

## Full Paper

**K-685, a TRPV1 Antagonist, Blocks PKC-Sensitized TRPV1 Activation and Improves the Inflammatory Pain in a Rat Complete Freund's Adjuvant Model**

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**Abstract.** Transient receptor potential vanilloid 1 (TRPV1) is a Ca<sup>2+</sup>-permeable non-selective cation channel that transmits pain signals. TRPV1 is activated by multiple stimuli such as capsaicin, acid, and heat. During inflammation, TRPV1 is reported to be sensitized by protein kinase C (PKC) in dorsal root ganglia (DRG) neurons, which leads to reduction in the threshold of the temperature for TRPV1 activation to body temperature. This sensitization is considered to contribute to chronic inflammatory pain. In a previous study, we discovered orally active 5,5-diarylpentadienamide TRPV1 antagonists. To examine the effects of our TRPV1 antagonists on PKC-sensitized TRPV1, we developed an in vitro assay system to monitor the TRPV1 sensitization by PKC. In this assay system, our TRPV1 antagonists, such as (2*E*,4*Z*)-*N*-[(3*R*)-3-hydroxy-2-oxo-1,2,3,4-tetrahydro-5-quinolyl]-5-(4-isopropoxyphenyl)-5-(4-trifluoromethylphenyl)-2,4-pentadienamide (K-685), inhibited the activation of TRPV1 sensitized by PKC. The potentiation of heat-induced inward currents by PKC was seen in rat DRG neurons, and K-685 attenuated these currents. Furthermore, K-685 reversed the thermal hyperalgesia and mechanical allodynia in a rat complete Freund's adjuvant-induced inflammatory pain model. These results therefore suggest that K-685 has a strong potential as a new analgesic drug for the treatment of inflammatory pain.

**Keywords:** TRPV1 antagonist, pain, protein kinase C (PKC), inflammation, complete Freund's adjuvant (CFA)

**Introduction**

Transient receptor potential vanilloid 1 (TRPV1) is a Ca<sup>2+</sup>-permeable non-selective cation channel that transmits pain signals induced by noxious stimuli (1–3). TRPV1 is activated by various stimuli such as vanilloid ligands (capsaicin) (1, 3), lipid ligands [anandamide (4), lipoxigenase products (5), and *N*-oleoyldopamine (6)], acid (pH < 6.0) (3), and heat (> 43°C) (3). Peripherally, TRPV1 is expressed in nonmyelinated C fibers and myelinated small Aδ fibers which contain substance P

and calcitonin gene-related peptide in the dorsal root ganglia (DRG) and trigeminal and vagal sensory ganglia (2, 7). The expression of TRPV1 in the central nervous system (CNS) has been also reported (8).

So far, the chronic pain due to inflammatory conditions such as arthritis has been considered to be intractable. TRPV1 has been suggested to be involved in inflammatory pain (8). For example, it was shown that thermal hyperalgesia was not developed under inflammatory conditions in TRPV1-knockout mice (9, 10). It has been reported that several chemical factors, such as nerve growth factor (11), bradykinin (11), prostaglandins (12), proteases (13), and ATP (14) are released during inflammation. The receptors for these proinflammatory chemical mediators are known to be colocalized with TRPV1 in the peripheral terminal of nociceptive neurons

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(8). It is known that stimulation by these mediators leads to the activation of the phospholipase C pathway, followed by the activation of protein kinase C (PKC). When TRPV1 is phosphorylated by PKC, the threshold in response to capsaicin, pH, and heat for the activation of TRPV1 is reported to be lowered (15 – 18). In particular, the temperature threshold is reported to be lowered to below 37°C, which suggests that TRPV1 is activated even at body temperature in such a pathological state, and this sensitization exacerbates pain symptoms (17, 18). Among the PKC isoforms, PKC $\epsilon$  is suggested to contribute to the sensitization of TRPV1 (18 – 20). In PKC $\epsilon$ -knockout mice, the hyperalgesia was reported to be decreased (19). On the other hand, the overexpression of dominant negative PKC $\epsilon$  was reported to prevent TRPV1 sensitization (20). These reports suggest that the sensitization of TRPV1 by PKC is responsible for the deterioration of pathological pain during inflammation. Therefore, inhibiting this pathological sensitization of TRPV1 has been hypothesized to have a favorable effect on inflammatory pain. However, it is difficult to monitor the TRPV1 activation at the temperature under the sensitized state in the conventional Ca<sup>2+</sup> assay system used in high throughput screening (HTS) systems because the temperature should be regulated between the lowered threshold and 43°C. In addition, the heat-induced Ca<sup>2+</sup> response through TRPV1 is smaller than the capsaicin- or acid-induced response. Thus, there have been few reports about HTS systems for detecting the heat-induced Ca<sup>2+</sup> response through PDBu-sensitized TRPV1, although there have been many reports about HTS systems for detection of capsaicin- or acid-induced Ca<sup>2+</sup> response through TRPV1.

There was one previous report that examined the effect of a TRPV1 antagonist, A-425619, on sensitized TRPV1 (21). In that study, the assay was performed by the addition of a hot solution (50°C) to the cell culture plate placed at room temperature since it is impossible to alter the temperature during the assay using the current Ca<sup>2+</sup> assay HTS systems. Therefore, it is possible that the precise regulation of the temperature was incomplete in the study. Furthermore, in that report, the effect of A-425619 on TRPV1 sensitized by phosphorylation was not examined under physiological conditions using neuronal cells. Although there have been several reports about TRPV1 antagonists which inhibited inflammatory pain (22 – 25), the effects of such antagonists on the activity of TRPV1 sensitized by PKC phosphorylation have not been examined sufficiently.

In a previous study, we discovered novel 5,5-diaryl-pentadienamides as orally active TRPV1 antagonists (26). These compounds inhibit the capsaicin-mediated Ca<sup>2+</sup> response through human and rat TRPV1 and

reversed pain behaviors in a rat neuropathic pain model. In the present study, we examined the effects of these TRPV1 antagonists on PKC-phosphorylated TRPV1. First, we developed an in vitro assay system to monitor the heat-activated TRPV1 phosphorylated by PKC. The assay was performed by adding phorbol ester to TRPV1-expressing cells at 40°C, which is a temperature between the normal threshold and the sensitized threshold. Then, the antagonist activity of our TRPV1 antagonists was evaluated in this assay system. Next, we examined whether similar sensitization was seen in rat DRG neurons. Then, we confirmed the effect of (2*E*,4*Z*)-*N*-[(3*R*)-3-hydroxy-2-oxo-1,2,3,4-tetrahydro-5-quinolyl]-5-(4-isopropoxyphenyl)-5-(4-trifluoromethylphenyl)-2,4-pentadienamamide (K-685), one of our TRPV1 antagonists, on PDBu-sensitized TRPV1 inward currents at 34°C, which is below the normal body temperature, using rat DRG neurons. Finally, the effect of K-685 on inflammatory pain was examined using a rat complete Freund's adjuvant (CFA) model.

## Materials and Methods

### Materials

The K-685 (Fig. 1) and *N*-(4-Tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetra-hydropyrazine-1(2*H*)-carbox-amide (BCTC) (23, 27) were synthesized in the Medicinal Chemistry Research Laboratories, Research Division, Kyowa Hakko Kirin Co., Ltd. All other chemicals and solvents were used in their analytical pure forms.

### Animals

Male Sprague-Dawley (SD) rats (5 – 6 weeks of age; Charles River Japan, Yokohama) were used in this study. The rats were kept in a specific pathogen-free animal facility that was maintained at a temperature of 19°C – 25°C, humidity of 30% – 70%, with a 12-h light/dark cycle and free access to food and water. The experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, and the experimental protocol used in this study was approved by the Committee for Animal Experiments at Kyowa Hakko Kirin Co., Ltd.

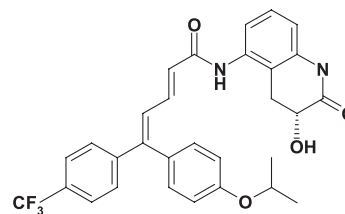


Fig. 1. The chemical structure of K-685.

### *Construction of expression vectors*

The human TRPV1 (hTRPV1) cDNA was purchased from GeneCopoeia (Rockville, MD, USA). A DNA fragment encoding hTRPV1 cDNA was enzymatically excised and inserted into the *Hind* III and *Bam* HI sites of pcDNA3.1(+)/Hygro. The established vector was termed pcDNA3.1(+)/Hygro-hTRPV1 and used in the subsequent expression study.

### *Cell culture and generation of cell lines stably expressing human TRPV1*

The 293EBNA cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 100 units/mL penicillin, and 100  $\mu$ g/mL streptomycin. To generate cell lines stably expressing hTRPV1, pcDNA3.1(+)/Hygro-hTRPV1 was transfected into HEK-293 cells using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). The transfected cells were selected with 300  $\mu$ g/mL hygromycin B. Hygromycin B-resistant cells were obtained by limited dilution and then were screened for hTRPV1-expressing clones by the  $\text{Ca}^{2+}$  mobilization assay described below. The most sensitive clone was termed as 293EBNA/hTRPV1, and this was used for the subsequent experiments.

### *Measurement of the antagonist activity at capsaicin-stimulated $\text{Ca}^{2+}$ response in 293EBNA/hTRPV1 cells*

The antagonist activity of the compounds for the capsaicin-stimulated  $\text{Ca}^{2+}$  response was determined with a  $\text{Ca}^{2+}$  influx assay by measuring the effect on the capsaicin-evoked increase in intracellular  $\text{Ca}^{2+}$  using 293EBNA/hTRPV1 cells. The  $\text{Ca}^{2+}$  response was monitored using a fluorescent probe in conjunction with a fluorometric imaging plate reader (FLIPR) (Molecular Devices, Sunnyvale, CA, USA) or a Functional Drug Screening System 6000 (FDSS6000) (Hamamatsu Photonics, Hamamatsu). The 293EBNA/hTRPV1 cells were seeded at  $2.5 \times 10^4$  cells/well into poly-D-lysine-coated 384-well plates and cultured overnight. The cells were loaded with FLIPR Calcium 3 assay kit dye solution in Hanks' balanced salt solution (HBSS) for 1 h. Then, the cells were preincubated with TRPV1 antagonists for 30 min at 37°C. After the preincubation, the cells were stimulated with capsaicin at the indicated temperature, and the fluorescence intensity was monitored. Curve fits of the data were solved using a four-parameter logistic equation using the XLfit software program (ID Business Solutions, Guilford, UK), and  $\text{IC}_{50}$  values were calculated.

### *Measurement of the antagonist activity for the acid-stimulated $\text{Ca}^{2+}$ response in 293EBNA/hTRPV1 cells*

The antagonist activity of the compounds for the acid-stimulated  $\text{Ca}^{2+}$  response was determined with a  $\text{Ca}^{2+}$  influx assay by measuring the effect on the pH 5.0-evoked increase in the intracellular  $\text{Ca}^{2+}$  response using 293EBNA/hTRPV1 cells. These experiments were performed using the previously reported method (27) with a slight modification. The  $\text{Ca}^{2+}$  response was monitored using a fluorescent probe in conjunction with FDSS6000. 293EBNA/hTRPV1 cells were seeded at  $3 \times 10^4$  cells/well into poly-L-lysine-coated 96-well plates and cultured overnight. After the medium was removed, the cells were loaded with Fluo-4 acetoxymethyl ester (Fluo-4-AM) in HBSS for 1 h. Then, the Fluo-4-AM solution was substituted with the HBSS containing 10 mM citric acid and 3.5 mM  $\text{CaCl}_2$ . The cells were preincubated with compounds for 5 min at 37°C, followed by stimulation with pH 5.0 at 37°C. The fluorescence intensity was monitored using the FDSS6000 instrument. Curve fits of the data were solved using a four-parameter logistic equation using the XLfit software program, and  $\text{IC}_{50}$  values were calculated.

### *Measurement of the antagonist activity for the heat-stimulated $\text{Ca}^{2+}$ response in PDBu-sensitized 293EBNA/hTRPV1 cells*

The antagonist activity of the compounds for the heat-stimulated  $\text{Ca}^{2+}$  response was determined with a  $\text{Ca}^{2+}$  influx assay by measuring the effect on the heat-evoked increase (stimulation with a temperature below the normal threshold) on the intracellular  $\text{Ca}^{2+}$  using PDBu-sensitized 293EBNA/hTRPV1 cells. The  $\text{Ca}^{2+}$  response was monitored using a fluorescent probe in conjunction with a FDSS6000 instrument. The 293EBNA/hTRPV1 cells were seeded at  $3.2 \times 10^4$  cells/well into poly-L-lysine-coated 96-well plates and cultured overnight. The cells were loaded with FLIPR Calcium 3 assay kit dye solution in HBSS for 1 h. Then, the cells were preincubated with TRPV1 antagonists for 30 min at 39°C. After the incubation, 1  $\mu$ M PDBu was added to the cells at 40°C, and the fluorescence intensity was monitored using the FDSS6000 instrument. Curve fits of the data were solved using a four-parameter logistic equation using the XLfit software program, and  $\text{IC}_{50}$  values were calculated.

### *Western blot analysis*

The 293EBNA/hTRPV1 cells were seeded at  $4 \times 10^5$  cells/well into 6-well plates and cultured overnight. The cells were cultured in serum-free DMEM for 24 h, and then were incubated with a PKC inhibitor (GF 109203X), a MEK inhibitor (PD98059), or TRPV1 antagonist for 30

min, followed by stimulation with PDBu for 10 min. After the medium was removed, the cells were rinsed with ice-cold PBS and mechanically detached in ice-cold RIPA buffer (ThermoScientific, Rockford, IL, USA) containing a phosphatase inhibitor cocktail (Nakalai Tesque, Kyoto). After the cells were homogenized and centrifuged at 15,000 rpm for 20 min at 4°C, the supernatants were collected. These cell lysates were subjected to SDS-PAGE and then were transferred to polyvinylidene difluoride membrane with the semi-dry method. The membrane was incubated with an anti-extracellular signal regulated protein kinase 1/2 (ERK1/2)-specific rabbit polyclonal antibody (Promega, Madison, WI, USA) or anti-phospho-ERK-specific rabbit polyclonal antibody (Promega), followed by incubation with a horseradish peroxidase-conjugated anti-rabbit IgG antibody (GE Healthcare, Buckinghamshire, UK). The bands were detected by SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific) and LAS-1000 (Fujifilm, Tokyo).

#### *Electrophysiology of rat DRG cells*

The DRGs (L4-L6 segments) isolated from SD rats were incubated with 0.1% trypsin for 10 min at 35°C–36°C, followed by the incubation with 0.1% collagenase for 40 min at 35°C–36°C. After being dispersed by pipetting, the DRG cells were seeded on 35-mm culture dishes containing 12-mm diameter glass coverslips coated with poly-D-lysine and then placed for more than 60 min in an atmosphere containing 5% CO<sub>2</sub> at 37°C. The dishes were positioned on the stage of an inverted microscope and superfused with a bath solution (150 mM NaCl, 5 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM D-glucose, and 10 mM HEPES, adjusted to pH 7.4 with NaOH). The membrane currents in the DRG cells were recorded using the conventional whole-cell patch clamp technique. Patch pipettes were fabricated from glass capillary tubes (GDC1.5; Narishige, Tokyo) by a vertical two-stage electrode puller (PP-83, Narishige). The patch pipettes had a resistance of 3–5 MΩ when filled with a pipette solution (140 mM KCl, 1 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 11 mM EGTA, 10 mM HEPES, adjusted to pH 7.4 with KOH). The membrane currents were recorded with an Axopatch 1D instrument (Axon Instruments, Inc., Foster City, CA, USA) using a pClamp6 data acquisition software package (Axon Instruments, Inc.). The sampling frequency for acquisition was 3.3 or 10 Hz. All experiments were performed at room temperature (22°C–26°C) under voltage clamping at –60 mV. The capacitance and series resistance were compensated by 80% without oscillations. All compounds were added to the bath solution and applied via continuous superfusion. During the stimulation with

low pH, the bath solution was replaced by a stimulation buffer (150 mM NaCl, 5 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM D-glucose, and 10 mM HEPES, adjusted to pH 5.5 with NaOH). During the stimulation with heat, cells were superfused with bath solution preheated to 37°C. During this stimulation, the actual temperature of the cells on coverslips was 34°C.

#### *CFA model*

CFA was injected into the left hind paws of SD rats. At 24 h after the injection of CFA, K-685 or diclofenac was administered orally. The pain behaviors were monitored at 1, 3, and 5 h after administration. Mechanical allodynia was monitored according to the method reported elsewhere (28). Briefly, the mechanical paw withdrawal threshold in the left hind paw of CFA-injected rats was monitored using a von-Frey filament. Rats with a 50% threshold less than 4 g were considered allodynic and were used for testing. The latency of the thermal pain-related behaviors for the left hind limb in CFA-injected rats was assessed using a 48°C Hot/Cold Plate 35100 (Ugo Basile, Comerio, Italy). Rats with latencies less than 70% of those of the normal rats were considered to be hyperalgesic and were used for testing.

The edema in the left hind paw was measured by a plethysmometer (TK-101; Muromachi Kikai, Tokyo) on the day after the injection of CFA. Edema was monitored hourly for 4 h.

#### *Statistical analyses*

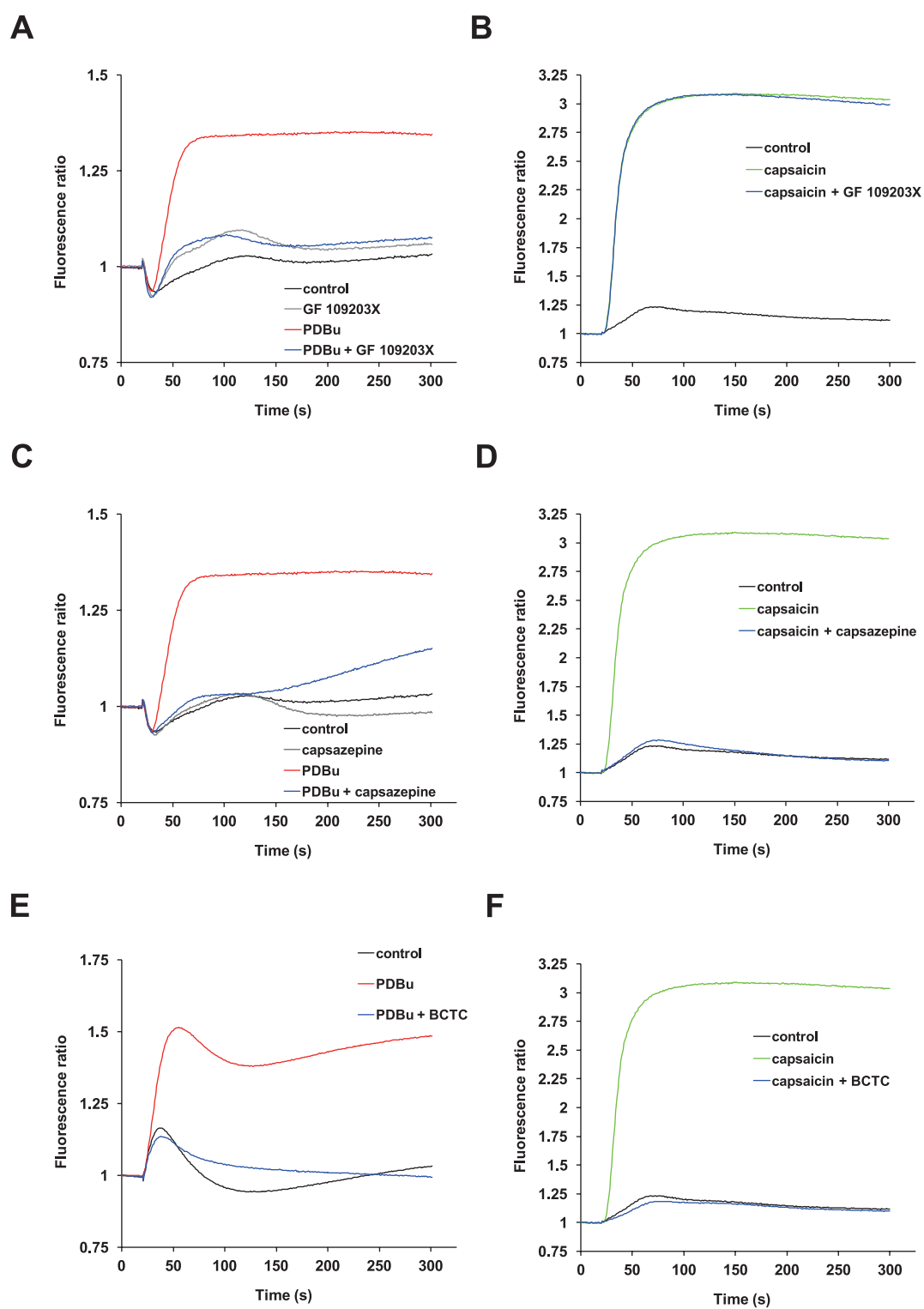
The data were presented as the means and standard errors of the means (SEM). Student's *t*-test or the Wilcoxon test was used for the analysis of any differences in parametric or non-parametric data between the two groups, respectively. Multiple comparisons of parametric or non-parametric data between treatment groups were assessed by Dunnett's test or the Steel test, respectively. Values of *P* < 0.05 were considered to be statistically significant. All statistical calculations were performed using the Statistical Analysis System (Release 9.1.3; SAS Institute, Cary, NC, USA) software package.

## **Results**

#### *PKC-mediated sensitization of hTRPV1*

Phorbol ester PDBu (1 μM) was added to the 293EBNA/hTRPV1 cells at 40°C, which is a temperature below the normal threshold of activation of TRPV1. The intracellular Ca<sup>2+</sup> level was increased by PDBu in 293EBNA/hTRPV1 cells, while the intracellular Ca<sup>2+</sup> in 293EBNA/hTRPV1 cells was not altered without PDBu at this temperature (Fig. 2: A, C, E). This PDBu-induced Ca<sup>2+</sup> increase was attenuated by TRPV1 antagonists



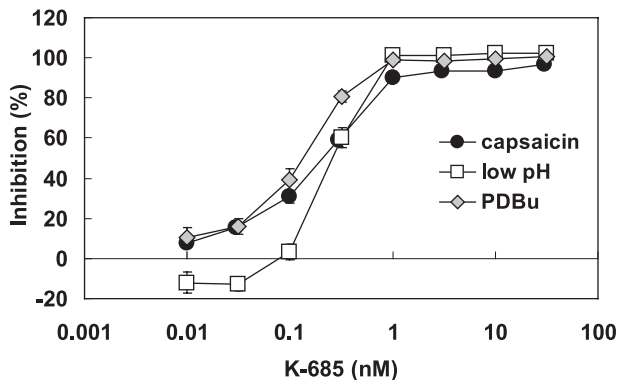


**Fig. 2.** The intracellular  $\text{Ca}^{2+}$  response in 293EBNA/hTRPV1 cells. The 293EBNA/hTRPV1 cells were preincubated with 5  $\mu\text{M}$  GF 109203X (A and B), 10  $\mu\text{M}$  capsazepine (C and D), or 1  $\mu\text{M}$  BCTC (E and F) for 30 min, followed by stimulation with 1  $\mu\text{M}$  PDBu (A, C, and E) or 1  $\mu\text{M}$  capsaicin (B, D, and F). The experiments were performed at 40°C. The data represent the means of 8 wells. Fluorescence signals were expressed as ratios to the initial fluorescence values.

(capsazepine and BCTC) or a PKC inhibitor (GF 109203X) (Fig. 2: A, C, E). Treatment with a PKC inhibitor (GF 109203X) did not influence the capsaicin-induced increase in intracellular  $\text{Ca}^{2+}$  in 293EBNA/hTRPV1 cells, while TRPV1 antagonists, capsazepine and BCTC, attenuated this capsaicin-induced  $\text{Ca}^{2+}$  increase (Fig. 2: B, D, F). While transient small changes in the fluorescence were seen in several experiments (Fig. 2: A, C, E) following the addition of PDBu, these changes were also found in the absence of PDBu. In 293EBNA cells, neither PDBu nor capsaicin increases the intracellular  $\text{Ca}^{2+}$  (data not shown).

#### Effect of K-685 on TRPV1 activation

In the assay system described above, our TRPV1 antagonists reported in the previous study attenuated the PDBu-induced increase in the intracellular  $\text{Ca}^{2+}$  in 293EBNA/hTRPV1 cells (data not shown). Our representative compound, K-685, whose  $\text{IC}_{50}$  for capsaicin (100 nM)-induced hTRPV1 activation was  $0.206 \pm 0.041$  nM (Fig. 3 and Table 1), inhibited this PDBu-mediated response with an  $\text{IC}_{50}$  of  $0.136 \pm 0.027$  nM (Fig. 3 and Table 1).

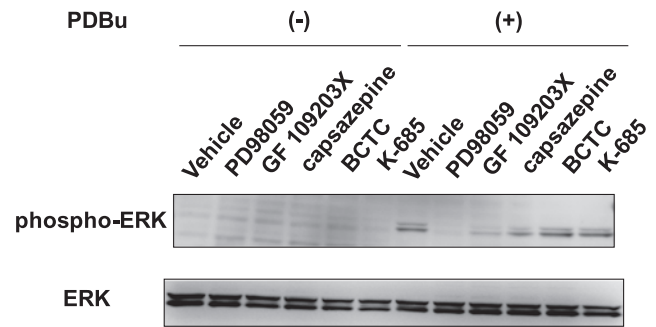


**Fig. 3.** The effect of K-685 on the capsaicin-, acid-, or PDBu-induced  $\text{Ca}^{2+}$  response in 293EBNA/hTRPV1 cells. The 293EBNA/hTRPV1 cells were preincubated with various concentrations of K-685, followed by stimulation with 100 nM capsaicin, pH 5.0 or 1  $\mu\text{M}$  PDBu at room temperature, 37°C, or 40°C, respectively. Each symbol represents the mean  $\pm$  S.E.M. (capsaicin,  $n = 4$ ; low pH,  $n = 3$ ; PDBu,  $n = 3$ ).

**Table 1.** The  $\text{IC}_{50}$  values (nM) of K-685 for the  $\text{Ca}^{2+}$  response in 293EBNA/hTRPV1 cells

Capsaicin (100 nM)	Acid (pH 5.0)	PDBu (1 $\mu\text{M}$ )
$0.206 \pm 0.041$	$0.261 \pm 0.042$	$0.136 \pm 0.027$

The 293EBNA/hTRPV1 cells were preincubated with various concentrations of K-685, followed by stimulation with 100 nM capsaicin, pH 5.0 or 1  $\mu\text{M}$  PDBu. The data represent the means  $\pm$  S.E.M. (capsaicin,  $n = 4$ ; acid,  $n = 3$ ; PDBu,  $n = 3$ ).



**Fig. 4.** The effects of compounds on the PDBu-induced activation of ERK. The 293EBNA/hTRPV1 cells were preincubated with 1  $\mu\text{M}$  PD98059, 5  $\mu\text{M}$  GF 109203X, 10  $\mu\text{M}$  capsazepine, 1  $\mu\text{M}$  BCTC, or 1  $\mu\text{M}$  K-685 for 30 min, followed by stimulation with 1  $\mu\text{M}$  PDBu for 10 min. Cell lysates were prepared and subjected to western blot analysis using anti-phospho-ERK or anti ERK1/2 antibodies. The experiment was repeated twice and a representative image of a blot is shown.

The acid (pH 5.0)-induced hTRPV1 activation was also attenuated by K-685. The  $\text{IC}_{50}$  for acid-induced hTRPV1 activation was  $0.261 \pm 0.042$  nM (Fig. 3 and Table 1).

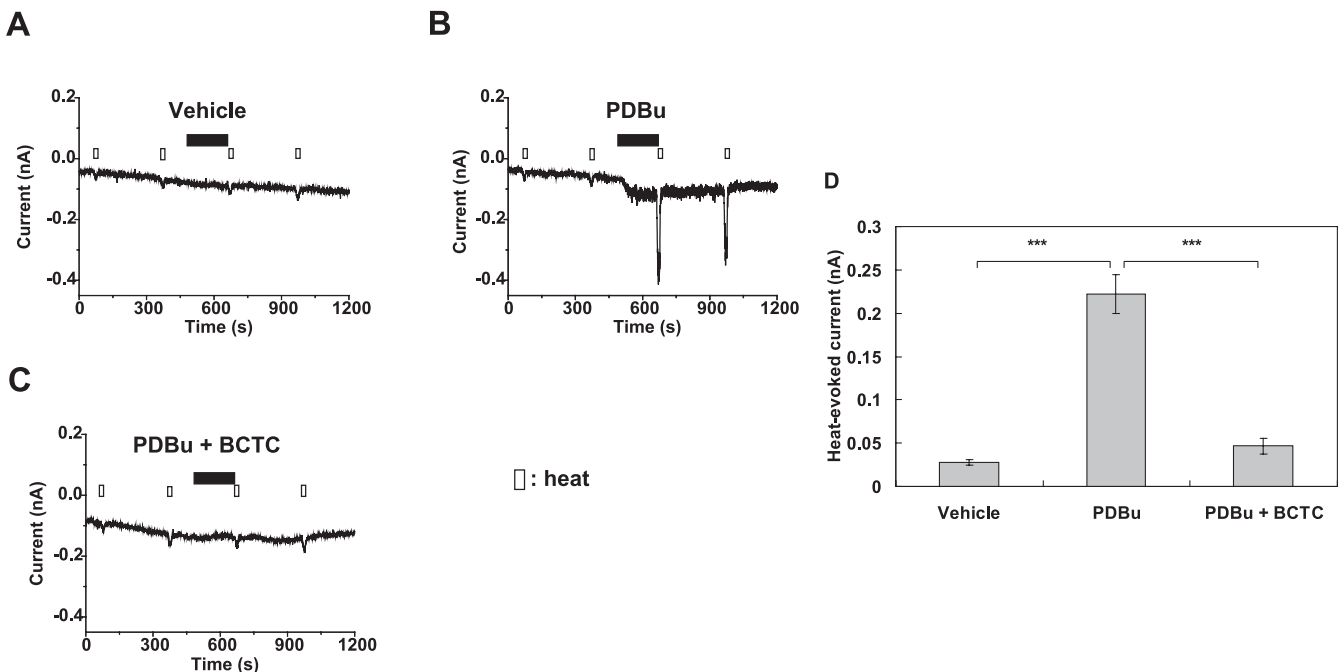
#### Effect of K-685 on PKC-dependent ERK activation

To investigate whether K-685 inhibited the PDBu-sensitized TRPV1 activation by inhibiting PKC activity, PDBu-induced ERK activation was monitored by a western blot analysis using a phospho-ERK specific antibody. As a result, K-685 did not alter the PDBu-induced ERK activation, as was seen following BCTC and capsazepine treatment (Fig. 4). In this experiment, the PKC inhibitor, GF 109203X, inhibited PDBu-induced ERK activation, as did a MEK inhibitor, PD98059 (Fig. 4).

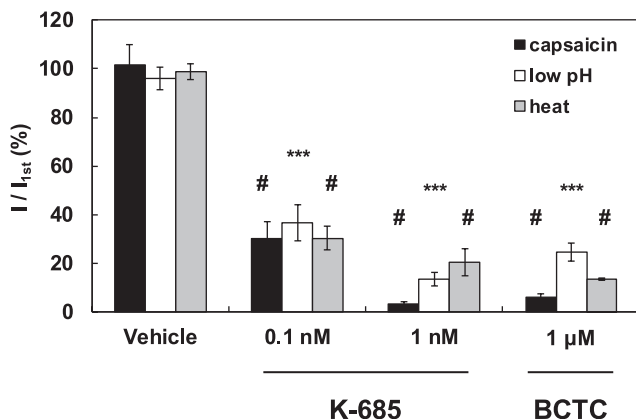
#### PKC-mediated sensitization of TRPV1 in rat DRG neurons

PDBu (1  $\mu\text{M}$ ) was added to rat DRG neurons, followed by stimulation with heat (34°C). The TRPV1-induced inward current was monitored by a whole-cell patch clamp. Heat stimulation at this temperature induced an inward current after pretreatment with PDBu (Fig. 5B), but not without pretreatment with PDBu (Fig. 5A). This PDBu-related sensitization was attenuated by BCTC (Fig. 5C).

The PDBu-sensitized heat-induced inward current in rat DRG neurons was inhibited in the presence of K-685 (Fig. 6). The current was especially abolished by treatment with 1 nM K-685 (Fig. 6). K-685 also significantly inhibited capsaicin- and acid-induced inward currents (Fig. 6).



**Fig. 5.** Sensitization of the inward currents by PDBu in rat DRG neurons. A – C) Representative traces of heat-evoked currents. Heat (34°C for 15 s) was applied successively. In some experiments, cells were treated with 1  $\mu$ M PDBu or 1  $\mu$ M BCTC for 3 min before the third heat stimulation. D) The bar graph represents the average of the third heat-evoked current amplitude observed in panel A. In all experiments, the basal temperature was room temperature (22°C – 26°C). Each column represents the mean  $\pm$  S.E.M. (vehicle,  $n = 4$ ; PDBu,  $n = 8$ ; PDBu + BCTC,  $n = 6$ ). \*\*\* $P < 0.001$ , significantly different from PDBu.



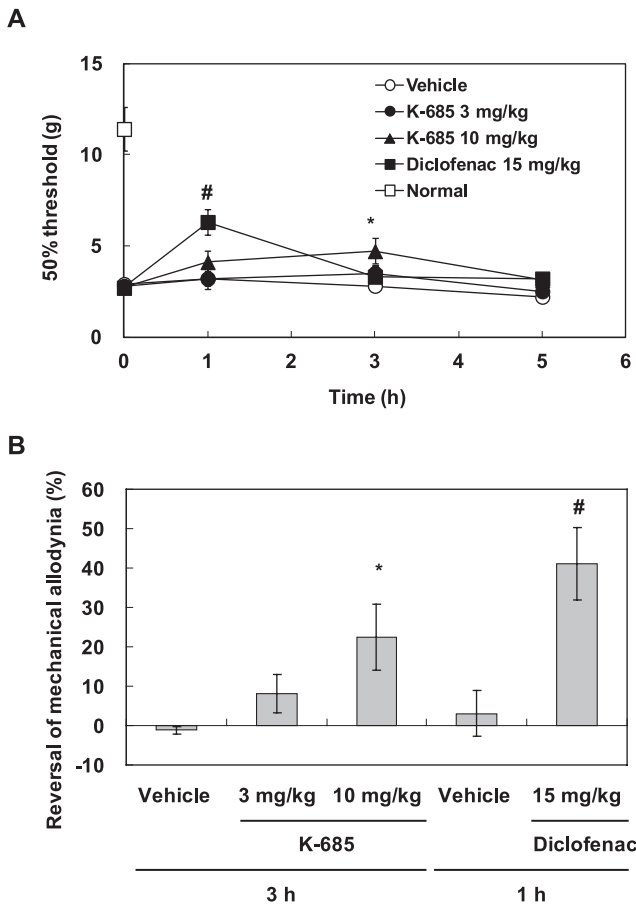
**Fig. 6.** The effects of TRPV1 antagonists on the inward currents in rat DRG neurons. Capsaicin (50 nM for 15 s) or acid (pH 5.5 for 15 s) was applied successively. Heat (34°C for 15 s) was also applied successively to PDBu-pretreated cells. Cells were treated with vehicle, K-685 (0.1, 1 nM) or BCTC (1  $\mu$ M) for 5 min before the second capsaisin stimulation. All experiments were performed at room temperature (22°C – 26°C) except for the heat stimulation. The data are shown as the ratio of the second/first capsaisin-, acid-, or heat-evoked currents. Each column represents the mean  $\pm$  S.E.M. ( $n = 5$ ). \*\*\* $P < 0.001$ , significantly different from the vehicle (Dunnett test); # $P < 0.05$ , significantly different from the vehicle (Steel test).

#### CFA model rats

The effect of K-685 on mechanical allodynia and thermal hyperalgesia was examined in a rat CFA model. K-685 (10 mg/kg) significantly reversed both mechanical allodynia (22.5%) and thermal hyperalgesia (68.9%) (Figs. 7 and 8). The degree of reversal of the thermal hyperalgesia was greater than that induced by diclofenac (15 mg/kg; 39.0%) (Fig. 8). On the other hand, K-685 did not affect the paw edema caused by CFA-induced inflammation, while diclofenac did decrease the edema (Fig. 9).

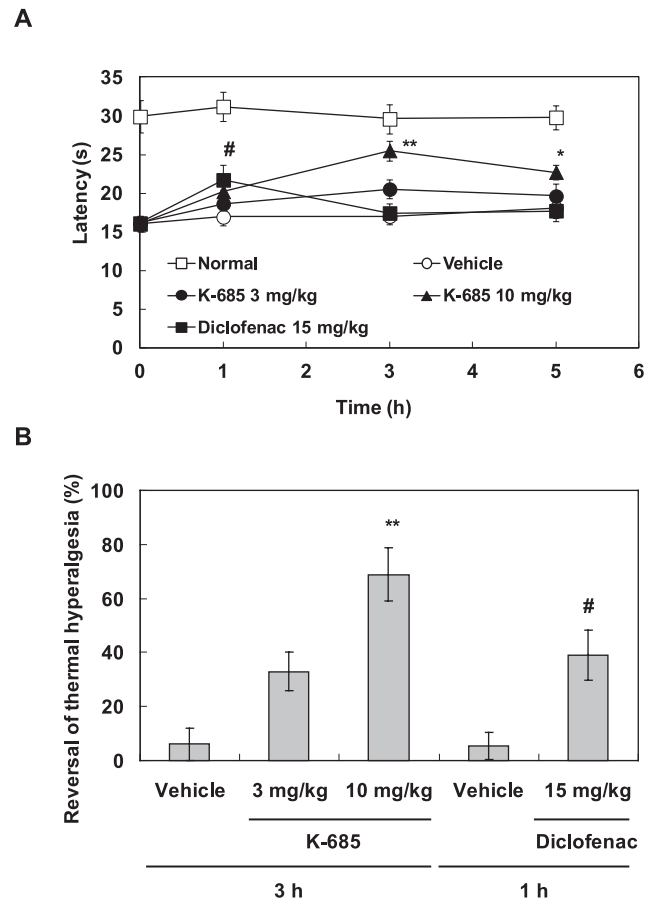
#### Discussion

In a previous study, we discovered novel 5,5-diaryl-pentadienamides, including K-685, that act as orally active TRPV1 antagonists (26). We also evaluated the binding affinities of K-685 to 12 pain-related receptors and 7 ion channels other than TRPV1 and confirmed that K-685 had more than 1000-fold selectivity for TRPV1 over these receptors and channels (data not shown). Therefore, K-685 was a highly TRPV1-selective antagonist. In the present study, the in vitro biological properties of K-685 were evaluated, especially on PKC-sensitized TRPV1. When phosphorylated by PKC, the



**Fig. 7.** The effect of K-685 on the mechanical allodynia in CFA-administrated rats. Rats were injected with CFA into the plantar region of the left hind paw 24 h prior to the experiment. Drugs and vehicle were administered orally. The mechanical paw withdrawal threshold in the left hind paw of CFA-injected rats was monitored using a von-Frey filament. Each symbol (A) or column (B) represents the mean  $\pm$  S.E.M. ( $n = 6$ ). The threshold in rats that were not injected with CFA (Normal) is also shown. \* $P < 0.05$ , significantly different from the vehicle (Steel-test); # $P < 0.05$ , significantly different from the vehicle (Wilcoxon test).

threshold temperature of TRPV1 is reported to drop from 43°C to below 37°C (17, 18). This sensitization suggests that TRPV1 is activated even at body temperature when it is phosphorylated by PKC. Using the FDSS6000 system, which has an internal incubator, the  $\text{Ca}^{2+}$  response was monitored following the addition of phorbol ester, PDBu, at 40°C, which is a temperature below the normal threshold. To facilitate stable detection of sensitization-induced response and stable evaluation of antagonist activity of compounds, we stimulated 293EBNA/TRPV1 cells at relatively higher temperature (40°C). In this in vitro assay system, PDBu increased the intracellular  $\text{Ca}^{2+}$  level in 293EBNA/hTRPV1 cells. On the other hand, the intracellular  $\text{Ca}^{2+}$  level was not increased in 293EBNA/hTRPV1 cells at 40°C without PDBu. This response

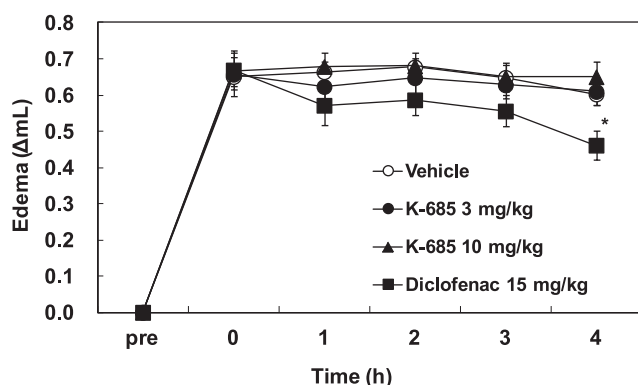


**Fig. 8.** The effect of K-685 on the thermal hyperalgesia in CFA-administrated rats. Rats were injected with CFA into the plantar region of the left hind paw 24 h prior to the experiment. Drugs and vehicle were administered orally. The latency of the thermal pain-related behaviors in the left hind limb in CFA-injected rats was assessed. Each symbol (A) or column (B) represents the mean  $\pm$  S.E.M. ( $n = 8$ ). The latency in rats that were not injected with CFA (Normal) is also shown. \* $P < 0.05$ , \*\* $P < 0.01$ , significantly different from the vehicle (Dunnett test); # $P < 0.01$ , significantly different from the vehicle ( $t$ -test).

was attenuated by treatment with a PKC inhibitor, GF 109203X, which was distinct from the capsaicin-induced response. The PDBu-induced intracellular  $\text{Ca}^{2+}$  increase was inhibited by TRPV1 antagonists. These results suggest that this PDBu-induced  $\text{Ca}^{2+}$  response is mainly dependent on PKC-sensitized TRPV1. Although there seems to be a small fraction of the  $\text{Ca}^{2+}$  response that GF 109203X did not inhibit, PDBu did not cause a  $\text{Ca}^{2+}$  response in the presence of GF 109203X compared with the response to vehicle in the cells pretreated with GF 109203X. Therefore, we concluded that the response to PDBu found in this study was mainly PKC-dependent, but was not direct agonistic activity against TRPV1.

In this assay system, our TRPV1 antagonist K-685, potentially attenuated the PDBu-dependent  $\text{Ca}^{2+}$  increase





**Fig. 9.** The effect of K-685 on the paw edema in CFA-administrated rats. Rats were injected with CFA into the plantar region of the left hind paw 24 h prior to the experiment. Drugs were administered orally. Each symbol represents the mean  $\pm$  S.E.M. ( $n = 8$ ). \* $P < 0.05$ , significantly different from the vehicle ( $t$ -test).

induced by heat stimulation with an  $IC_{50}$  of  $0.136 \pm 0.027$  nM. The potency of K-685 for inhibiting PDBu-induced TRPV1 activation was comparable to, and slightly higher than, those of capsaicin- and acid-stimulated TRPV1 activation, respectively. Moreover, the potency of K-685 was 10-fold higher than that of A425619, whose  $pIC_{50}$  for heat-induced activation of TRPV1 sensitized by PDBu was reported to be 8.70 (21). Since K-685 did not inhibit the PDBu-induced ERK phosphorylation, which is a PKC-dependent event (29), it is suggested that the inhibition of the PDBu-dependent  $Ca^{2+}$  increase by K-685 was dependent on the TRPV1 antagonist activity, not on the inhibition of PKC. This result suggests that K-685 has the potential to inhibit TRPV1 activity due to sensitization with PKC.

The PDBu-sensitized TRPV1 activation that was seen in 293EBNA/hTRPV1 cells was also seen by electrophysiological studies using rat DRG neurons. K-685 inhibited the PDBu-sensitized inward current in rat DRG neurons. In the present study, rat DRG neurons responded to the  $34^{\circ}\text{C}$  condition in the presence of PDBu. These results suggest that our compounds have the potential to attenuate TRPV1 activity in DRG neurons sensitized by PKC during inflammation. Moreover, K-685 also inhibited the capsaicin- and acid-induced inward currents in rat DRG neurons, which showed that K-685 has a broad spectrum of antagonist activity on rat TRPV1 under physiological conditions.

In the previous report, when TRPV1 was phosphorylated by bradykinin or PKC and then the temperature was increased gradually, an inward current was detected below  $30^{\circ}\text{C}$ , which was not detected under normal conditions (17, 18). In the present study, the application of PDBu at room temperature ( $22^{\circ}\text{C} - 26^{\circ}\text{C}$ ) caused a small

shift of the baseline, and this shift was not seen in the presence of BCTC, which suggested that there was activation of TRPV1. Since the small shift of the baseline in this study coincided with this inward current in the previous report, we consider that the phosphorylation of TRPV1 by PKC might influence the TRPV1 function even at room temperature. Thus, the phosphorylation of TRPV1 by PKC is suggested to have a major impact on the activity of TRPV1, especially under inflammation, where many mediators to activate PKC in neuronal cells are released. Therefore, K-685, which inhibited the inward current through TRPV1 sensitized with PKC, may ameliorate inflammatory pain.

The effects of K-685 on nociceptive behaviors were examined in a rat CFA inflammatory model. The results showed that K-685 reversed the mechanical allodynia and thermal hyperalgesia. The effect of K-685 on the thermal hyperalgesia and mechanical allodynia was significant at 3 h after oral administration. Our pharmacokinetics studies suggest that the long time required to reach peak plasma level of this compound in rats (data not shown) might contribute to the delay in the effect. Since K-685 did not affect the paw edema in this model, K-685 showed antinociceptive effects through its blockade of TRPV1 activity, not through the inhibition of inflammation. Of note, K-685 improved the thermal hyperalgesia with greater efficacy than diclofenac. This difference in efficacy between mechanical allodynia and thermal hyperalgesia in the CFA model has also been reported for other TRPV1 antagonists (30). In this report, the  $ED_{50}$  of TRPV1 antagonist A-784168 for the reversal of mechanical allodynia was about nine-times greater than that of thermal hyperalgesia (30). Furthermore, it was also reported that TRPV1 antagonist A-795614 with poor CNS penetration leads to limited improvement of mechanical allodynia (30). It is possible that the TRPV1 in CNS neurons is involved in the mechanical allodynia in the CFA model. These experiments suggest that TRPV1 antagonists with CNS penetration are likely to have more favorable effects on the improvement of mechanical allodynia in the CFA model. Although it is unclear whether the PKC-mediated sensitization of TRPV1 also occurred in CNS neurons, the CNS permeability of K-685 (26) might also contribute to its effect on mechanical allodynia.

These results suggest that K-685 has potential for use as a therapeutic drug for inflammatory chronic pain. It has been reported that some TRPV1 antagonists led to an elevation of the body temperature and the heat pain threshold in clinical studies (31, 32). Since it is not clear whether K-685 will have similar effects, these safety issues will need to be examined for K-685 prior to its being considered for clinical application.

In this study, it was revealed that K-685 has potent antagonistic activity for TRPV1 in vitro (PKC-sensitized TRPV1 activity) and in vivo (rat CFA model) under inflammatory condition. It has been suggested that the sensitization of TRPV1 by PKC has a role in inflammatory pain and TRPV1 antagonists that inhibit TRPV1 activity in such a sensitized state may be effective for the treatment of inflammatory pain.

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