition of 0.5 N sulfuric acid and optical absorbance was measured at 450 nm using an Envision 2102 Multilabel Reader (PerkinElmer, Waltham, MA, USA).

Immunohistochemistry

Right side L4 DRGs were removed and frozen in Optimal Cutting Temperature compound (Sakura Finetek, Torrance, CA, USA). Transverse sections (10 μm) were cut by cryostat (CM1850; Leica, Nussloch, Germany) and thaw-mounted onto glass slides (Matsunami Glass, Osaka, Japan). Sections were incubated with 4% paraformaldehyde in PBS for 10 min. After rinsing in PBS for 5 min, sections were incubated with blocking solution containing 3% (v/v) bovine serum albumin (BSA) for 1 h at room temperature and incubated with primary antibodies or isotype controls (Table II) in a medium containing 3% (v/v) BSA overnight at 4°C. After washing with PBS, sections were reacted with Alexa Fluor 488-conjugated anti-goat IgG antibody (1:500, Invitrogen, for TRPV1) and Alexa Flour 594-conjugated anti-rabbit IgG antibody (1:500, Invitrogen, for phospho-PKCε) for 2 h at room temperature. After washing, sections were mounted with Vectashield (Vector Labs, Burlingame, CA, USA) and coverslipped. Immunohistochemical images were obtained using a Keyence microscope (Biorevo, BZ-9000, Osaka, Japan).

Statistical analysis

All data are expressed as means and corresponding 95% confidence intervals (CI). $P < 0.05$ was considered statistically significant. Gaussian distribution was analyzed by Shapiroe–Wilk normality test. When departures from the assumptions were detected, non-parametric analysis was used. Statistical significance of changes in levels of TRPV1, p-PKCε and TRPV1 phosphorylation, or sensitivity to capsaicin in DRG neurons between sham and MIA rats, were analyzed by Mann–Whitney *U*-test. Analysis of time course effects on grip strength was conducted using repeated measures analysis of variance. Bonferroni test was performed as a post-hoc comparison. The Steel–Dwass test was used to determine differences in capsaicin-induced pain-related behavior between MIA and sham rats, PMA-treated and vehicle-treated rats, or protein kinase inhibitor-treated and vehicle groups. Analyses were performed with SAS software version 9.4 (SAS Institute, Tokyo, Japan) and GraphPad Prism6 software (GraphPad, USA).

Results

Increased capsaicin-induced pain-related behavior in MIA rats

This study used a MIA-induced joint pain model as an experimental OA pain model, and examined grip strength to confirm knee joint pain. As previously reported[17], intra-articular injection of MIA reduced grip strength of hind limbs for up to 28 days [Fig. 1(A), $(F)(9, 144) = 8.16$, $P < 0.001$]. Because the reduction plateaued on day 14 after sham or MIA injection, further investigation was performed on day 14 rats. Reduced grip strength of MIA injected rats was dose-dependently reversed by celecoxib (a clinically used

Table I
List of primary antibodies used in western blotting

Primary antibodies	Supplier	Species	Type	Dilution	Reference
Anti-TRPV1	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	Rabbit	Polyclonal	1:300	sc-28759, Lot#G3010
Anti-phospho-PKCε Ser729	Santa Cruz Biotechnology	Goat	Polyclonal	1:200	sc-12355, Lot#C2113
Anti-PKCε	Santa Cruz Biotechnology	Goat	Polyclonal	1:200	sc-214-G, Lot#J3002
Anti-β-actin (Clone AC-15)	Sigma–Aldrich (St. Louis, MO, USA)	Mouse	Monoclonal	1:2000	A1978, Lot#011M4812

Table II
List of primary antibodies and isotype control used in immunohistochemistry

	Supplier	Species	Type	Dilution	Reference
Primary antibodies					
Anti-TRPV1	Neuromics (Edina, MN, USA)	Goat	Polyclonal	1:300	GT15129, Lot#401557
Anti-phospho-PKCε Ser729	Abcam (Cambridge, UK)	Rabbit	Polyclonal	1:25	ab63387, Lot#GR43554-4
Isotype controls					
Goat control IgG	Neuromics	Goat	Polyclonal	1:300	GT15900, Lot#400923
Rabbit control IgG	Abcam	Rabbit	Polyclonal	1:5	ab27478, Lot#GR166285-18

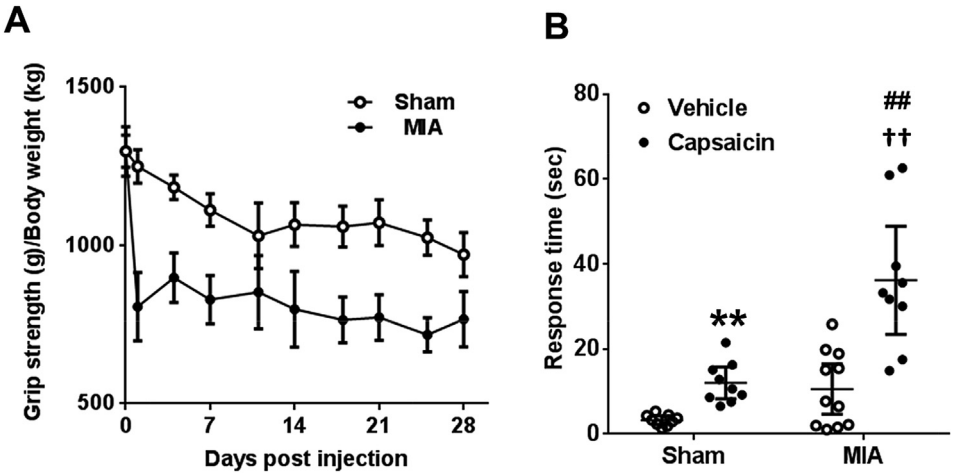

Fig. 1. Increased capsaicin-induced pain-related behavior in MIA rats. (A) Time course of changes in hind limb grip strength in sham and MIA rats ($F(1,16) = 59.94$, $P < 0.001$ for injection group; $F(9144) = 39.79$, $P < 0.001$ for time; $F(9144) = 8.16$, $P < 0.001$ for interaction, sham:$n = 8$, MIA:$n = 10$). (B) On day 14 after MIA or saline injection, 10 μg of capsaicin or vehicle (10% ethanol in saline) were intra-articularly injected into sham or MIA-treated ipsilateral knee joints, and the response time spent in pain-related behavior was measured for 10 min. Data were analyzed by the Steel–Dwass test ($^{**}P = 0.002$ vs vehicle-treated sham rats, $^{\dagger\dagger}P = 0.009$ vs vehicle-treated MIA rats, $^{\#\#}P = 0.002$ vs capsaicin-treated sham rats, Sham-Vehicle:$n = 9$, Sham-Capsaicin:$n = 9$, MIA-Vehicle:$n = 11$, MIA-Capsaicin:$n = 9$).

analgesic for OA pain) (Supplementary Fig. 1). Significant cartilage degeneration ($P = 0.024$) and synovitis ($P = 0.012$) were also observed in MIA injected rats (Supplementary Fig. 2). Thus, MIA rats developed features (joint degeneration with cartilage loss) of OA pain.

To investigate functional changes of TRPV1 in MIA rats, capsaicin was administered to knee joints, and pain-related behavior was evaluated. Although capsaicin administration significantly induced pain-related behavior in sham and MIA rats [Fig. 1(B); Sham; $P = 0.002$, MIA; $P = 0.009$], duration of pain-related behavior significantly increased in MIA rats compared with sham rats ($P = 0.002$). In contrast, pain-related behavior induced by vehicle administration was not significantly different between MIA and sham rats ($P = 0.92$). Increased capsaicin-induced pain-related behavior in MIA rats suggested that TRPV1 functions were enhanced in knees of MIA rats.

Increased sensitivity to capsaicin in DRG neurons of MIA rats

The sensory nerves of knee joints are thought to be the target of intra-articularly injected capsaicin and to transmit pain signals through TRPV1. We investigated sensitivity changes to capsaicin in DRG neurons innervating the knee joint using the whole-cell patch clamp system. Capsaicin increased inward currents in a concentration-dependent manner in back-labeled DRG neurons of both sham and MIA rats. However, EC_{50} values of inward currents induced by capsaicin were significantly different between sham and MIA rats [Fig. 2(A), (B); sham (3.6 μM; 1.6, 5.5), MIA (1.3 μM; 0.70, 2.0), $P = 0.031$]. Increased sensitivity to capsaicin was observed in DRG neurons of MIA rats. In contrast, current density of inward currents induced by high concentration of capsaicin (30 μM), indicating functional membrane expression of TRPV1, remained unchanged between sham and MIA rats [Fig. 2(C), (D); sham (−43 pA/pF; −77, −8.0), MIA (−66 pA/pF; −125, −6.6), $P = 0.65$].

Protein expression levels of TRPV1 in DRGs of MIA rats

TRPV1 protein levels in ipsilateral L3–4 DRGs were not significantly different between sham and MIA rats [Fig. 3(A), (B); sham (100%; 52, 148), MIA (107%; 72, 142), $P = 0.94$]. Thus, increased sensitivity to capsaicin in DRG neurons of MIA rats was not due to increased TRPV1 protein expression.

TRPV1 phosphorylation in DRG neurons of MIA rats

Next, we analyzed functional changes of TRPV1 by measuring TRPV1 phosphorylation. We developed novel antibodies against phosphorylated TRPV1 at Ser502 or Ser800, and generated an ELISA system. Phospho-Ser502 and phospho-Ser800 of TRPV1 increased dose-dependently in TRPV1-CHO cells, but not in control CHO cells, following PMA stimulation, which induces TRPV1 phosphorylation (Supplementary Fig. 3)[22]. Absorption studies using each antigen indicated ELISA specificity, not only for TRPV1-CHO cells, but also for DRG (Supplementaty Fig. 4). Analysis of phosphorylated TRPV1 levels in DRG indicated that phosphorylated TRPV1 at Ser800 was significantly increased in MIA rats compared with sham rats. However, phosphorylated TRPV1 at Ser502 remained unchanged between sham and MIA rats [Fig. 4(A); sham (0.25; 0.23, 0.27), MIA (0.27; 0.24, 0.30), $P = 0.31$, [Fig. 4(B)]; sham (0.30; 0.26, 0.33), MIA (0.56; 0.53, 0.60), $P = 0.002$].

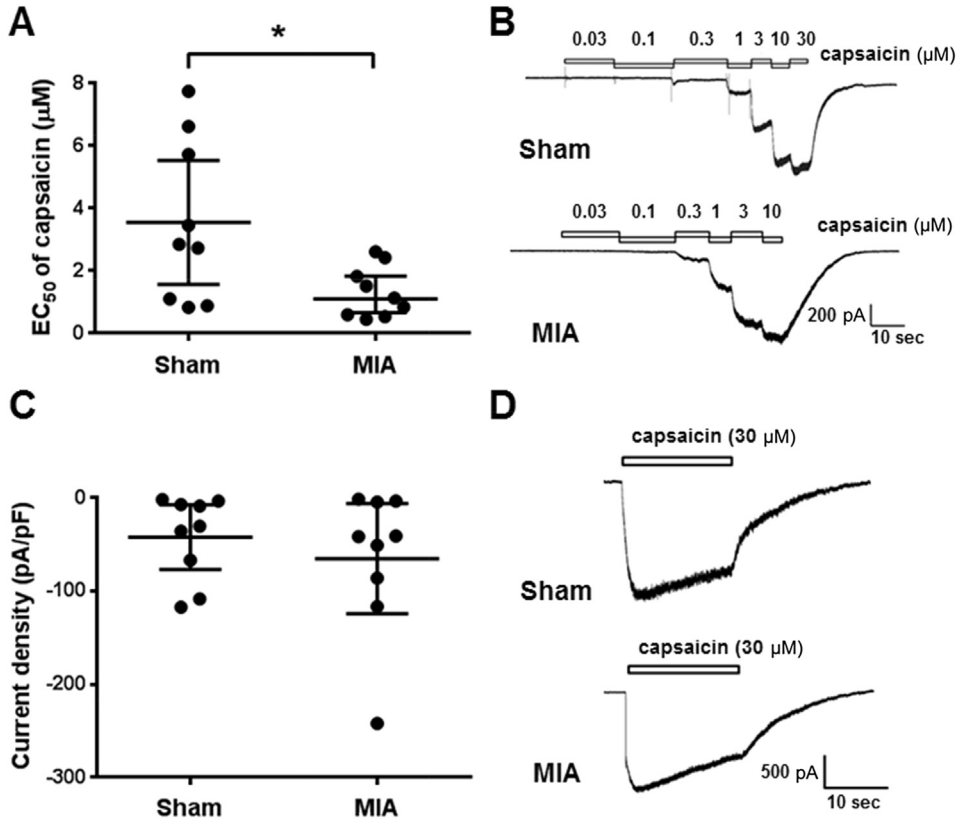

Fig. 2. Capsaicin-induced inward currents in DRG neurons. WGA-Alexa 488 back-labeled DRG neurons from the knee joints of sham and MIA rats were selected and the EC_{50} of capsaicin-induced inward currents was determined. Capsaicin was cumulatively applied to DRG neurons until the current reached a plateau. (A) EC_{50} of capsaicin-induced inward currents in DRG neurons was significantly decreased in MIA rats compared with sham rats. (B) Representative traces of capsaicin-induced inward currents from DRG neurons from sham and MIA rats. (C) The current density induced by high concentration of capsaicin (30 μM) remained unchanged between sham and MIA rats. (D) Representative traces of 30-μM capsaicin-induced inward currents from sham and MIA rats. (A, C) Dots indicate results of single DRG neuron ($n = 9$), and horizontal bars indicate the mean. Data were obtained from four independent experiments, and in each experiment DRG neurons were pooled from three rats. Data were analyzed by Mann–Whitney *U*-test (*$P = 0.031$ vs sham rats).

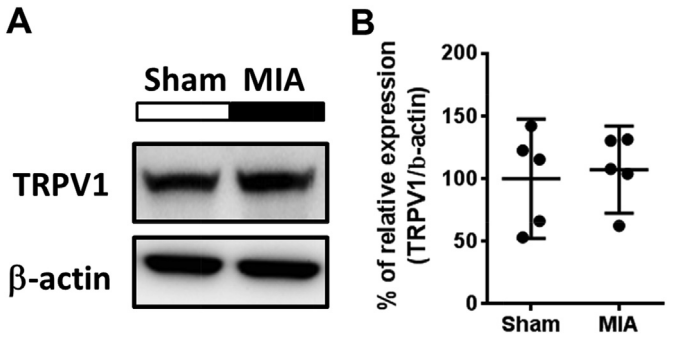

Fig. 3. Western blot analysis of TRPV1 protein expression in DRGs. DRG protein lysates were prepared from the right side L3 and L4 DRGs of rats. Ten micrograms of protein lysates were separated by SDS-PAGE and analyzed by western blot. (A) Representative western blot analysis of TRPV1 (upper) and β-actin (lower). (B) Quantitative results of TRPV1 protein expression. TRPV1 protein expression was quantified as described in the Materials and Methods, and normalized to β-actin levels. Data represent relative expression to sham rats. TRPV1 protein expression in DRG was unchanged between sham and MIA rats. Data were analyzed by Mann–Whitney *U*-test (not significant, $P = 0.94$ vs sham rats, $n = 5$/group).

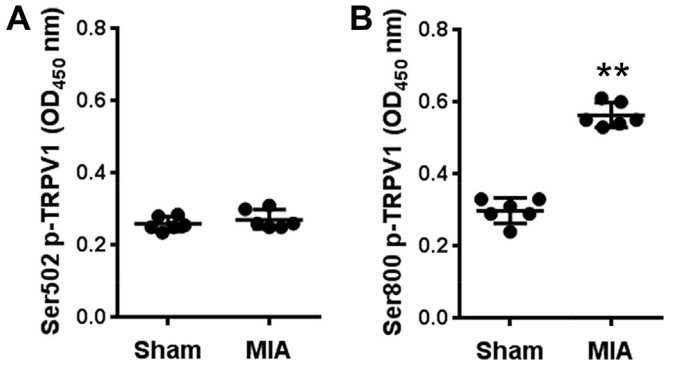

Fig. 4. ELISA of phosphorylated TRPV1 in DRGs. (A) Phospho-TRPV1 at Ser502 was unchanged between sham and MIA rats. (B) Increased phospho-TRPV1 at Ser800 in DRG of MIA rats. DRG protein lysates were prepared as described in Fig. 3. Five nanograms of each lysate were analyzed by ELISA. Data represent absorbance at 450 nm obtained by ELISA. Data were analyzed by Mann–Whitney *U*-test (**$P = 0.002$ vs sham rats, $n = 6$/group).

PKCε expression and co-localization with TRPV1 in DRG neurons

Because increased phosphorylated TRPV1 at Ser800 in DRG was observed in MIA rats, we examined whether PKC activity was potentiated in MIA rats. PKCε is an isoform of PKC that phosphorylates TRPV1 at Ser800[22–26], and phosphorylated PKCε at Ser729 is a marker of PKCε activation[27]. Western blot analysis using anti-phospho PKCε (Ser729) antibody showed phosphorylated PKCε was significantly increased in DRG of MIA rats compared with sham rats [Fig. 5(A), (B); sham (100%; 85, 115), MIA (146%; 115, 177), P=0.008]. Immunohistological analyses showed expression of phosphorylated PKCε co-localized with TRPV1 in small-to-medium diameter DRG neurons [Fig. 5(C)].

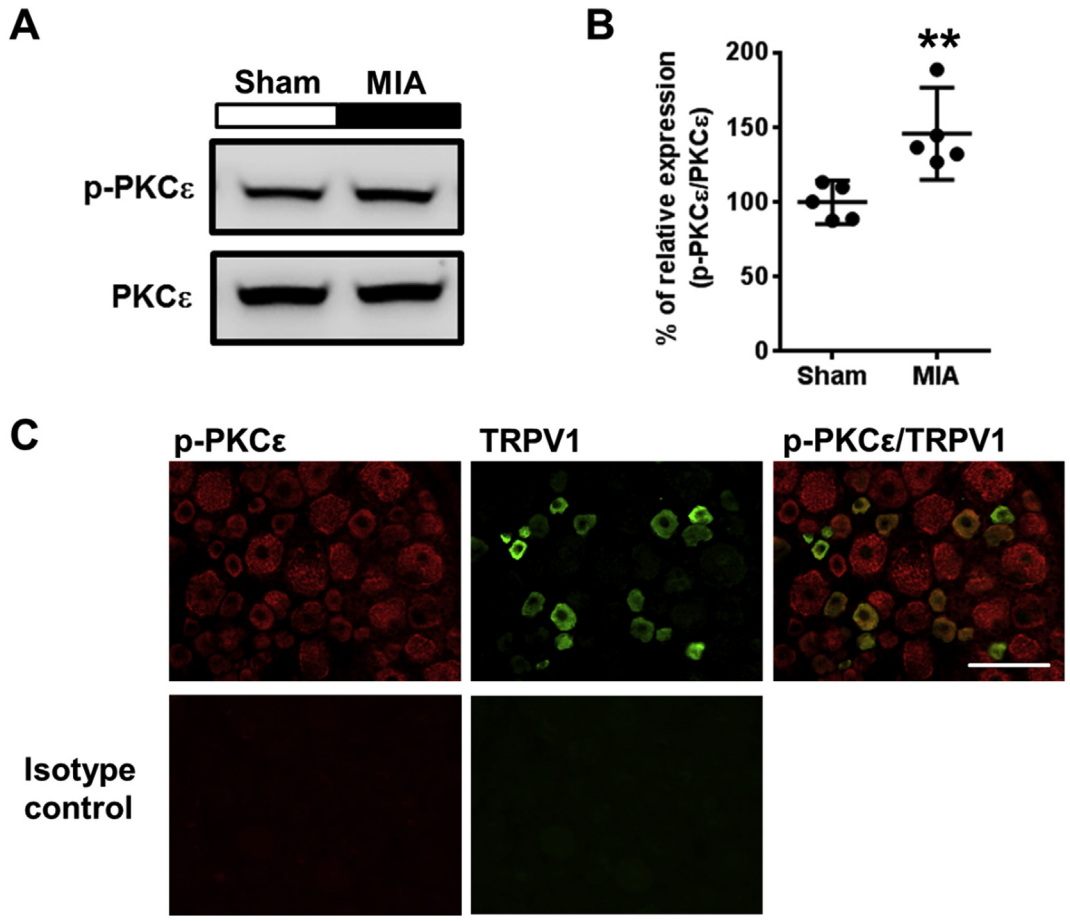

Fig. 5. Increased phosphorylated PKCε in DRGs of MIA rats. Ten micrograms of protein lysates, prepared as in Fig. 3, were analyzed by western blot. (A) Representative western blots of phospho-PKCε (p-PKCε; upper) and total PKCε (lower). (B) Quantitative results of phosphorylated PKCε levels in DRGs, normalized by total PKCε. Data represent relative expression to sham rats. Data were analyzed by Mann–Whitney *U*-test (**$P = 0.008$ vs sham rats, $n = 5$/group). (C) Immunohistochemical analysis of phospho-PKCε and TRPV1 in DRG neurons of MIA rats. Expression of TRPV1 (green, 29 ± 4.4% of DRG neurons) and phospho-PKCε (red, 82 ± 6.7% of DRG neurons), and merged image (yellow, 19 ± 3.2% of DRG neurons) in DRG of MIA rats are indicated. Figure shows a representative image from three independent experiments. Scale bar represents 100 μm.

Effects of intra-articular injection of protein kinase inhibitors on capsaicin sensitivity in MIA rats

The mechanism of TRPV1 sensitization in MIA rats might involve TRPV1 phosphorylation at Ser800 by PKC signaling, in particular PKCε. Therefore, we next examined the effect of PKC inhibitor on the development of increased capsaicin sensitivity in MIA rats. Intra-articular injection of PKC inhibitor (bisindolylmaleimide I; BIS) but not PKA inhibitor (KT5720) suppressed increased capsaicin sensitivity in MIA rats [Fig. 6(A), (B)]. In contrast, neither inhibitor affected capsaicin-induced pain-related behaviors in sham rats. Thus, increased capsaicin sensitivity in MIA rats was mediated by PKC-induced phosphorylation of TRPV1. However, both the PKC and PKA inhibitor reversed the reduction of grip strength in MIA rats (Supplementary Fig. 5) suggesting the dosage of PKA inhibitor was sufficient to block PKA activity.

Effects of PKC activator on capsaicin-induced pain-related behavior

To examine the involvement of PKC on increased capsaicin sensitivity in MIA rats, we investigated whether administration of a PKC activator (PMA) increased responses to capsaicin in normal rats [Fig. 7]. Pain-related behavior induced by capsaicin injection were significantly increased following pre-administration of PMA ($P = 0.006$). However, PMA administration alone did not induce pain-related behavior in normal rats ($P = 0.51$) suggesting PKC activation was sufficient to induce increased capsaicin sensitivity at knee joints.

Discussion

The present study investigated the sensitization of TRPV1 in MIA rats by examining capsaicin-induced responses *in vivo* and *in vitro*. As previously reported[13–16], MIA rats developed OA-like pain caused by joint degeneration with cartilage loss, and responded to celecoxib, which is used clinically for OA pain. They were highly reproducible, 85% of MIA-injected rats developed the joint pain. Increased sensitivity for capsaicin *in vivo* after intra-articular injection of capsaicin and in DRG neurons on capsaicin-induced inward currents indicates TRPV1 is sensitized in MIA rats.

Increased TRPV1 functions under pain conditions could be resulted from total- or membrane-located expressional changes of TRPV1, or functional changes of TRPV1 by phosphorylation[18,28,29]. Previous studies indicated changes in DRG TRPV1 expression in MIA rats[11,30]. One study showed decreased TRPV1 protein expression in DRG by ELISA at the ipsilateral side of MIA injection[30], and another showed increased numbers of TRPV1-immunopositive neurons in back-labeled DRG from knee joints of MIA rats[11]. Here, we showed not only TRPV1 protein expression, but also TRPV1 functional membrane expression in back-labeled DRG neurons

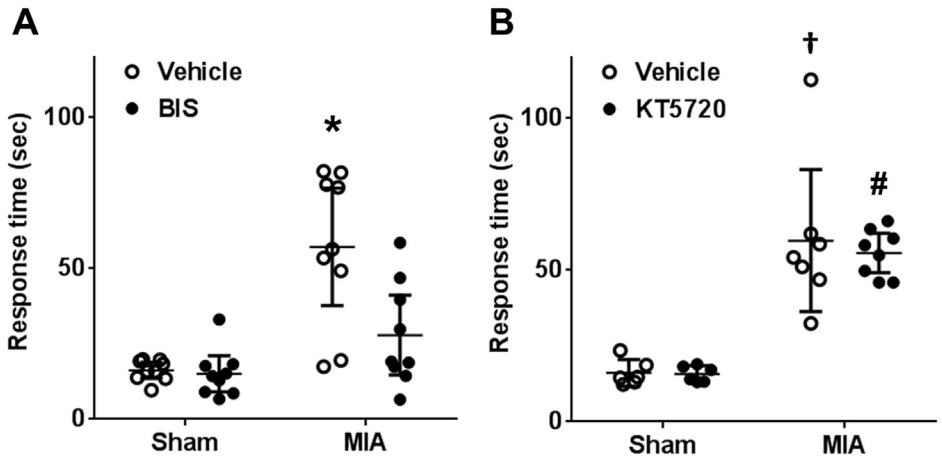

Fig. 6. Effect of protein kinase C (PKC) and protein kinase A (PKA) inhibitors on capsaicin-induced, pain-related behavior in MIA rats. Data were analyzed by the Steel–Dwass test. (A) Pre-administration of PKC inhibitor, bisindolylmaleimide I (BIS), inhibited the capsaicin-induced pain-related behavior in MIA rats ($^{*}P = 0.012$ vs vehicle-treated sham rats, $n = 9$/group). (B) Pre-administration of PKA inhibitor, KT5720, did not inhibit the capsaicin-induced pain-related behavior in MIA rats ($^{\dagger}P = 0.014$ vs vehicle-treated sham rats, $^{\#}P = 0.011$ vs KT5720-treated sham rats, Sham-Vehicle:$n = 6$, Sham-KT5720:$n = 6$, MIA-Vehicle:$n = 7$, MIA-KT5720:$n = 8$). (A, B) Ten micrograms of capsaicin were intra-articularly injected into sham or MIA-treated ipsilateral knee joints, and the duration of pain-related behavior was recorded for 10 min. BIS (5 nmol) and KT5720 (10 nmol) were pre-administered intra-articularly 1 h before capsaicin injection.

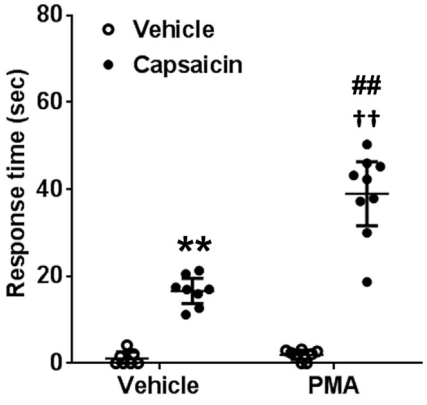

Fig. 7. Effect of phorbol 12-myristate 13-acetate (PMA) on capsaicin-induced, pain-related behavior in normal rats. Ten picomoles of PMA or vehicle (10% ethanol in saline) were administered intra-articularly 15 min before capsaicin injection. Ten micrograms of capsaicin were injected intra-articularly, and the duration of pain-related behavior was recorded for 10 min. Data were analyzed by the Steel–Dwass test ($^{**}P = 0.006$ vs vehicle treated with vehicle injection group, $^{\#\#}P = 0.003$ vs PMA treated with vehicle injection group, $^{\dagger\dagger}P = 0.006$ vs vehicle treated with capsaicin injection group, Vehicle–Vehicle:$n = 7$, Vehicle-Capsaicin:$n = 8$, PMA-Vehicle:$n = 8$, PMA-Capsaicin:$n = 9$).

from knee joints, were unchanged in MIA rats. These results clearly indicate that changes in TRPV1 expression were not involved in the enhancement of TRPV1 function in MIA rats. However, increased TRPV1 translocation to afferent terminals in knee joints without expression changes in the DRG neurons might occur. Because changes in TRPV1 expression were not observed, we examined functional changes of TRPV1. The balance of TRPV1 phosphorylation and dephosphorylation modulates TRPV1 function[26,31,32]. In addition, inflammatory cytokines, such as TNF-α and NGF, which are increased in OA, phosphorylate and potentiate TRPV1 function[29,33,34]. To quantitate phosphorylated TRPV1, we developed novel antibodies against phosphorylated TRPV1 and generated an ELISA. Phosphorylated TRPV1 at Ser800 was significantly increased in DRG of MIA rats. This is the first study to detect changes of TRPV1 phosphorylation at specific residue sites directly *in vivo* and in an animal pain model. However, phosphorylated TRPV1 at Ser502 was unchanged between sham and MIA rats. In contrast to Ser800, phosphorylated Ser502 is dephosphorylated by capsaicin stimulation[31,35]. Phosphorylation and dephosphorylation of TRPV1 at Ser502 and Ser800 might be regulated independently in pathological conditions. Further studies on the phosphorylation of TRPV1 in other pain models, such as neuropathic pain, and comparison to MIA rats might identify the regulatory mechanisms of each TRPV1 phosphorylated residue. Although we confirmed the specificity of our novel ELISA system by absorption studies of each antigen in both TRPV1-CHO cells and DRG, the created antibodies were not suitable for western blotting or immunohistology (data not shown), suggesting the recognition site of phosphorylated TRPV1 is impaired by immobilization.

Next, we addressed the mechanism of TRPV1 phosphorylation at Ser800. PKCε phosphorylates TRPV1 at Ser800[35] and sensitizes nociceptors[36,37]. PKCε is activated by phosphorylation, and the frequency of phosphorylated PKCε–immunopositive neurons was increased in DRGs of carrageenan- or complete Freund's adjuvant-induced inflammation pain model[27]. Here, we showed phosphorylated PKCε was quantitatively increased in DRG of MIA rats and co-localized with TRPV1 indicating TRPV1 phosphorylation at Ser800 is induced by PKCε activation in MIA rats. Interestingly, the frequency of phosphorylated PKCε–immunopositive neurons decreased in DRGs in a chronic constriction injury neuropathic pain model[27]. Thus, MIA rats are similar to inflammatory pain model compared with neuropathic pain model at the molecular level. To confirm involvement of PKCε in the development of enhanced TRPV1 function in knee joints of MIA rats, we used PKC inhibitors.

Intra-articular pretreatment with a PKC inhibitor suppressed increased capsaicin sensitivity in MIA rats, suggesting PKC enhances TRPV1 function in knee joints of MIA rats. The PKC inhibitor bisindolylmaleimide I inhibits other kinases such as PKA and glycogen synthase kinase-3 at high concentrations[38,39]. Here, bisindolylmaleimide I did not affect capsaicin-induced pain-related behavior in sham rats, and a PKA inhibitor did not attenuate capsaicin-induced pain-related behavior in MIA rats, suggesting bisindolylmaleimide I were PKC specific. In addition, intra-articular injection of PMA, a PKC activator, induced increased capsaicin-induced pain-related behavior in normal rats. PMA sensitizes TRPV1[22], and other receptors that modulate pain[40]. However, because PMA administration itself did not induce pain-related behavior in normal rats, increased pain-related behavior induced

by capsaicin imply TRPV1 sensitization. These results support the involvement of PKC in the development of enhanced TRPV1 function in knee joints. Furthermore, treatment with a PKA inhibitor, KT5720, did not affect capsaicin-induced pain-related behavior in sham or MIA rats. PKA increases capsaicin-induced responses by increasing TRPV1 translocation to the cell surface membrane[41,42]. No significant changes in functional DRG TRPV1 membrane expression were observed by electrophysiological analysis indicating PKA is not involved in enhanced TRPV1 function in MIA rats. However, bisindolylmaleimide I and KT5720 treatment were analgesic and reversed reduced grip strength in MIA rats suggesting PKA activates nociceptors that mediate joint pain in MIA rats independently from TRPV1. PKA-mediated phosphorylation of TRPV1 involves inflammatory hyperalgesia in trigeminal ganglia neurons[43]. Involvement of PKC and PKA for the development of hyperalgesia might differ between types of diseases or neurons. Previous studies showed TRPV1 functional changes pathological pain[9,18,28,44]. Increased capsaicin-induced CGRP release in spinal cord[44], sensitization of joint afferent neurons in OA model rats[9], and the involvement of PKC in diabetic neuropathy model rats and in bone cancer pain model rats[18,28] were reported. Therefore, TRPV1 phosphorylation might be involved in the development of hypersensitization in OA pain. However, these studies did not identify phosphorylated TRPV1 residues or examine the effects of PKC inhibition *in vivo*, especially in OA pain. Here, we found direct evidence of TRPV1 phosphorylation at Ser800 *in vivo*, as well its connection to enhanced TRPV1 function in MIA rats. Ser800 is an important TRPV1 sensitization site, which increases the response to capsaicin, heat, or acid[45]. Thus, TRPV1 functional changes might be involved in painful OA of the knee. However, they are insufficient to explain the importance of TRPV1 changes for causing and maintaining OA pain. Recently, TRPV1 antagonists were shown to have strong analgesic effects in OA pain models[9,44]; inflammatory cytokines, such as TNF-α, induced TRPV1 phosphorylation; and phosphorylated TRPV1 was gated by normal body temperature[46]. Additionally, a SNP of TRPV1, resulting in reduced sensitivity to capsaicin and thermal stimuli in humans, was associated with reduced risk of symptomatic knee OA[10]. Therefore, TRPV1 might have an important role in OA pain. Further studies focusing on *in vivo* functions of enhanced TRPV1 function, and its correlation with clinical OA, are required to understand the significance of results from this study.

In conclusion, TRPV1 function was enhanced in DRG neurons and in knee joints of MIA rats *via* TRPV1 phosphorylation by PKCε. These findings will enhance our understanding of OA pain mechanisms in the knee, and the use of TRPV1 antagonists as potent novel analgesics for OA pain.

Author contributions

KK and KH contributed equally to this work. KK collected and analyzed data, designed the study, and wrote the paper. KH collected and analyzed data, participated in study design, and helped to prepare the paper. KO and KT performed all electrophysiological studies. SI created and performed the ELISA study. YS performed histological studies of knee joints damage of MIA rats. HO, MF and MY gave technical support and participated in study design. IF designed and validated antibodies. SY gave advice for the ELISA study. AM gave advice for antibody validation. TA and TK developed the concept and gave advice. GS gave conceptual advice. YM supervised the project and edited the paper. All authors discussed the results and implications and commented on the manuscript at all stages.

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Competing interests

All authors were all employees of Shionogi & Co., Ltd. at the time of data collection and manuscript writing.

Supplementary data

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References

1. Jordt SE, Tominaga M, Julius D. Acid potentiation of the capsaicin receptor determined by a key extracellular site. Proc Natl Acad Sci U. S. A 2000;97(14):8134–9.
2. Smart D, Gunthorpe MJ, Jerman JC, Nasir S, Gray J, Muir AI, *et al*. The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). Br J Pharmacol 2000;129(2):227–30.
3. Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, *et al*. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 2000;288(5464):306–13.
4. Davis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P, *et al*. Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. Nature 2000;405(6783):183–7.
5. Gavva NR, Tamir R, Qu Y, Klionsky L, Zhang TJ, Immke D, *et al*. AMG 9810 [(E)-3-(4-t-butylphenyl)-N-(2,3-dihydrobenzo[b][1,4] dioxin-6-yl)acrylamide], a novel vanilloid receptor 1 (TRPV1) antagonist with antihyperalgesic properties. J Pharmacol Exp Ther 2005;313(1):474–84.
6. Honore P, Wismer CT, Mikusa J, Zhu CZ, Zhong C, Gauvin DM, *et al*. A-425619 [1-isoquinolin-5-yl-3-(4-trifluoromethyl-benzyl)-urea], a novel transient receptor potential type V1 receptor antagonist, relieves pathophysiological pain associated with inflammation and tissue injury in rats. J Pharmacol Exp Ther 2005;314(1):410–21.
7. Lehto SG, Tamir R, Deng H, Klionsky L, Kuang R, Le A, *et al*. Antihyperalgesic effects of (R,E)-N-(2-hydroxy-2,3-dihydro-1H-inden-4-yl)-3-(2-(piperidin-1-yl)-4-(trifluoromethyl) phenyl)-acrylamide (AMG8562), a novel transient receptor potential vanilloid type 1 modulator that does not cause hyperthermia in rats. J Pharmacol Exp Ther 2008;326(1):218–29.
8. Tafesse L, Kanemasa T, Kurose N, Yu J, Asaki T, Wu G, *et al*. Structure-activity relationship studies and discovery of a potent transient receptor potential vanilloid (TRPV1) antagonist 4-[3-chloro-5-[(1S)-1,2-dihydroxyethyl]-2-pyridyl]-N-[5-(trifluoromethyl)-2-pyridyl]-3,6-dihydro-2H-pyridine-1-carboxamide (V116517) as a clinical candidate for pain management. J Med Chem 2014;57(15):6781–94.
9. Kelly S, Chapman RJ, Woodhams S, Sagar DR, Turner J, Burston JJ, *et al*. Increased function of pronociceptive TRPV1 at the level of the joint in a rat model of osteoarthritis pain. Ann Rheum Dis 2013 Oct 23, http://dx.doi.org/10.1136/annrheumdis-2013-203413.
10. Valdes AM, De Wilde G, Doherty SA, Lories RJ, Vaughn FL, Laslett LL, *et al*. The Ile585Val TRPV1 variant is involved in risk of painful knee osteoarthritis. Ann Rheum Dis 2011;70(9):1556–61.
11. Fernihough J, Gentry C, Bevan S, Winter J. Regulation of calcitonin gene-related peptide and TRPV1 in a rat model of osteoarthritis. Neurosci Lett 2005;388(2):75–80.
12. Adães S, Mendonça M, Santos TN, Castro-Lopes JM, Ferreira-Gomes J, Neto FL. Intra-articular injection of collagenase in the knee of rats as an alternative model to study nociception associated with osteoarthritis. Arthritis Res Ther 2014;16(1).

13. Malfait AM, Little CB, McDougall JJ. A commentary on modelling osteoarthritis pain in small animals. Osteoarthritis Cartilage 2013;21(9):1316–26.
14. Guingamp C, Gegout-Pottie P, Philippe L, Terlain B, Netter P, Gillet P. Mono-iodoacetate-induced experimental osteoarthritis: a dose-response study of loss of mobility, morphology, and biochemistry. Arthritis Rheum 1997;40(9):1670–9.
15. Guzman RE, Evans MG, Bove S, Morenko B, Kilgore K. Mono-iodoacetate-induced histologic changes in subchondral bone and articular cartilage of rat femorotibial joints: an animal model of osteoarthritis. Toxicol Pathol 2003;31(6):619–24.
16. Suokas AK, Sagar DR, Mapp PI, Chapman V, Walsh DA. Design, study quality and evidence of analgesic efficacy in studies of drugs in models of OA pain: a systematic review and a meta-analysis. Osteoarthritis Cartilage 2014;22(9):1207–23.
17. Chandran P, Pai M, Blomme EA, Hsieh GC, Decker MW, Honore P. Pharmacological modulation of movement-evoked pain in a rat model of osteoarthritis. Eur J Pharmacol 2009;613(1–3):39–45.
18. Hong S, Wiley JW. Early painful diabetic neuropathy is associated with differential changes in the expression and function of vanilloid receptor 1. J Biol Chem 2005;280(1):618–27.
19. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol 2010;8(6):e1000412.
20. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. Osteoarthritis Cartilage 2012;20(4):256–60.
21. Pomonis JD, Boulet JM, Gottshall SL, Phillips S, Sellers R, Bunton T, *et al*. Development and pharmacological characterization of a rat model of osteoarthritis pain. Pain 2005;114(3):339–46.
22. Numazaki M, Tominaga T, Toyooka H, Tominaga M. Direct phosphorylation of capsaicin receptor VR1 by protein kinase C epsilon and identification of two target serine residues. J Biol Chem 2002;277(16):13375–8.
23. Bhave G, Hu HJ, Glauner KS, Zhu W, Wang H, Brasier DJ, *et al*. Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). Proc Natl Acad Sci U. S. A 2003;100:12480–5.
24. Cesare P, Dekker LV, Sardini A, Parker PJ, McNaughton PA. Specific involvement of PKC-epsilon in sensitization of the neuronal response to painful heat. Neuron 1999;23(3):617–24.
25. Li L, Hasan R, Zhang X. The Basal Thermal sensitivity of the TRPV1 ion channel is determined by PKCβII. J Neurosci 2014;34(24):8246–58.
26. Premkumar LS, Ahern GP. Induction of vanilloid receptor channel activity by protein kinase C. Nature 2000;408(6815):985–90.
27. Zhou Y, Li GD, Zhao ZQ. State-dependent phosphorylation of epsilon-isozyme of protein kinase C in adult rat dorsal root ganglia after inflammation and nerve injury. J Neurochem 2003;85(3):571–80.
28. Pan HL, Zhang YQ, Zhao ZQ. Involvement of lysophosphatidic acid in bone cancer pain by potentiation of TRPV1 via PKCε pathway in dorsal root ganglion neurons. Mol Pain 2010;6:85.
29. Zhang X, Huang J, McNaughton PA. NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. EMBO J 2005;24(24):4211–23.
30. Han P, Korepanova AV, Vos MH, Moreland RB, Chiu ML, Faltynek CR. Quantification of TRPV1 protein levels in rat tissues to understand its physiological roles. J Mol Neurosci 2013;50(1):23–32.
31. Jung J, Shin JS, Lee SY, Hwang SW, Koo J, Cho H, *et al*. Phosphorylation of vanilloid receptor 1 by Ca^{2+}/calmodulin-dependent kinase II regulates its vanilloid binding. J Biol Chem 2004;279(8):7048–54.
32. Mohapatra DP, Nau C. Regulation of Ca2+-dependent desensitization in the vanilloid receptor TRPV1 by calcineurin and cAMP-dependent protein kinase. J Biol Chem 2005;280(14):13424–32.
33. Constantin CE, Mair N, Sailer CA, Andratsch M, Xu ZZ, Blumer MJ, *et al*. Endogenous tumor necrosis factor alpha (TNFalpha) requires TNF receptor type 2 to generate heat hyperalgesia in a mouse cancer model. J Neurosci 2008;28(19):5072–81.
34. Orita S, Ishikawa T, Miyagi M, Ochiai N, Inoue G, Eguchi Y, *et al*. Pain-related sensory innervation in monoiodoacetate-induced osteoarthritis in rat knees that gradually develops neuronal injury in addition to inflammatory pain. BMC Musculoskelet Disord 2011;12:134–45.
35. Mandadi S, Tominaga T, Numazaki M, Murayama N, Saito N, Armati PJ, *et al*. Increased sensitivity of desensitized TRPV1 by PMA occurs through PKCepsilon-mediated phosphorylation at S800. Pain 2006;123(1–2):106–16.
36. Aley KO, Messing RO, Mochly-Rosen D, Levine JD. Chronic hypersensitivity for inflammatory nociceptors sensitization mediated by the e isozyme of protein kinase C. J Neurosci 2000;20:4680–5.
37. Khasar SG, Lin YH, Martin A, Dadgar J, McMahon T, Wang D, *et al*. A novel nociceptors signaling pathway revealed in protein kinase C e mutant mice. Neuron 1999;24:253–60.
38. Hers I, Tavaré JM, Denton RM. The protein kinase C inhibitors bisindolylmaleimide I (GF 109203x) and IX (Ro 31-8220) are potent inhibitors of glycogen synthase kinase-3 activity. FEBS Lett 1999;460(3):433–6.
39. Jacobson PB, Kuchera SL, Metz A, Schächtele C, Imre K, Schrier DJ. Anti-inflammatory properties of Gö 6850: a selective inhibitor of protein kinase C. J Pharmacol Exp Ther 1995;275(2):995–1002.
40. Fan HC, Zhang X, McNaughton PA. Activation of the TRPV4 ion channel is enhanced by phosphorylation. J Biol Chem 2009;284(41):27884–91.
41. Bhave G, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau 4th RW. cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. Neuron 2002;35:721–31.
42. Vetter I, Cheng W, Peiris M, Wyse BD, Roberts-Thomson SJ, Zheng J, *et al*. Rapid, opioid-sensitive mechanisms involved in transient receptor potential vanilloid 1 sensitization. J Biol Chem 2008;283(28):19540–50.
43. Schnizler K, Shutov LP, Van Kanegan MJ, Merrill MA, Nichols B, McKnight GS, *et al*. Protein kinase A anchoring via AKAP150 is essential for TRPV1 modulation by forskolin and prostaglandin E2 in mouse sensory neurons. J Neurosci 2008;28(19):4904–17.
44. Puttfarcken PS, Han P, Joshi SK, Neelands TR, Gauvin DM, Baker SJ, *et al*. A-995662 [(R)-8-(4-methyl-5-(4-(trifluoromethyl)phenyl)oxazol-2-ylamino)-1,2,3,4-tetrahydronaphthalen-2-ol], a novel, selective TRPV1 receptor antagonist, reduces spinal release of glutamate and CGRP in a rat knee joint pain model. Pain 2010;150(2):319–26.
45. Wang S, Joseph J, Ro JY, Chung MK. Modality-specific mechanisms of protein kinase C-induced hypersensitivity of TRPV1: S800 is a polymodal sensitization site. Pain 2015;156(5):931–41.
46. Sugimoto Y, Kojima Y, Inayoshi A, Inoue K, Miura-Kusaka H, Mori K, *et al*. K-685, a TRPV1 antagonist, blocks PKC-sensitized TRPV1 activation and improves the inflammatory pain in a rat complete Freund's adjuvant model. J Pharmacol Sci 2013;123(3):256–66.