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Novel agonistic action of mustard oil on recombinant and endogenous porcine transient receptor potential V1 (pTRPV1) channels

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ABSTRACT

Neurogenic components play a crucial role in inflammation and nociception. Mustard oil (MO) is a pungent plant extract from mustard seed, horseradish and wasabi, the main constituent of which is allylisothiocyanate. We have characterized the action of MO on transient receptor potential V1 (TRPV1), a key receptor of signal transduction pathways in the nociceptive system, using fura-2-based $[Ca^{2+}]_i$ imaging and the patch-clamp technique in a heterologous expression system and sensory neurons. In human embryonic kidney (HEK) 293 cells expressing porcine TRPV1 (pTRPV1), MO evoked increases of $[Ca^{2+}]_i$ in a concentration-dependent manner. A high concentration of MO elicited irreversible cell swelling. Capsazepine, ruthenium red and iodoresiniferatoxin dose-dependently suppressed the MO-induced $[Ca^{2+}]_i$ increase. MO elicited outward rectified currents in pTRPV1-expressing HEK 293 cells with a reversal potential similar to that of capsaicin. $[Ca^{2+}]_i$ responses to MO were completely abolished by the removal of external Ca^{2+}. MO simultaneously elicited an inward current and increase of $[Ca^{2+}]_i$ in the same cells, indicating that MO promoted Ca^{2+} influx through TRPV1 channels. In cultured porcine dorsal root ganglion (DRG) neurons, MO elicited a $[Ca^{2+}]_i$ increase and inward current. Among DRG neurons responding to MO, 85% were also sensitive to capsaicin. The present data indicate that MO is a novel agonist of TRPV1 channels, and suggest that the action of MO in vivo may be partly mediated via TRPV1. These results provide an insight into the TRPV1-mediated effects of MO on inflammation and hyperalgesia.

1. Introduction

A neurogenic component has been reported to play a pivotal role in the pathological mechanisms of numerous inflammatory diseases [1,2] and nociception [3]. The vanilloid receptor 1 (VR1), which is now referred to as transient receptor potential V1 (TRPV1), is a non-selective cation channel found in sensory neurons and functions as a molecular integrator of pain perception. TRPV1 is activated by protons, heat and capsaicin, which is the pungent agent in chilli peppers which produces itching, picking and burning sensations [4–6]. Because mice genetically lacking the TRPV1 receptor exhibit impaired nociception [7,8], TRPV1 is considered to be a key component of signal transduction pathways in the nociceptive system.

Many naturally occurring pungent compounds have been reported to be activators of TRPV1 [9]. These include the

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vanilloid compounds such as the cactus extract resiniferatoxin, the ginger extracts zingerone and gingerol, and the nutmeg and clove oil constituent eugenol [10]. More recently, some endogenous activators of TRPV1, such as endovanilloids, have been identified [11]. Both agonist and antagonist strategies are thought to have merit for the treatment of a whole range of conditions ranging from inflammatory and neuropathic pain, to bladder dysfunction and irritable bowel syndrome [12].

Mustard oil (MO) is one of the pungent components of mustard seed, horseradish and wasabi [13] and its major active constituent is allylisothiocyanate [14]. Topical application of MO to the skin activates underlying sensory nerve endings, resulting in the production of burning pain, inflammation and hypersensitivity to thermal and mechanical stimuli [15–17]. MO causes a neurogenic inflammation, which is not observed in a denervated component of the rat hind paw skin [18]. However, the cellular targets and mechanisms of action of MO are still not fully elucidated. A recent report indicates that ankylin transmembrane protein 1 (ANKTM1) or TRPA1 [19], a member of the TRP channel family implicated in the detection of noxious cold, is activated by MO [13]. Similar to capsaicin, instillation of MO into the colon induces visceral pain behavior, hyperalgesia and plasma extravasation [20–22]. It is still a matter of debate whether this occurs through a direct action on sensory neurons or through a specific membrane receptor [9]. Since TRPVI-deficient mice still retain sensitivity to MO [23], capsaicin and MO have been suggested to excite nociceptors through distinct molecular mechanisms. On the other hand, it is suggested that MO and capsaicin act through convergent cellular signaling pathways, because inflammatory responses produced by these irritants show partial cross-desensitization [24]. In fact, in a mouse model of visceral pain induced by colonic instillation of MO, similar activation of spinal extracellular-regulated kinases (ERK1/2) in response to capsaicin is observed [25]. Using this model, a TRPV1 antagonist causes significant inhibition of MO-induced nociception [26]. Therefore, it is of interest to examine whether MO stimulates TRPV1 channels directly or indirectly.

Recently, we cloned a porcine orthologue of TRPV1 (pTRPV1) and analyzed its functional properties using a heterologous expression system [27]. We showed that pTRPV1 was a non-selective cation channel sensitive to a number of vanilloid agonists, including capsaicin and endovanilloids, heat and protons. From pharmacological characteristics, it has been shown that there were remarkable species differences; e.g., PPAHV, an agonist for TRPV1, can stimulate rodent [28–31] and porcine TRPV1 [27] but not human [29] and guinea pig orthologues [32]. Capsazepine, a TRPV1 antagonist, does not antagonize proton- and heat-induced responses in rodent TRPV1 [29,30] but does antagonize in human [29], guinea pig [32] and porcine TRPV1 [27]. It is apparent that chicken TRPV1 is not sensitive to capsaicin [33] and rabbit TRPV1 is less sensitive to capsaicin [34]. Although it has been reported that rat TRPV1-transfected cells are insensitive to MO [13,35], due to the presence of significant evidence for species differences in the molecular pharmacology, it is worth investigating whether MO stimulates other TRPV1 orthologues.

In the present study, to elucidate the molecular mechanisms underlying the action of MO on nociceptors, we investigated whether MO activates TRPV1 in a heterologous expression system and sensory neurons, using pTRPV1-expressing human embryonic kidney (HEK) 293 cells and porcine dorsal root ganglion (DRG) neurons, respectively. To examine TRPV1 activity, we used fura-2-based $[Ca^{2+}]_i$ imaging and whole-cell patch-clamp techniques.

2. Materials and methods

2.1. Cell preparation and transfection

All protocols for experiments on animals were approved by the Committee on Animal Experimentation, Graduate School of Veterinary Medicine, Hokkaido University. Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin G (Meiji-Seika, Japan) and 100 μg/ml streptomycin (Banyu, Japan). Cells were transfected with porcine TRPV1 (pTRPV1) combined with green fluorescent protein (GFP) cDNA [27] using a transfection reagent (Lipofectamine 2000, Invitrogen, USA) and used 36–48 h after transfection. TRPV1-expressing cells were identified by GFP fluorescence with an appropriate filter.

Dorsal root ganglion (DRG) neurons were obtained from male pigs (2–4 weeks after birth, 5–10 kg) as described previously [27]. In brief, pigs were deeply anesthetized by sodium pentobarbital (50 mg/kg, i.p.) after tranquilization with ketamine (15 mg/kg, i.m.) and then killed by bloodletting from the cervical artery. Lumbar DRG were removed, dissected and freed from connective tissue under optical control using fine forceps and scissors in ice-cold phosphate-buffered saline (PBS: in mM, 134.8 NaCl, 8.1 Na_2HPO_4, 1.5 KH_2PO_4, 2.7 KCl) supplemented with penicillin G and streptomycin. Isolated ganglia were cut into small pieces and enzymatically dissociated with collagenase – (1 mg/ml, type II, Worthington, USA) and DNase – (1 mg/ml, Roche, USA) containing PBS for 30 min at 37 °C. Then, ganglia were further digested with trypsin (10 mg/ml, Sigma, USA) and DNase (1 mg/ml) for 30 min at 37 °C. After enzyme digestion, ganglia were immersed in culture medium; M199 (Sigma) supplemented with fetal bovine serum (10%, Sigma), penicillin G (100 U/ml) and streptomycin (100 μg/ml). DRG neurons were obtained by gentle trituration with a fine-polished Pasteur pipette. Then the cell suspension was centrifuged (800 rpm, 5 min, 4 °C) and the pellet containing the cells was resuspended in the culture medium. Aliquots were placed onto glass coverslips coated with poly-D-lysine (Sigma) and cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. In the present experiment, cells cultured within 1 day were used.

2.2. Measurement of intracellular Ca^{2+} concentration

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in individual cells was measured with a fluorescent Ca^{2+} indicator, fura-2 [36], by dual excitation using a fluorescent imaging system controlling illumination and acquisition as described previously [37]. To load fura-2, cells were incubated for 1 h at room temperature with 10 μM fura-2 acetoxymethyl ester (fura-2 AM) in normal external solution (in mM): 134 NaCl, 6

KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 glucose, 10 HEPES (pH 7.4 with NaOH). A coverslip with fura-2-loaded cells was placed in an experimental chamber mounted on the stage of an inverted microscope (Olympus IX71, Japan) equipped with an image acquisition and analysis system (Aqua Cosmos, Hamamatsu Photonics, Japan). Cells with GFP fluorescence were selected for measurement of $[Ca^{2+}]_i$ and the patch-clamp experiment. Cells were illuminated every 2.5 s with light at 340 and 380 nm and the respective fluorescence signals of 500 nm were detected. The fluorescence emitted was projected to a CCD camera (ORCA-ER, Hamamatsu Photonics, Japan) and the ratio of fluorescent signals (F340/F380) was stored on the hard disc of a PC (Pro-2500, EPSON, Japan). Calibration of fura-2 was performed with a Ca^{2+} calibration buffer solution (Molecular Probes, USA) containing 5 μM fura-2. Cells were continuously superfused with the external solution at a flow rate of 1 ml/min through a Y-tube pipette placed close to the cells. All experiments were carried out at room temperature (20–24 °C).

2.3. Whole-cell current recording

Membrane currents were recorded using the conventional whole-cell configuration of the patch-clamp technique with a patch-clamp amplifier (Axopatch 200B, Axon Instruments, USA). The resistance of patch electrodes ranged from 3 to 4 MΩ. The currents were filtered at 1 kHz and sampled at 5 kHz by an A/D converter (Digidata, Axon Instruments) in conjunction with a personal computer. During the experiments and analysis, pClamp 6 software (Axon Instruments) was used. The standard pipette solution contained (in mM): 120 CsCl, 20 tetraethylammonium Cl, 1.2 $MgCl_2$, 2 $ATPNa_2$, 0.2 $GTPNa_3$, 10 HEPES and 10 EGTA (pH 7.2 with CsOH). For simultaneous measurement of $[Ca^{2+}]_i$ with the current responses to capsaicin and MO, EGTA was omitted and fura-2 (0.1 mM) was added to the pipette solution. A Y-tube system similar to that used for $[Ca^{2+}]_i$ measurement was used for drug application and external perfusion.

2.4. Immunocytochemistry

To identify MO-sensitive neurons expressing TRPV1 receptors, cultured porcine DRG cells were subjected to immunostaining with an anti-TRPV1 antibody. After $[Ca^{2+}]_i$ responses to capsaicin and MO were observed, cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature and subsequently rinsed with 10 mM phosphate-buffered saline with 0.3% Triton X-100 (Triton-PBS). Non-specific binding sites were blocked with 10% normal goat serum in Triton-PBS for 1 h. The cells were next incubated with a rabbit antiserum for TRPV1 (diluted 1:100, Santa Cruz, USA) for 1 h at room temperature. After several rinses with Triton-PBS, the antibody was visualized by incubation with Alexa® 488-labeled goat anti-rabbit IgG (10 μg/ml, Molecular Probes) in Triton-PBS for 1 h for the detection of TRPV1. Finally cells were extensively rinsed with Triton-PBS, then with distilled water. Preparations were examined with a confocal laser microscope (FV500, Olympus, Japan) using software (Fluoview, Olympus).

2.5. Chemicals

Capsaicin from Sigma was dissolved in ethanol at a high concentration as a stock solution (1 mM). Capsazepine, icilin, iodoresiniferatoxin, menthol and ruthenium red (Sigma) were dissolved in dimethylsulfoxide (DMSO) to make a stock solution (0.1 M). The drugs were diluted to their final concentration with use of the external solution. The final concentrations of ethanol and DMSO were less than 0.001 and 0.01% (v/v), respectively, which did not affect membrane currents and $[Ca^{2+}]_i$. We used pure allylisothiocyanate (Nakarai Tesque, Japan) as mustard oil, which was dissolved in the external solution and sonicated before use. These drugs did not change the pH of the external solution. All other drugs used were of analytical grade.

2.6. Data analysis

The data are presented as the mean ± S.E.M. (n = number of observations). Comparisons were made by the paired Student's t-test and differences with a p-value of less than 0.05 were considered significant. Pairwise associations were examined by Pearson's linear regression. Values of the 50% effective concentration (EC_{50}) and 50% inhibitory concentration (IC_{50}) were determined using Origin software (OriginLab, USA). The present data were obtained from at least four different transfections or from seven different pigs per experiment.

3. Results

3.1. Mustard oil increased $[Ca^{2+}]_i$ in porcine TRPV1-expressing HEK 293 Cells

We have reported that capsaicin and other vanilloids are capable of evoking increases of $[Ca^{2+}]_i$ in HEK 293 cells heterologously expressing porcine TRPV1 [27]. We examined whether mustard oil was able to stimulate TRPV1 channels using fura-2-based $[Ca^{2+}]_i$ imaging in these cells (Fig. 1). Cells were first stimulated by capsaicin (30 μM) and subsequently by MO (3 mM). Representative time-lapse $[Ca^{2+}]_i$ imaging clearly showed that MO elicited a $[Ca^{2+}]_i$ increase in cells responding to capsaicin (Fig. 1). The time-to-peak of $[Ca^{2+}]_i$ increase induced by MO was significantly slower (81.5 ± 6.1 s, $n = 57$, $p < 0.01$) than that by capsaicin (27.2 ± 1.6 s). When applied MO at a high concentration (30 mM), $[Ca^{2+}]_i$ temporarily increased. About 1 min after its application, fluorescent signals excited at both 340 and 380 nm (F340, F380) gradually declined with marked cell swelling (Fig. 1A(c)) and these shape changes were not recovered with extensive washout of MO.

The $[Ca^{2+}]_i$ increase was induced by MO in pTRPV1-expressing cells, regardless of the order of stimulation by capsaicin (Fig. 1D).

Fig. 2A shows a comparison of the structures of capsaicin and MO. Unlike capsaicin, MO does not possess a vanilloid structure, but both agents have an allyl base in their structures. There was a significantly positive relation between

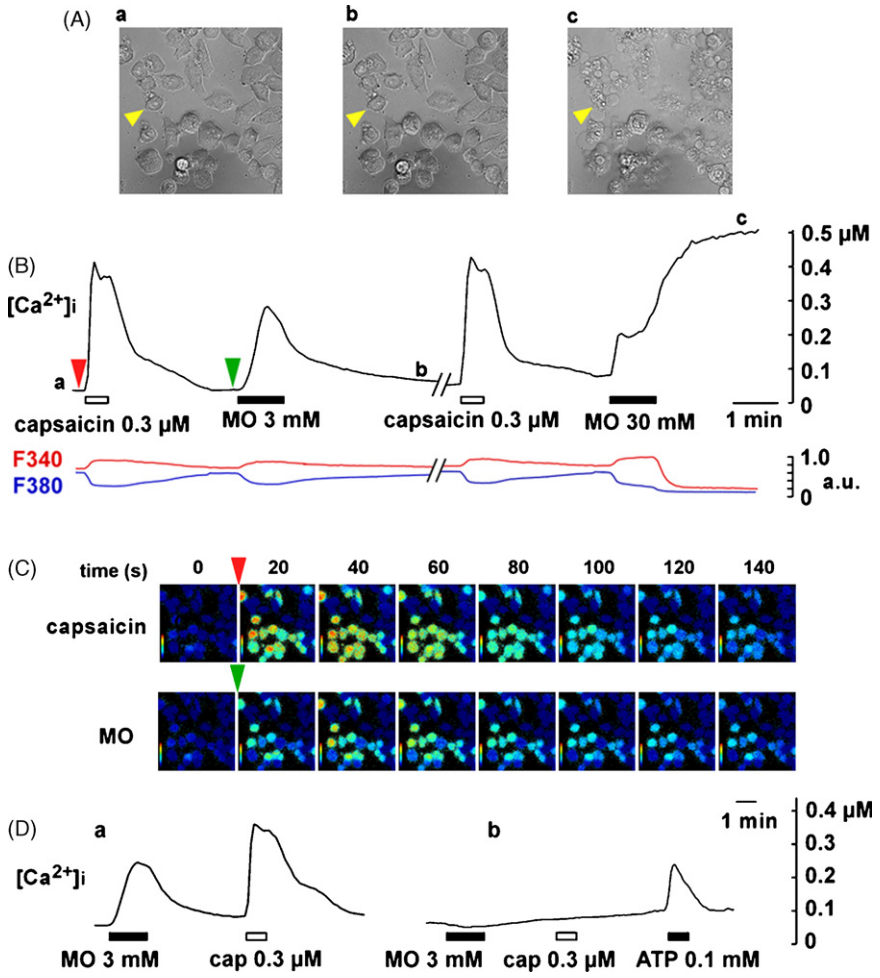

Fig. 1 – MO increases $[Ca^{2+}]_i$ in porcine TRPV1-expressing HEK 293 cells. (A) Images under transmitted light corresponding to the time points in (B) (a–c). (B) Representative $[Ca^{2+}]_i$ increases induced by capsaicin (0.3 μM) and mustard oil (MO; 3, 10 mM) in the same cells marked with a yellow triangle in (A). The upper panel shows $[Ca^{2+}]_i$ and the lower one fluorescent signals of fura-2 (a.u., arbitrary unit) exited at 340 and 380 nm. (C) Pseudcolor time-lapse images from microscope-based ratiometric analysis of $[Ca^{2+}]_i$ corresponding to the time points in (B). Red reflects high $[Ca^{2+}]_i$, and blue low $[Ca^{2+}]_i$. Note that mustard oil induced $[Ca^{2+}]_i$ increases in cells responded to capsaicin. (D): (a) Representative $[Ca^{2+}]_i$ responses to MO applied first, then applied capsaicin. (b) In pTRPV1-untransfected HEK 293 cells, MO and capsaicin (cap) did not evoke $[Ca^{2+}]_i$ increases, but ATP (0.1 mM) did.

the amplitude of the $[Ca^{2+}]_i$ increase induced by capsaicin and that by MO in each pTRPV1-expressing HEK 293 cell ($r = 0.80$, $p < 0.01$). MO evoked $[Ca^{2+}]_i$ increases in pTRPV1-expressing HEK 293 cells in a concentration-dependent manner with an EC_{50} of 1.8 ± 0.3 mM (Fig. 2C and D). The application of MO (3 mM) to naive HEK 293 cells did not increase $[Ca^{2+}]_i$ (Fig. 1D(b)), but small non-specific effects occurred with high concentrations (>10 mM).

Since it has been reported that MO is an agonist for TRPA1 [13,35], we examined whether recombinant pTRPV1 had a relation to TRPA1 function. As shown in Fig. 2E, pTRPV1 did not respond to icilin (0.1 mM), a TRPA1 agonist [19] or menthol (0.1 mM), a TRPM8 agonist [38]. Cold stimulation (5 C), which activates TRPA1 [19,35], was also ineffective in stimulating pTRPV1. It was confirmed that these cells responded to MO (3 mM). These results indicate that MO activates pTRPV1 unrelated to the functional properties of TRPA1.

3.2. *Effects of vanilloid antagonists on $[Ca^{2+}]_i$ responses to MO*

The TRPV1 antagonists capsazepine [39], iodoresiniferatoxin [40] and ruthenium red [10] were tested for their ability to inhibit the MO-induced $[Ca^{2+}]_i$ increase. As shown in the inset of Fig. 3, the sustained $[Ca^{2+}]_i$ increase in the presence of MO (3 mM) was clearly inhibited by the addition of iodoresiniferatoxin (0.01 μM). All antagonists applied cumulatively caused concentration-dependent inhibition of the MO-induced $[Ca^{2+}]_i$ increase. The values of IC_{50} were 2.2 ± 0.09 nM for iodoresiniferatoxin, 0.11 ± 0.004 μM for ruthenium red and 0.23 ± 0.01 μM for capsazepine.

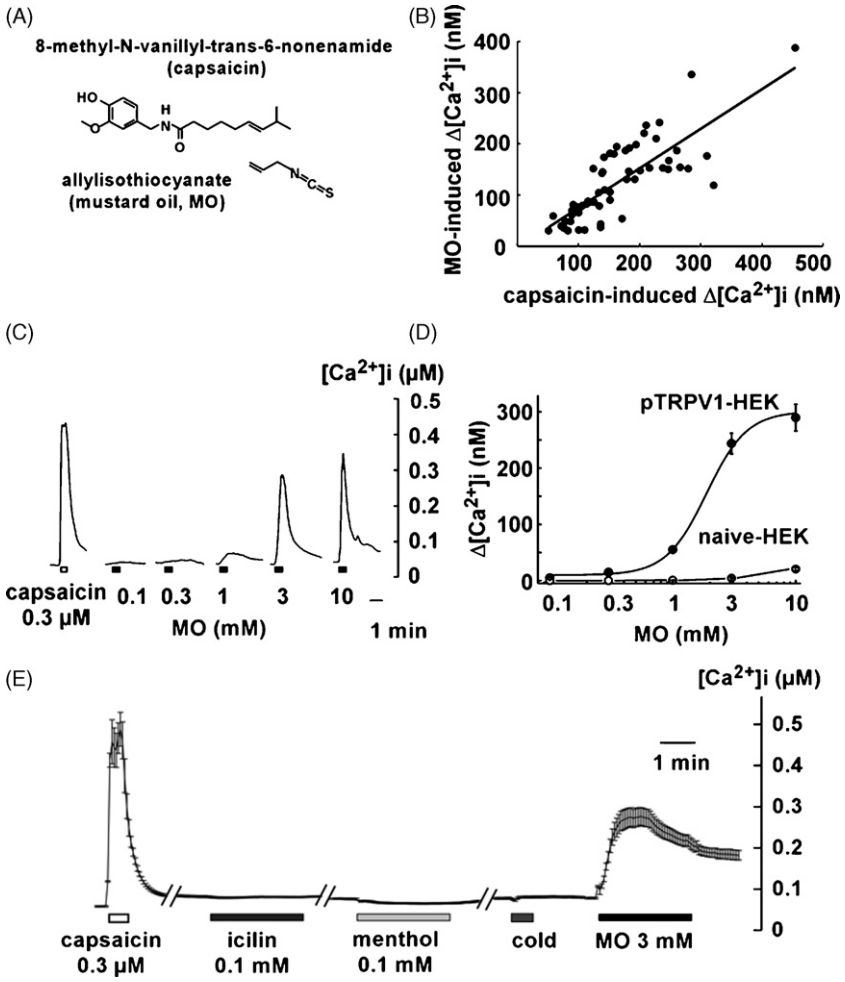

Fig. 2 – Concentration-dependent $[Ca^{2+}]_i$ increases induced by MO in pTRPV1-expressing HEK 293 cells. (A) The chemical structures of capsaicin and mustard oil (MO, allylisothiocyanate). MO does not have a vanillyl group but both have an allyl base in their structures. (B) Relation between the amplitudes of capsaicin- and MO-induced $[Ca^{2+}]_i$ increases in individual cells ($n = 57$). (C) Typical changes in $[Ca^{2+}]_i$ in response to MO at different concentrations. Cells were first stimulated with capsaicin (0.3 μM) for 30 s and then various concentrations of MO were applied for 1 min at intervals of 8 min. (D) Concentration–response relation for MO on pTRPV1-expressing HEK 293 cells ($n = 83$–116) and non-transfected HEK 293 cells ($n = 51$–66). (E) Icilin (0.1 mM), menthol (0.1 mM) and noxious cold stimulation (5 °C) did not elicit any $[Ca^{2+}]_i$ changes in pTRPV1-expressing HEK 293 cells ($n = 42$). Symbols with vertical lines show mean ± S.E.M.

3.3. *Comparison of membrane current responses to capsaicin and MO*

To examine whether MO activates functional ion channels, pTRPV1-expressing HEK 293 cells were whole-cell voltage-clamped and the effect of MO on membrane currents was examined. At a holding potential of −60 mV, capsaicin (0.3 μM) evoked an inward current as reported previously [27]. In the same cell, MO (3 mM) applied subsequently elicited a small inward current with increases in current noise. To confirm that MO-activated currents were indeed mediated via TRPV1, we characterized their current–voltage (*I–V*) relationship using a voltage-ramp protocol (−80 to +80 mV for 100 ms) applied every 15 s. MO-activated currents were found to be indistinguishable from those of capsaicin. The *I–V* relation obtained for MO was outwardly rectifying similar to capsaicin and exhibited a reversal potential close to 0 mV ($E_{rev} = +7.7 \pm 1.2$ mV compared to $+8.2 \pm 1.4$ mV for capsaicin, $n = 26$) as expected for non-selective cation channels. The average inward currents at −60 mV induced by capsaicin and MO was summarized in Fig. 4C.

3.4. *Simultaneous measurement of membrane current and $[Ca^{2+}]_i$ responses to MO*

It is known that the capsaicin-evoked $[Ca^{2+}]_i$ increase results from an influx of divalent cations directly through TRPV1 channels [41]. To examine whether MO also promotes Ca influx through TRPV1 channels, we simultaneously measured $[Ca^{2+}]_i$ and current responses to MO under voltage-clamp conditions. Time-lapse $[Ca^{2+}]_i$ imaging of capsaicin- and MO-induced responses in a voltage-clamped cell are shown in Fig. 5A. At −60 mV, capsaicin (0.3 μM) elicited an inward current with a concomitant rise of $[Ca^{2+}]_i$ after some seconds'

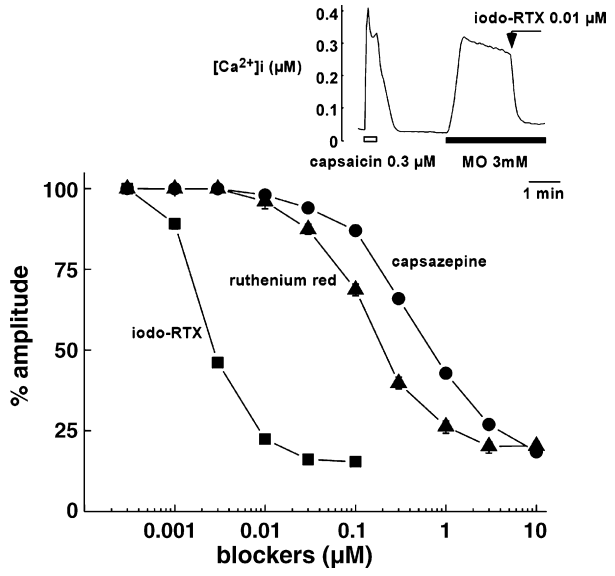

Fig. 3 – Inhibitory effects of TRPV1 antagonists on MO-induced increases of $[Ca^{2+}]_i$ in pTRPV1-expressing HEK 293 cells. Cumulative concentration–inhibition curves for iodoresiniferatoxin (iodo-RTX), ruthenium red and capsazepine on the MO (3 mM)-induced $[Ca^{2+}]_i$ increase. Responses were estimated as a percentage of the amplitude of the MO-induced $[Ca^{2+}]_i$ increase in the absence of these antagonists (capsazepine, $n = 28$; ruthenium red, $n = 32$; iodo-RTX, $n = 26$). Symbols with vertical lines show mean ± S.E.M. Inset shows a representative inhibitory effect of iodo-RTX at 0.01 μM on the MO-induced $[Ca^{2+}]_i$ increase.

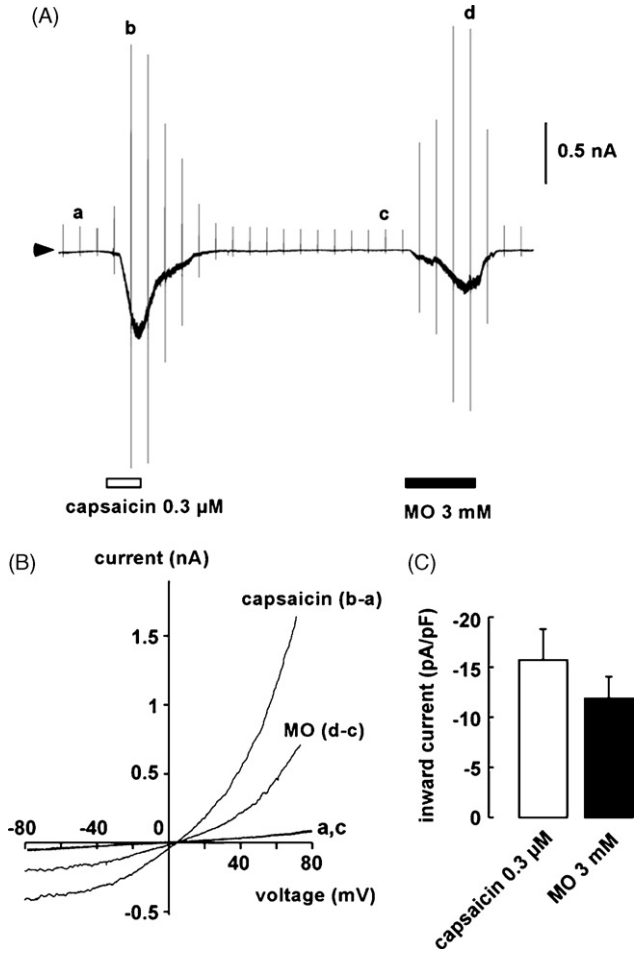

Fig. 4 – Current responses to capsaicin and mustard oil in voltage-clamped HEK 293 cells expressing pTRPV1. (A) At a holding potential of −60 mV, capsaicin (0.3 μM) was applied for 30 s and mustard oil (MO, 3 mM) for 1 min. Before, during and after these stimuli, ramp voltages from −80 to +80 mV for 100 ms every 15 s were applied at the points shown as vertical lines in the current trace. (B). Current–voltage relations induced by capsaicin and MO obtained with a ramp protocol are plotted as the difference from currents before their application (trace 'b' minus 'a'; trace 'd' minus 'c'). (C) Comparison of inward current density evoked by capsaicin and MO at −60 mV. Vertical lines with symbols show mean ± S.E.M. ($n = 16$).

delay. In the same cell, MO (3 mM) produced a small inward current together with a $[Ca^{2+}]_i$ increase but the onset was somewhat slower than that by capsaicin (Fig. 5B). In voltage-clamped cells at −60 mV, the changes in the $[Ca^{2+}]_i$ increase and inward current in response to MO were 0.15 ± 0.03 μM and 6.5 ± 1.2 pA/pF ($n = 9$), respectively, similar to those to capsaicin (0.25 ± 0.05 μM for the $[Ca^{2+}]_i$ increase and 12.0 ± 2.6 pA/pF for inward current, $n = 9$). These results clearly indicate that MO activates functional ion channels with Ca^{2+} permeability in pTRPV1-expressing HEK 293 cells.

3.5. *Effects of external Ca^{2+} removal on MO-induced $[Ca^{2+}]_i$ increase*

As shown in Fig. 5, MO was able to activate pTRPV1 and promoted Ca^{2+} influx into pTRPV1-expressing cells. In addition, some reports have demonstrated that Ca^{2+} release from internal stores is involved in the $[Ca^{2+}]_i$ increase following TRPV1 activation [42,43]. Therefore, to elucidate further characteristics of MO-induced $[Ca^{2+}]_i$ increases, the effects of external Ca^{2+} removal on $[Ca^{2+}]_i$ responses to MO were examined. Typical $[Ca^{2+}]_i$ responses to capsaicin and MO with and without external Ca^{2+}, and summarized data (inset) are depicted in Fig. 6. During the sustained $[Ca^{2+}]_i$ increases in the presence of capsaicin and MO, the removal of external Ca^{2+} by switching of normal external solution to Ca^{2+}-free external solution containing 0.5 mM EGTA produced declines of the $[Ca^{2+}]_i$ levels. The reduced $[Ca^{2+}]_i$ level was restored after readministration of Ca^{2+}. The effects of Ca^{2+} removal before MO application were examined to determine the possible involvement of Ca^{2+} mobilization from internal stores in MO-induced $[Ca^{2+}]_i$ increases. As shown in Fig. 6B, no $[Ca^{2+}]_i$ increase occurred with MO in the absence of external Ca^{2+}, but after readministration of Ca^{2+} together with MO, a robust $[Ca^{2+}]_i$ increase occurred with a rapid rise. The increase of $[Ca^{2+}]_i$ was not generated by readministration of Ca^{2+} alone (Fig. 6B(c)). Moreover, depletion of intracellular Ca^{2+} stores by thapsigargin (1 μM), a smooth endoplasmic reticulum Ca-ATPase (SERCA) pump blocker, did not inhibit the MO-induced

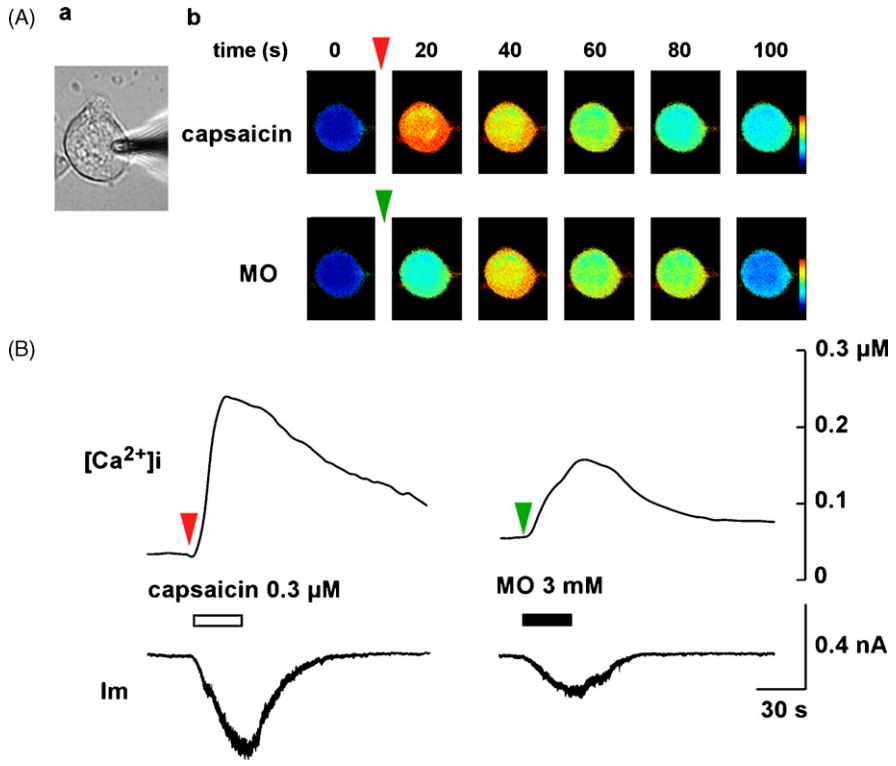

Fig. 5 – Simultaneous measurement of current and $[Ca^{2+}]_i$ responses to capsaicin and mustard oil in the same pTRPV1-expressing HEK 293 cell. (A): (a) Image under transmitted light of the patch-clamped cell. (b) Pseudcolour time-lapse Ca^{2+} images corresponding to the time points from before to after 60 s application of capsaicin (0.3 μM, upper) and mustard oil (MO, 3 mM, lower). (B) $[Ca^{2+}]_i$ and current responses to capsaicin and MO in the same cell at a holding potential of −60 mV.

$[Ca^{2+}]_i$ increase (Fig. 6B(d)). These results suggest that $[Ca^{2+}]_i$ responses to MO are solely mediated by Ca^{2+} influx across the plasma membrane.

3.6. *$[Ca^{2+}]_i$ and current responses to MO in cultured porcine DRG neurons*

Finally, to determine whether MO had an agonistic action on endogenous TRPV1, the effects of MO on cultured porcine DRG neurons were examined. In this study, cells were first stimulated with capsaicin (0.3 μM) to identify DRG neurons expressing TRPV1. Typical $[Ca^{2+}]_i$ responses to capsaicin and MO in the same cells and TRPV1-immunoreactivity are shown in Fig. 7A. Cells responding to capsaicin showed positive immunoreactivity to the anti-TRPV1 antibody. Similar to the recombinant pTRPV1, capsaicin and MO produced $[Ca^{2+}]_i$ increases and there was a positive relation between them ($r = 0.70$, $p < 0.01$, Fig. 7B). MO evoked increases of $[Ca^{2+}]_i$ in 79 of 88 DRG neurons, which were estimated to be neural cells based on the increase of $[Ca^{2+}]_i$ with 60 mM KCl. Some porcine DRG neurons responding to MO also responded to capsaicin. Among 79 neurons responding to MO, 65 were capsaicin-sensitive (82.3%) and the remainder (14 neurons, 17.7%) were capsaicin-insensitive. The concentration–response relations for MO are depicted in Fig. 7A(b) and the EC_{50} value was estimated to be 1.73 ± 0.51 mM.

We also examined the current responses to MO in porcine DRG neurons. In agreement with the results in a heterologous expression system (Fig. 4), MO elicited inward currents in porcine DRG at −60 mV with kinetics similar to pTRPV1-expressing HEK 293 cells. The amplitudes of inward current induced by capsaicin and MO were 65.6 ± 13.3 pA/pF and 19.9 ± 4.1 pA/pF ($n = 12$), respectively. These results clearly indicate that MO plays an agonistic action not only on recombinant pTRPV1 channels but also on endogenous ones.

4. Discussion

Since TRPV1 is an important molecular gateway to the pain sensation [10], the development of therapeutic and analgesic drugs, that target this molecule has been desired. Various naturally occurring substances have been considered as possible candidates targeting TRPV1 channels [9]. In the present experiment, we have clearly shown that mustard oil (MO, allylisothiocyanate) is an effective agonistic compound for the porcine orthologue of TRPV1 (pTRPV1) channels. Since capsaicin and MO have the ability to evoke a painful burning sensation, TRPV1 is one of the targets for MO in nociceptive actions.

In a heterologous expression system, MO evoked a $[Ca^{2+}]_i$ increase in cells responding to capsaicin and there was a positive correlation between the amplitudes of the $[Ca^{2+}]_i$ increases induced by capsaicin and MO. The MO-induced $[Ca^{2+}]_i$ increase was effectively suppressed by three different TRPV1 antagonists and the order of inhibitory potency was

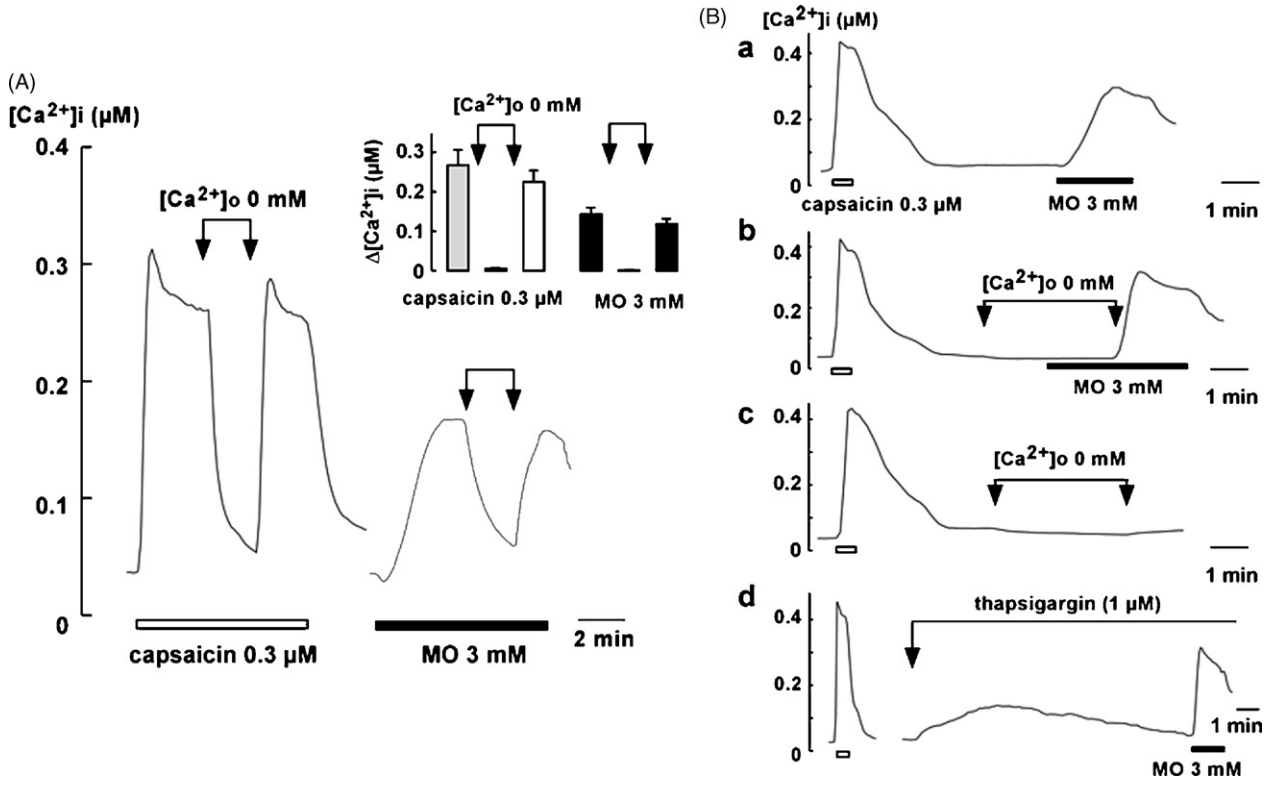

Fig. 6 – Effects of external Ca^{2+} removal of MO-induced $[Ca^{2+}]_i$ increase in pTRPV1-expressing HEK 293 cells. (A) Actual traces of $[Ca^{2+}]_i$ responses to capsaicin (0.3 μM) and mustard oil (3 mM) in the same cell. During the $[Ca^{2+}]_i$ increase generated by these agents, the external Ca^{2+} was removed. Insets show summarized changes in peak $[Ca^{2+}]_i$ levels before and during removal of external Ca^{2+}, and after its reintroduction (capsaicin, $n = 32$; MO, $n = 41$). Symbols with vertical lines show mean ± S.E.M. (B): (a) Representative $[Ca^{2+}]_i$ responses to MO (3 mM) in the presence of external Ca^{2+}. (b) External Ca^{2+} was removed by changing normal external solution to Ca^{2+}-free solution containing 1 mM EGTA ($[Ca^{2+}]_o$ 0 mM) 1 min before stimulation with MO and was readministered during MO stimulation. (c) No response occurred after readministration of external Ca^{2+} without MO. (d) Thapsigargin (1 μM) did not suppress the MO-induced $[Ca^{2+}]_i$ increase. Time scale in (d) is different from in (a–c).

similar to that for capsaicin reported previously [27]. We found that MO evoked inward currents with slower activation and smaller amplitude than those of capsaicin. The current–voltage relationships in responses to MO and capsaicin were almost identical, with marked outward rectification and similar values for reversal potentials, suggesting that both activated the same ion channels. Similar to capsaicin, the $[Ca^{2+}]_i$ increase induced by MO was reduced by the removal of external Ca^{2+}; no increases of $[Ca^{2+}]_i$ occurred in the absence of external Ca^{2+}, and thapsigargin did not affect the MO-induced $[Ca^{2+}]_i$ rise. Moreover, like capsaicin, the MO-induced $[Ca^{2+}]_i$ increase was clearly mediated by Ca^{2+} influx through the activation of TRPV1, since the $[Ca^{2+}]_i$ increase was elicited even in voltage-clamped cells. These properties of the action of MO, closely resembling capsaicin, demonstrated that MO-activated TRPV1 channels in a heterologous expression system. This was also the case in porcine sensory neurons expressing endogenous TRPV1 receptors.

In a mouse model of visceral pain induced by colonic instillation of MO, ruthenium red, a non-competitive TRPV1 antagonist cause significant inhibition of MO-induced nociception [26]. The neurogenic inflammatory response to MO in the mouse ear is produced by release of the same mediators as for capsaicin [17], and the inflammatory responses to capsaicin and MO show partial cross-desensitization [44]. Depletion of sensory neuropeptides by pretreatment with capsaicin causes a decrease in the inflammatory response to MO in the mouse ear [15], rat bladder [45] and rat skin [46]. These data provide the hypothesis that MO produces its effects through activation of capsaicin-sensitive primary afferent fibers. The present results suggest that these inflammatory and nociceptive responses to MO in vivo are in part mediated through TRPV1 activation. In contrast, some reports suggest that capsaicin and MO act via distinct molecular mechanisms. For instance, MO does not activate rat TRPV1-expressing CHO [35] and HEK 293 cells [13]. TRPV1-deficient mice retain sensitivity to MO [7] and trigeminal neurons from normal and TRPV1-deficent mice are indistinguishable in their sensitivity to MO [14]. In addition, MO-induced neurogenic inflammation is observed in TRPV1 knockout mice [23].

It is known that there are obvious pharmacological species variations in the sensitivity of agonist for TRPV1. Relative insensitivity to capsaicin in chicken TRPV1 [33] and lesser sensitivity in rabbit TRPV1 [34] have been reported. PPAHV, a vanilloid agonist, is capable of activating rat [29–31], mouse

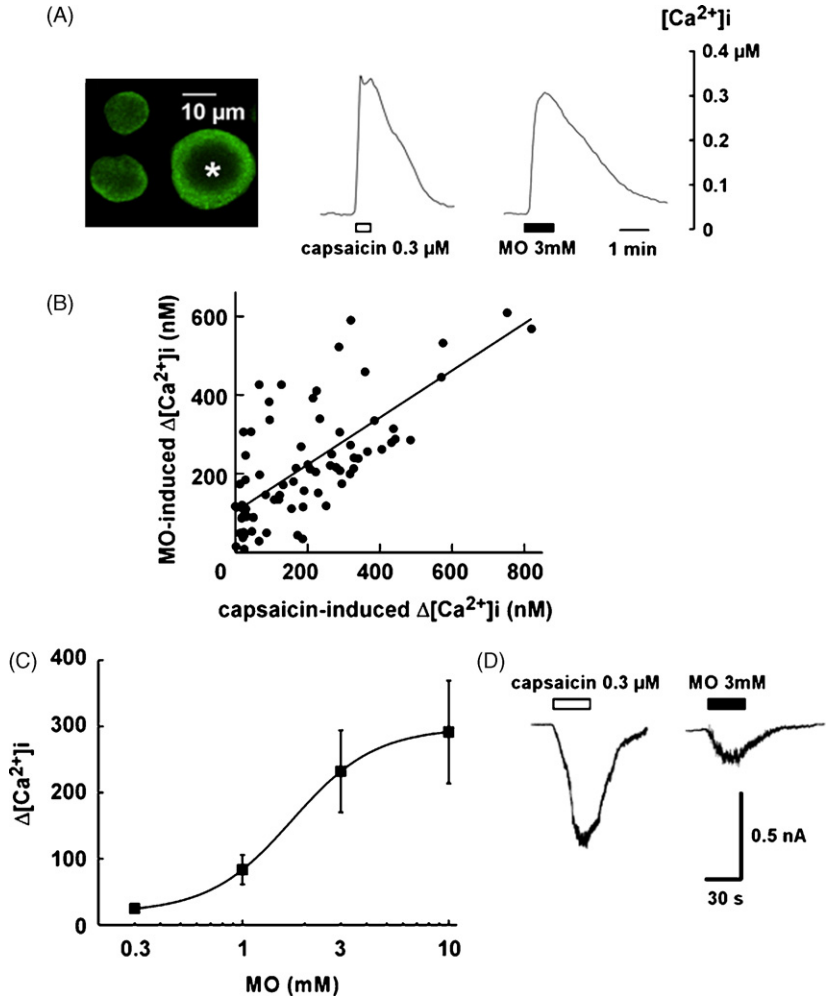

Fig. 7 – Effects of capsaicin and MO on endogenous pTRPV1 in cultured porcine DRG neurons. (A) Immunocytochemistry for TRPV1 receptor protein in porcine DRG neurons. After $[Ca^{2+}]_i$ responses were measured with capsaicin (0.3 μM) and mustard oil (MO, 3 mM), cells were subjected to immunostaining with a TRPV1 antibody. $[Ca^{2+}]_i$ response to capsaicin and the subsequent response to MO in the cell marked with an asterisk. (B) Relationship between the amplitudes of capsaicin- and MO-induced $[Ca^{2+}]_i$ increases in individual DRG neurons (n = 79 from 7 animals). (C) Concentration–response relation for the peak $[Ca^{2+}]_i$ levels induced by MO. Symbols with vertical lines show mean ± S.E.M. (n = 14). (D) Representative current responses to capsaicin and MO in the same DRG neuron at a holding potential of −60 mV.

[30], and porcine TRPV1 [27], but not human [29] and guinea pig TRPV1 [32]. Therefore, differences of MO action among species may be related to species difference in molecular structure of TRPV1. As we reported previously, pTRPV1 has highly homologous to other mammalian orthologues, but is characterized by some different amino acid residues [27]. One of remarkable points is that a threonine (T550) within the S3 to S4 region, reported to be responsible for sensitivity to capsaicin [34], is substituted to alanine (A) in pTRPV1. Since pTRPV1 is activated by various vanilloids including capsaicin and endovanilloids, it is conceivable that T550 was not necessarily essential for vanilloid sensitivity. Therefore, it may be possible that difference of amino acids in pTRPV1 from other TRPV1orthologues are responsible for the difference of MO actions among TRPV1 species. Amino acid sequences required for the sensitivity of MO will be an interesting area for future work.

Recent reports have demonstrated that MO is an agonist for rat TRPA1 (formerly called ANKTM1) [13,35], which forms Ca^{2+} permeant non-selective cation channels as thermoreceptors sensitive to noxious cold [19,35]. Regarding cold sensation, however, some reports indicate that noxious cold does not activate mouse TRPA1 [13,47]. In the present experiments, the possibility that pTRPV1 shared TRPA1 properties could be excluded, since pTRPV1 did not respond to icilin, menthol or noxious cold. It has also been reported that capsaicin has no effect on rat TRPA1 (ANKTM1)-expressing HEK 293 cells [13].

In cultured porcine DRG neurons, capsaicin could activate the vast majority (85%) of neurons responding to MO. Similarly, in adult rat DRG neurons, 97% of neurons positive for TRPA1 express TRPV1 [19]. In contrast, in rat trigeminal ganglia, MO responsive cells represented a subset (50%) of capsaicin-responding cells [14]. Therefore, there appears to be different percentages of overlap for MO- and capsaicin-sensitivity among

different animal species. Furthermore, in mouse DRG neurons, it has been suggested that MO appears to be less specific, as it activates 63% of the cold-sensitive population and 12% of the cold-insensitive one [35].

MO and capsaicin are used as tools for the development of visceral pain models [20–22,25] and inflammation models [15,17,23]. The present data provide evidence that MO-induced TRPV1 activation may be one of the underlying mechanisms in these models. It has been reported that MO produces not only neurogenic but non-neurogenic inflammation [23]. Since high concentrations of MO produced irreversible cell-damage such as swelling, part of the non-neurogenic inflammation could be explained to by these effects. In the current study, relatively high concentrations of MO were required to activate heterologously and endogenously expressed pTRPV1 compared to capsaicin. In an inflammatory mouse model, MO was reported to be less potent than capsaicin as a stimulator of plasma extravasation [17].

It is known that the active structure is a vanillyl group for capsaicin as well as other vanilloid agonists [10]. MO has an allyl base but not a vanillyl group in its structure. Since capsaicin also possesses this structure, we speculate that the allyl base is one of target molecules for synthesis of pharmacological agents for TRPV1. Recently, allicin, a chemical constituent of garlic extracts containing allyl base, has shown cross-reactivity between TRPV1 and TRPA1 [48]. The difference of responsiveness to MO between porcine TRPV1 (present data) and rat TRPV1 [13,15] may be based on a difference of recognition sites for the allyl base.

In conclusion, we have demonstrated that MO is an agonist for porcine TRPV1, but it remains to be determined if TRPV1 agonists bearing such properties will contribute to the design of new and improved therapeutic agents. As has occurred with most important current therapeutic drugs, the use of natural substances will probably continue to be an important strategy in the elucidation of functions of complex biological systems and thereby in the identification of new targets for the discovery of new clinically relevant drugs.

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