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RESEARCH PAPER

Activation of transient receptor potential A1 by a non-pungent capsaicin-like compound, capsiate

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BACKGROUND AND PURPOSE

Capsiate is produced by 'CH-19 Sweet' (*Capsicum annuun* L.), a non-pungent cultivar of red pepper. Like capsaicin, capsiate is thought to enhance energy metabolism by activating the sympathetic nervous system and suppressing inflammation, but the underlying mechanisms for this are uncertain. We previously reported that capsiate could activate transient receptor potential vanilloid 1 (TRPV1), a capsaicin receptor. The purpose of the present study is to investigate whether capsinoids activate other TRP channels.

EXPERIMENTAL APPROACH

Using Ca^{2+} imaging and whole-cell patch-clamp methods, we analysed the response of TRP channels to three kinds of capsinoids, capsiate, dihydrocapsiate and nordihydrocapsiate, in HEK293T cells expressing TRP channels or in primary cultures of mouse dorsal root ganglion neurons.

KEY RESULTS

We found that in both cell types TRP ankyrin 1 (TRPA1) had a slightly weaker response to capsinoids compared with TRPV1, with the capsiate EC_{50} for TRPA1 activation being more than that for TRPV1 activation, and that the capsinoid-evoked action was blocked by a specific TRPA1 antagonist. TRPA1 was activated by capsinoids, but not by their degradation products. Amino acids known to participate in TRPA1 activation following cysteine covalent modification or zinc treatment were not involved in the activation of TRPA1 by capsinoid.

CONCLUSIONS AND IMPLICATIONS

Taken together, these results indicate that capsinoids activate TRPA1 by an as yet unknown mechanism, and TRPA1 could be involved in physiological phenomena associated with capsinoid treatment.

Abbreviations

2-APB, 2-aminoethoxydiphenyl borate; AITC, allyl isothiocyanate; DRG, dorsal root ganglion; HEK, human embryonic kidney; HEK293T cell, HEK-derived 293T cell; TRPA1, transient receptor potential ankyrin 1; TRPV1, transient receptor potential vanilloid 1

Introduction

Capsiate, 4-hydroxy-3-methoxybenzyl (*E*)-8-methyl-6-nonenoate, is the main ingredient of 'CH-19 Sweet' (*Capsicum annuun* L.) produced by selective breeding of red pepper, and it structurally resembles capsaicin (Kobata *et al.*, 1998). The capsiate analogues dihydrocapsiate and nordihydrocapsiate were also identified from CH-19 Sweet, and these

compounds are known generally as capsinoids (Kobata *et al.*, 1999). Capsiate is known to enhance energy metabolism via activation of the sympathetic nervous system (Ohnuki *et al.*, 2001a,b) and contributes to the metabolic system (Ohnuki *et al.*, 2001a,b; Kawabata *et al.*, 2006; Snitker *et al.*, 2009; Josse *et al.*, 2010). In addition, a single intragastric administration of capsiate was found to increase adrenaline secretion and oxygen consumption in mice (Ohnuki *et al.*, 2001a,b). Capsiate also had highly protective effects against angiogenesis via inhibition of reactive oxygen species production and c-Src kinase activity (Lee *et al.*, 2010). However, the mechanisms underlying these effects of capsiate have not been elucidated.

Vanilloid compounds, including capsaicin, activate transient receptor potential vanilloid subfamily 1 (TRPV1) channels (Julius and Basbaum, 2001). Transient receptor potential (*TRP*) was first identified as a gene whose mutation was responsible for the photoreceptor mutant in *Drosophila* (Montell and Rubin, 1989). TRP channels have six transmembrane domains with an ankyrin repeat domain in the N-terminus and the functional channel is a tetramer of four subunits (Nilius *et al.*, 2007). Generally, TRP channels are characterized as non-selective cation channels and have high permeability to Ca^{2+}. The mammalian TRP channel super family is composed of six subfamilies, including TRPV, TRPM, TRPA, TRPP, TRPML and TRPC (Nilius *et al.*, 2007). Some family members respond to thermal or mechanical stimuli, as well as chemicals. For example, TRPV1 acts as a heat sensor above 42°C, although the activation mechanism by thermal stimuli has not been well clarified, and TRPA1 is sensitive to various reagents and cold stimuli below 17°C.

We previously reported that capsiate activated TRPV1, although capsiate-evoked responses *in vivo* lasted longer than those produced by capsaicin (Iida *et al.*, 2003). However, we also observed that capsiate is unstable in aqueous conditions, which would not be consistent with its observed long action (Iida *et al.*, 2003). The long-lasting action of capsiate can be partially explained by its high lipophilicity (octanol/water separation coefficient: 4.51), which would allow capsiate to remain with membrane lipids for longer periods. Alternatively, capsiate may be able to activate other TRP channels, which could also explain its long-term action. Therefore, in this study, we investigated whether capsiate activates other TRP channels and found that capsiate activated TRPA1.

Methods

Animals

Male C57BL/6NCr mice (4–6 weeks old; SLC, Hamamatsu, Japan) were used for a control. TRPV1/TRPA1 double-deficient mice were obtained from a mating between TRPV1-deficient and TRPA1-deficient mice (both were generously provided by Dr David Julius, UCSF, San Francisco, CA, USA) (Caterina *et al.*, 2000; Bautista *et al.*, 2006), which were backcrossed on a C57BL/6NCr background. TRPA1-deficient mice were used in a behavioural test. Mice were housed in a controlled environment (12 h light/12 h dark cycle; room temperature, 22–24°C; 50–60% relative humidity) with free access to food and water. All procedures involving the care and use of animals were approved by the Institutional Animal Care and Use Committee of National Institutes of Natural Sciences and carried out in accordance with the National Institutes of Health *Guide for the care and use of laboratory animals* (NIH publication No. 85-23. Revised 1985).

Construction of hTRPV1 and hTRPA1 mutants

Two types of hTRPV1 mutants (Y511A and S512Y) and three types of hTRPA1 mutants (C621S/C641S/C665S, C641S/C1021S and H983A) were made using a modified QuickChange Site-Directed Mutagenesis method (Agilent Technologies Inc., Santa Clara, CA, USA). The entire sequence including the desired substitutions in the mutants was confirmed.

Ca^{2+} imaging

Human embryonic kidney (HEK)-derived 293T (HEK293T) cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Wako Pure Chemical Industries, Ltd, Osaka, Japan) containing 10% FBS (Biowest SAS, Caille, France), 100 units·mL^{-1} penicillin (Invitrogen Corp., Carlsbad, CA, USA), 100 mg·mL^{-1} streptomycin (Invitrogen Corp.), and 2 mM L-glutamine (GlutaMAX, Invitrogen Corp.) at 37°C in 5% CO_2. For the Ca^{2+} imaging experiments, HEK293T cells transfected with either empty vectors or TRP channel cDNAs in OPTI-MEM medium (Invitrogen Corp.) or primary cultures of dorsal root ganglia (DRG) on coverslips were incubated for 30 min at 37°C in culture medium containing 5 μM Fura-2-acetoxymethyl ester (Molecular Probes, Invitrogen Corp.). The cover slips were washed with a standard bath solution containing 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES and 10 mM glucose at pH 7.4 adjusted with NaOH, and Fura-2 fluorescence was measured in a standard bath solution. The coverslips were mounted in a chamber connected to a gravity flow system to deliver various stimuli. Chemical stimulation was applied by running a bath solution containing various chemical reagents. Cell viability was confirmed by responses to 5 μM ionomycin (Dojindo Laboratories, Kumamoto, Japan).

Electrophysiology

For whole-cell patch-clamp recordings, 1 μg human, rat or mouse TRP channel expression vector and 0.1 μg pGreen Lantern 1 vector were transfected into HEK293T cells cultured in 35 mm dishes using Lipofectamine and Plus reagents (Invitrogen Corp.). The standard bath solution was the same as that used for the Ca^{2+} imaging experiments. The pipette solution contained 140 mM KCl, 5 mM EGTA and 10 mM HEPES at pH 7.4 adjusted with KOH. For recording, data were sampled at 10 kHz and filtered at 5 kHz for analysis (Axopatch 200B amplifier with pClamp software; Molecular Devices, Sunnyvale, CA, USA). The membrane potential was clamped at –60 mV in the whole-cell configuration. All of the patch-clamp experiments were performed at room temperature. Chemical stimulation was applied as described earlier for the Ca^{2+} imaging experiments. The current–voltage relationship during stimulation was obtained using voltage ramps (–100 to +100 mV in 40 ms).

DRG cultures

DRG from C57BL/6 NCr mice were rapidly dissected and dissociated by incubation for 25 min at 37°C in a solution of

culture medium (Earle's balanced salts solution with 10% FBS, 0.5% penicillin–streptomycin, 1% glutamax and 1% vitamin solution) containing 1.3% collagenase type XI. Cells were gently triturated using fire-polished Pasteur pipettes and centrifuged in culture medium to separate cells from debris. Cells were resuspended and plated onto cover slips coated with poly-D-lysine. Ca^{2+} imaging experiments were performed 18 h after plating.

Pain related behavioural test

Wild-type (WT) and TRPA1-deficient mice were placed individually in transparent cages (20 × 12 × 12 cm) for 1 h before experiments. An intraplantar injection of 20 μL capsiate (0.6 mM, solvent: 10% dimethyl sulfoxide containing saline) was then made into the left hind paw. The time spent licking and biting the injected paw was measured for 10 min after injection.

Chemicals

Capsiate, dihydrocapsiate, nordihydrocapsiate, vanillyl alcohol (6*E*)-8-methyl-6-nonenoic acid, 8-methylnonanoic acid, and 7-methyloctanoic acid were provided by Ajinomoto Co., Inc. (Tokyo, Japan) Allyl isothiocyanate (AITC), carvacrol and menthol were from Wako Pure Chemical Industries, Ltd. 2-Aminoethoxydiphenyl borate (2-APB) was from Merck (Darmstadt, Germany). The other chemicals were from Sigma-Aldrich (St. Louis, MO, USA). Octanol/water partition coefficients for dihydrocapsiate and nordihydrocapsiate were 4.96 and 4.43, respectively.

Data analysis and statistics

Octanol/water partition coefficients were calculated and expressed as logP values using LogP DB v.12 soft ware (Advanced Chemistry Development, Inc., Toronto, Canada). Values for Ca^{2+} imaging and patch-clamp experiments are presented as means ± SEM from three or more independent experiments. Student's *t*-test or one-way ANOVA followed by two-tailed multiple *t*-test with Bonferroni correction was used. $P < 0.05$ was considered statistically significant.

Drug/molecular target nomenclature

Drug/molecular target nomenclature conforms to the *British Journal of Pharmacology*'s Guide to Receptors and Channels (Alexander *et al.*, 2011).

Results

Capsinoids increased cytosolic Ca^{2+} concentrations through both TRPA1 and TRPV1

We previously reported that capsiate caused TRPV1 activation but observed smaller capsiate-evoked responses after oral administration, probably because of the high lipophilicity and low stability of capsiate in aqueous solution (Iida *et al.*, 2003). Next, we investigated the role of capsinoids in nociception. Initially, we determined whether capsinoids activate several other TRP channels, which are known to respond to chemical, thermal and mechanical stimuli (Nilius *et al.*, 2007). We used HEK293T cells transfected with cDNAs for different TRP channels expressed in sensory neurons and examined their responses using a Fura-2-based Ca^{2+} imaging method. Capsinoid, 10 μM, increased cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) in HEK293T cells expressing human TRPA1 (hTRPA1, provided by Dr Ardem Patapoutian, Scripps Research Institute, La Jolla, CA, USA) (Figure 1A and B) in addition to human TRPV1 (hTRPV1, provided by Dr Yasuo Mori, Kyoto University, Kyoto, Japan), which also responded to capsaicin (1 μM), whereas such $[Ca^{2+}]_i$ increases were not observed in cells expressing rat TRPM8 (rTRPM8, provided by Dr David Julius, UCSF) (Figure 1B). Because the agonist sensitivity of human and mouse TRPV2 clones is known to differ, we examined the effects of capsinoids in HEK293 cells expressing human TRPV2 (hTRPV2, provided by Dr Shigeo Wakabayashi, National Cardiovascular Center Research Institute, Suita, Japan) or mouse TRPV2 (mTRPV2, cloned by ourselves), and found that capsinoids do not activate either human or mouse TRPV2 (Figure 1B). The presence of functional rTRPM8, hTRPV2 and mTRPV2 was confirmed by application of menthol (500 μM) and 2-APB (500 μM), respectively (data not shown). These results suggest that capsinoids act on hTRPV1 and hTRPA1, but not rTRPM8, hTRPV2 or mTRPV2. Capsinoid action on hTRPA1 was confirmed by the similar responses produced by the hTRPA1-specific agonist, AITC (60 μM) (Figure 1A). We confirmed that vector-transfected HEK293T cells did not respond to capsinoids (Figure 1B), further supporting the specific actions of capsinoids on hTRPV1 and hTRPA1. Interestingly, we observed that 10 μM capsinoids (capsiate, dihydrocapsiate or nordihydrocapsiate) caused similar $[Ca^{2+}]_i$ increases (Figure 1B), suggesting that the efficacy of the capsinoids on hTRPV1 and hTRPA1 activation is similar. Taken together, these results indicate that capsinoids increase $[Ca^{2+}]_i$ via activation of hTRPA1, as well as hTRPV1. The increased $[Ca^{2+}]_i$ did not return to basal levels after agonist washout (Figure 1A) probably because increased $[Ca^{2+}]_i$ caused further TRPA1 activation (Doerner *et al.*, 2007; Zurborg *et al.*, 2007).

Capsinoids evoked inward currents in hTRPA1-expressing HEK293T cells

To study whether the observed responses in the Ca^{2+} imaging experiments were caused by hTRPA1 activation in the plasma membrane, we performed whole-cell patch-clamp experiments in HEK293T cells expressing hTRPA1. We observed membrane currents at −60 mV with repetitive voltage-ramp pulses (40 ms) from −100 mV to +100 mV every 5 s. These whole-cell recordings showed that capsinoids evoked hTRPA1-derived inward currents at −60 mV with an outwardly rectifying current–voltage relationship (Figure 2A–C). Capsiate-evoked current activation was also observed in HEK293 cells expressing mouse TRPA1 (mTRPA1) (Supporting Information Figure S1), indicating that TRPA1 activation by capsiate is not human clone specific. Dose-response curves for hTRPA1 activation by capsinoids showed that EC_{50} values for activation by capsiate, dihydrocapsiate and nordihydrocapsiate were similar (2.76 ± 0.08 μM, 2.94 ± 0.19 μM, and 2.82 ± 0.16 μM, respectively) (Figure 2D), consistent with the similar $[Ca^{2+}]_i$ increases produced by the capsinoids (Figure 1B). Potency for hTRPA1 activation by capsinoids was weaker than that for rat TRPV1 (rTRPV1) with EC_{50} values for

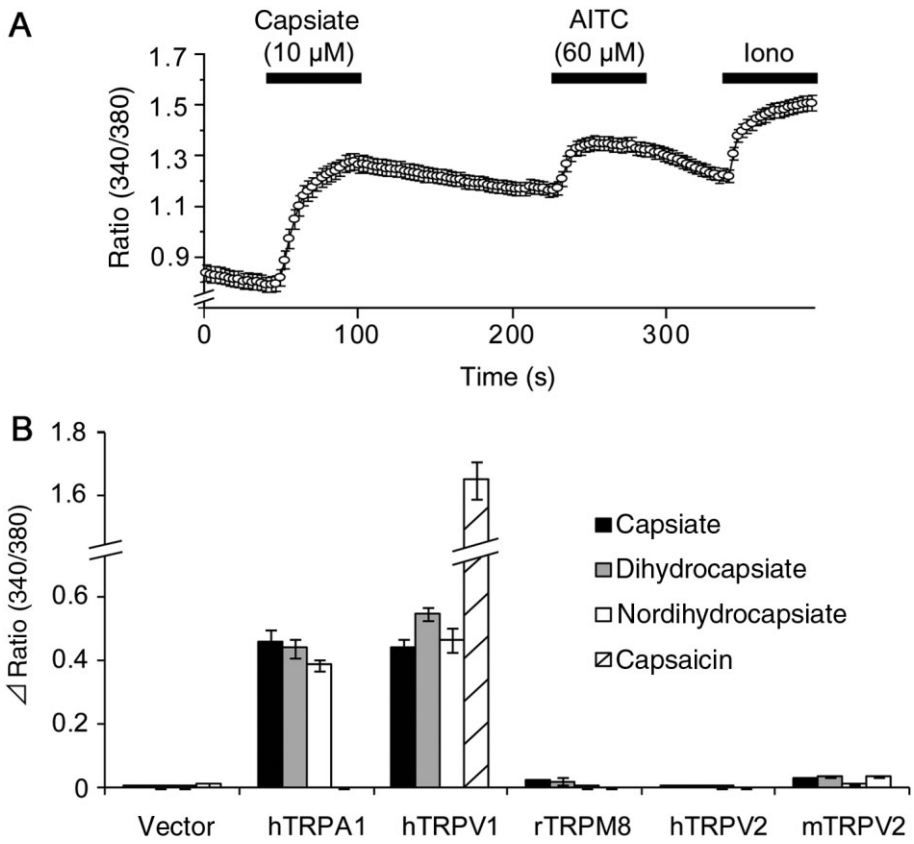

Figure 1

Capsinoids increased cytosolic Ca^{2+} concentrations through both TRPA1 and hTRPV1. (A) Both capsiate (10 μM) and AITC (60 μM) increased cytosolic Ca^{2+} concentrations (the ratio of 340/380 nm values) in HEK293T cells expressing hTRPA1. Data are expressed as the mean ± SEM of 48 cells. Ionomycin (Iono) was applied to confirm cell viability. Bars indicate duration of chemical applications. (B) Changes in cytosolic Ca^{2+} concentrations (mean ± SEM of 19–48 cells) elicited by 10 μM capsinoids (capsiate, dihydrocapsiate and nordihydrocapsiate) in HEK293T cells transfected with cDNAs for hTRPA1, hTRPV1, rTRPM8, hTRPV2, mTRPV2, and empty vector. hTRPV1 responses to capsaicin (1 μM) were also examined.

hTRPA1 and rTRPV1 capsiate activation of 2.76 μM, and 290 nM, respectively (Figure 2D and Iida *et al.*, 2003). Efficacy for hTRPA1 activation by dihydrocapsiate was slightly greater than that for capsiate or nordihydrocapsiate (Figure 2D). On the other hand, peak responses of hTRPA1 to capsiate appeared to be delayed (Figure 2A–C), so we compared the time to peak capsiate response for hTRPV1 and hTRPA1. Figure 3A shows a representative current trace for the stimulant effect of capsiate (about saturating concentrations for hTRPV1 and hTRPA1) in HEK293 cells expressing hTRPV1 (grey) or hTRPA1 (black). The times to peak response of hTRPA1 were significantly longer than those of hTRPV1 both at EC_{50} concentrations (0.3 μM for hTRPV1 and 3 μM for hTRPA1) and saturating concentrations (1 μM for hTRPV1 and 10 μM for hTRPA1) (Figure 3B), suggesting that the apparent delayed activation is not due to differences in solution flow. Menthol, which activates TRPA1, is also known to work as a TRPA1 antagonist (Karashima *et al.*, 2007). In order to examine the possibility that bimodal effects of capsiate cause the apparent delayed activation, we checked whether capsiate inhibits TRPA1 activation by AITC. Although AITC (20 μM)-activated hTRPA1 currents were desensitized during AITC application, such channel activity was not affected by capsiate (30 μM) (Supporting Information Figure S2A). We also excluded the possibility that capsiate (30 μM) inhibits TRPM8 by the addition of 500 μM menthol (Supporting Information Figure S2B). These results suggest that capsiate is a selective TRPA1 and TRPV1 agonist.

Next, we determined whether the inward currents induced by the capsinoids in whole-cell recordings could be inhibited by the specific TRPA1 antagonist, HC-030031. No current activation was induced by capsinoids in the presence of HC-030031 (30 μM) while the capsinoid-evoked current activation was initiated upon HC-030031 washout (Figure 4), further supporting TRPA1 activation by capsinoids. Taken together, these results suggest that capsinoid-evoked inward currents were derived from TRPA1 activation.

Capsinoids activated native mouse TRPA1

To further examine whether capsinoids activate TRPA1 in native cells, mouse DRG neurons were analysed using a Ca^{2+} imaging method. Cell viability was confirmed by the responses to high K^+ solution and ionomycin. As shown in Figure 5A, there appeared to be three kinds of capsiate-sensitive cells: (i) 13.9% (69/497) of WT DRG neurons responded to capsaicin but not AITC and as such, probably

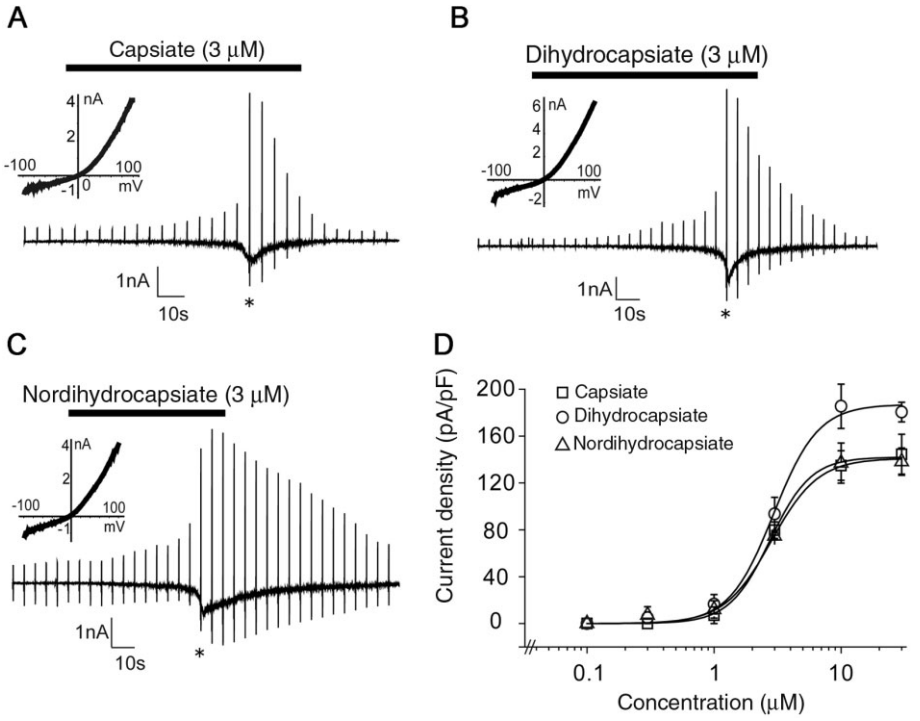

Figure 2

Capsinoids evoked inward currents in hTRPA1-expressing HEK293T cells. Representative hTRPA1-mediated whole-cell current traces upon application of capsiate (A), dihydrocapsiate (B) and nordihydrocapsiate (C) in hTRPA1-expressing HEK293T cells recorded at –60 mV with voltage-ramp pulses (–100 to +100 mV in 40 ms) every 5 s. Current–voltage curves at the time point shown as * (inset). (D) Dose-response curves for the hTRPA1 current responses to capsinoids. Data are shown as the mean ± SEM of four to six cells.

express TRPV1 alone (orange); (ii) cells that responded to both AITC and capsaicin (13.3%, 66/497), which probably express both TRPA1 and TRPV1 (blue); and (iii) although at a much lower incidence (4.2%, 21/497), cells that responded to AITC but not to capsaicin, which probably express TRPA1 alone (green). Interestingly, all the AITC- or capsaicin-sensitive cells responded to capsiate, which can be explained by the ability of capsiate to activate both TRPV1 and TRPA1. DRG neurons from mice lacking both TRPV1 and TRPA1 showed no responses to capsiate, AITC or capsaicin, further supporting the concept that capsiate activates both TRPV1 and TRPA1 (Figure 5B). Previously, we reported that intraplantar injection of capsiate caused pain-related licking and biting behaviour (Iida *et al.*, 2003). Therefore, we performed a pain-related behavioural test to examine to what extent TRPA1 is involved in capsiate-evoked pain-related behaviours. Comparison of pain-related behaviours (licking and biting) upon intraplantar injection of 20 μL capsiate (0.6 mM) into the hind paws of WT and TRPA1-deficient mice showed that TRPA1-deficient mice had a partial but significant reduction in pain-related behaviours (0–10 min, Figure 5C). There was also a tendency for a reduction in pain-related behaviours that was more predominant between 5 and 10 min after capsiate injection compared with the period between 0 and 5 min, although no statistical difference was achieved. These results indicate that capsiate causes pain-related behaviours through activation of TRPA1 endogenously expressed in native DRG neurons, and suggest that the residual pain-related behaviours observed in the TRPA1-deficient mice are due to TRPV1 activation by capsiate.

Capsinoid degradation products did not evoke inward currents in hTRPA1-expressing HEK293T cells

Our previous report demonstrated that capsiate is degraded rapidly under aqueous conditions and that the amount of capsiate remaining in standard bath solutions is less than 20% after 50 s, as evidenced by HPLC analysis (Iida *et al.*, 2003). Hence, we investigated whether delayed TRPA1 activation could be mediated by the degradation products (Supporting Information Figure S3) or only the capsinoids themselves. We observed capsiate-evoked inward currents only when capsiate was prepared just before initiating the experiment (Figure 6A). Moreover, capsiate degradation products (e.g. vanillyl alcohol (6*E*)-8-methyl-6-nonenoic acid, 8-methylnonanoic acid and 7-methyloctanoic acid) did not activate TRPA1 even when freshly prepared, although TRPA1 activation was confirmed by capsiate or another TRPA1 agonist, carvacrol (Figure 6B and Supporting Information Figure S4). These results indicate that only intact capsinoids themselves can activate TRPA1.

Capsiate and capsaicin might activate TRPV1 through the same sites

The vanilloid moiety of capsaicin was previously reported to interact with Y511 and S512 in the cytosolic region linking

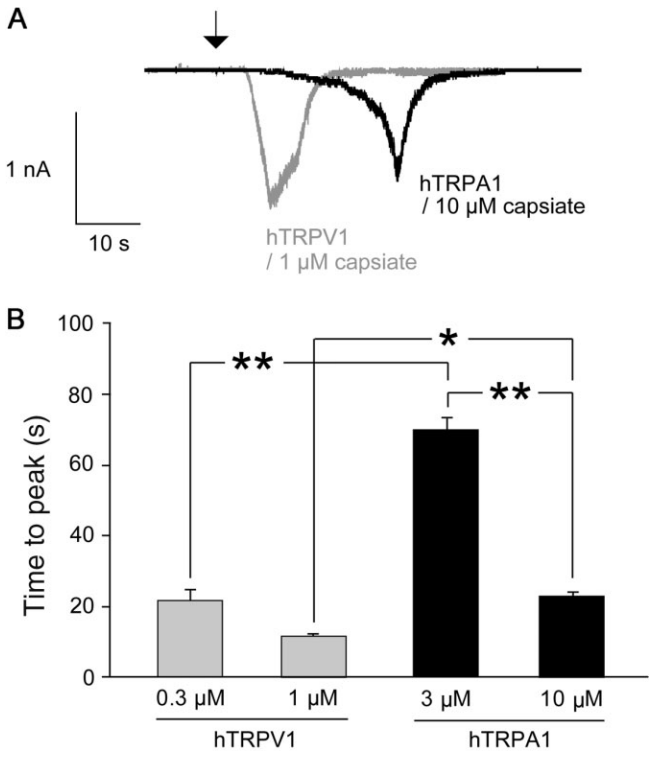

Figure 3

Time-to-peak responses of hTRPA1 to capsiate were longer than those of TRPV1. (A) Representative hTRPA1 (black) or hTRPV1 (grey)-mediated whole-cell current trace upon capsiate application. The arrow indicates the time of capsiate application. (B) Times to peak response of hTRPA1 and hTRPV1 to capsiate. Concentrations of capsiate used were around EC_{50} values (0.3 μM for hTRPV1 and 3 μM for hTRPA1) and saturating concentrations (1 μM for hTRPV1 and 10 μM for hTRPA1). Data are shown as the mean ± SEM of five cells. *$P < 0.05$; **$P < 0.01$.

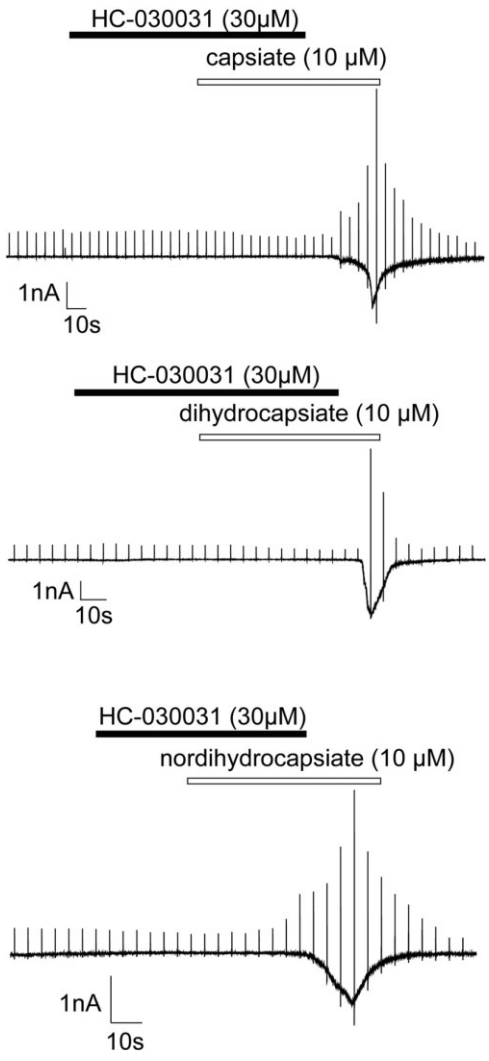

Figure 4

Capsinoid-evoked currents were blocked by a selective TRPA1 antagonist. Representative hTRPA1-mediated whole-cell current traces upon application of capsiate (A), dihydrocapsiate (B) and nordihydrocapsiate (C) in hTRPA1-expressing HEK293T cells in the presence or absence of HC-030031 (30 μM). Membrane currents were recorded at −60 mV with voltage-ramp pulses (−100 to +100 mV in 40 ms) every 5 s.

the second and third transmembrane domains of rat TRPV1 (Jordt and Julius, 2002; Gavva *et al.*, 2004). Based on the close similarity of capsinoids to capsaicin, we hypothesized that capsiate activates TRPV1 by the same mechanism as capsaicin. To evaluate this hypothesis, we produced Y511A and S512Y hTRPV1 mutants (the two amino acids that are conserved between the rat and human versions), and evaluated their responses to capsinoids using a whole-cell patch-clamp technique. We first confirmed that the hTRPV1 mutants did not respond to capsaicin (Figure 7A and B). We then examined whether capsiate evoked inward currents in these hTRPV1 mutants (Figure 7A and B). Capsiate did not activate either hTRPV1 mutant, suggesting that capsiate and capsaicin activate hTRPV1 by binding to the same sites.

The binding site of capsinoids in TRPA1 is distinguishable from those for allyl isothiocyanate and zinc

hTRPA1 required more time for activation by capsinoids relative to hTRPV1 (Figure 3), and this result could not be attributed to capsinoid degradation (Figure 6B and Supporting Information Figure S4). Since capsiate binds to the same sites as capsaicin to activate hTRPV1, capsiate may act on sites much deeper in the cytosolic regions of hTRPA1. It was previously found that structurally unrelated electrophilic compounds activated TRPA1 through the covalent modification of cytosolic cysteine residues (Hinman *et al.*, 2006; Macpherson *et al.*, 2007) and that zinc could activate TRPA1 by acting on a specific cytosolic histidine residue (Hu *et al.*, 2009). Involvement of cysteine and histidine residues in hTRPA1 activation by capsiate might explain the delay of capsiate action on hTRPA1. Therefore, we produced a triple hTRPA1 mutant (C621S/C641S/C665S), a double hTRPA1 mutant (C641S/C1021S) and a single hTRPA1 mutant (H983A), which targeted residues reported to be involved in TRPA1 activation, and examined their responses to capsinoids. The

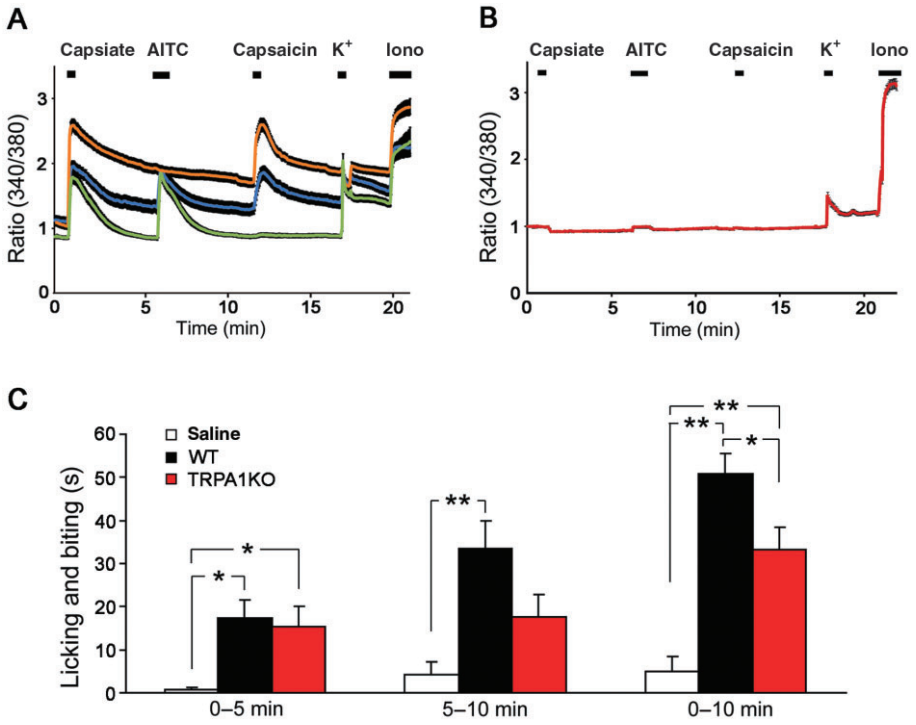

Figure 5

Capsiate activated TRPA1, as well as TRPV1 in mouse DRG neurons caused pain-related behaviours in mice. Traces for changes in cytosolic Ca^{2+} concentrations (the ratio of 340/380 nm mean values ± SEM of 150–170 cells) in mouse DRG neurons from WT (A) and TRPA1/TRPV1 knockout (B) mice. Treatments with capsiate (10 μM), AITC (300 μM), capsaicin (1 μM), KCl (K^+, 40 mM) and ionomycin (Iono, 5 μM) are indicated by the bars. The three kinds of capsiate-sensitive neurons are shown in orange, blue and green. (C) Pain-related behaviours induced by intraplantar injection of 20 μL saline or capsiate (20 μL of 0.6 mM) into the hind paw of wild-type (WT) and TRPA1-deficient (TRPA1KO) mice. Data show the time spent licking or biting for 5 min (0–5 and 5–10 min) or 10 min (0–10 min) in WT (n = 6–8) or TRPA1-deficient mice (n = 8). *P < 0.05, **P < 0.01.

activity of hTRPA1 mutants was confirmed with the agonists 2-APB and carvacrol, which are not involved in covalent modification. We observed that capsinoids evoked inward currents in all the TRPA1 mutants (Figure 8), suggesting that capsinoids activate TRPA1 through an as yet unknown mechanism.

Discussion and conclusions

The data in this study suggest the following: (i) capsiate, dihydrocapsiate and nordihydrocapsiate are novel agonists for hTRPA1, which is known to be activated by a lot of plant products including phytocannabinoids (De Petrocellis *et al.*, 2011), pungent compounds from Sichuan and Melegueta peppers (Riera *et al.*, 2009) and thymol, a major component of thyme and oregano (Lee *et al.*, 2008); (ii) capsiate and capsaicin might act on the same site in the hTRPV1 protein; and (iii) the binding site for capsinoids in hTRPA1 protein differs from that for AITC or zinc, both of which have binding sites in the hTRPA1 cytosolic region.

The finding that capsiate can activate both hTRPA1 and hTRPV1 could account for the long-lasting features of capsiate-evoked pain-related behaviours (Figure 5C), although it could also be due to the high lipophilicity of the compound (Iida *et al.*, 2003). However, the time to peak responses for capsiate differed between hTRPV1 and hTRPA1 (Figure 3A), which suggests that capsiate may activate the two channels via different activation mechanisms. There are two possibilities to explain these apparently different actions: (i) capsiate degradation products might activate TRPA1 and (ii) the action sites in the two channels could be structurally different. The first possibility can be discounted because only undegraded capsiate, dihydrocapsiate and nordihydrocapsiate were able to activate hTRPA1 (Figure 6 and Supporting Information Figure S4). Regarding the second possibility, the distance between the extracellular solution and the capsiate binding site in TRPA1 might account for the differences in the time to peak capsinoid-evoked current responses for hTRPV1 and hTRPA1. As expected, capsiate, which is structurally very close to capsaicin, activated hTRPV1 by acting at the same amino acids as capsaicin (Figure 7). While searching for hTRPA1 amino acids involved in capsiate action, we examined whether the residues involved in hTRPA1 activation by electrophilic compounds and zinc are also capsiate targets. However, none of the hTRPA1 proteins with the relevant cysteine and histidine residues mutated showed reduced responses to capsiate (Figure 8), indicating that those amino acids are not the targets of capsiate action. Although the structural basis for capsiate hTRPA1 activation remains

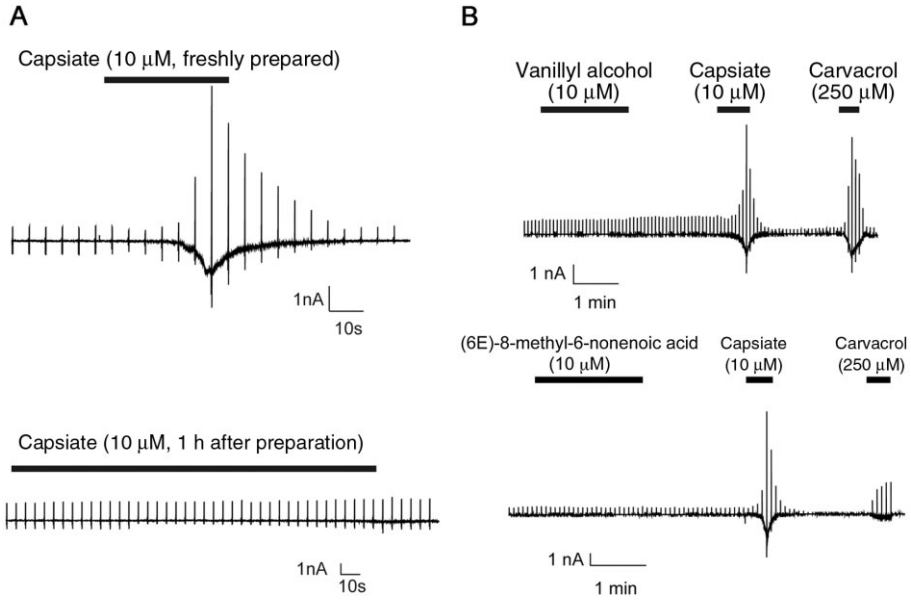

Figure 6

Capsinoid degradation products did not evoke membrane currents in hTRPA1-expressing HEK293T cells. (A) Representative whole-cell current traces in hTRPA1-expressing HEK293T cells at –60 mV with voltage-ramp pulses (–100 to +100 mV in 40 ms) every 5 s upon capsiate application (freshly prepared, upper; 1 h after preparation, lower). (B) Representative whole-cell current traces in hTRPA1-expressing HEK293T cells upon application of capsiate degradation products (vanillyl alcohol, upper; (6E)-8-methyl-6-nonenoic acid, lower). hTRPA1 activities were confirmed by application of carvacrol (250 μM).

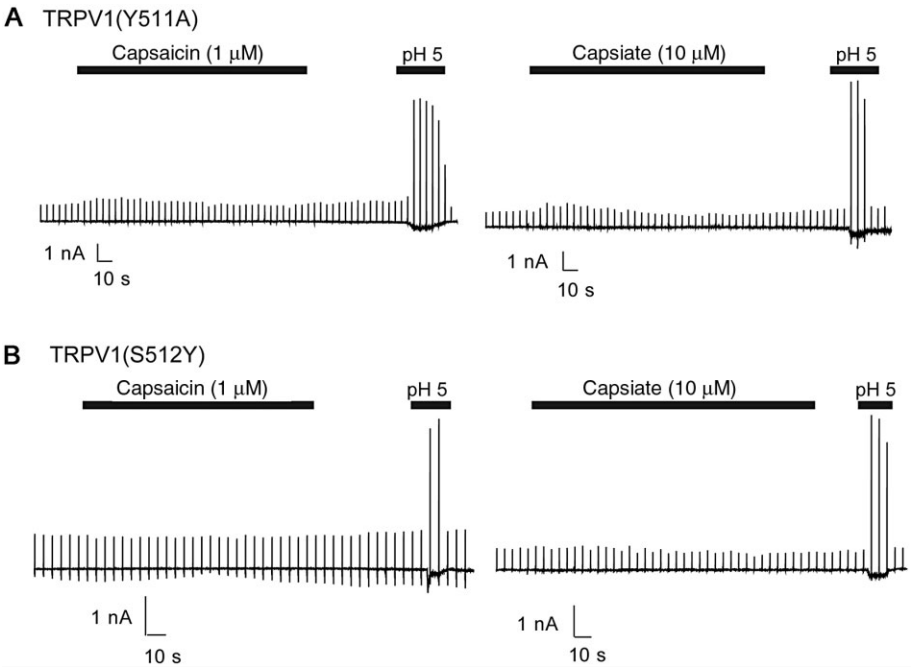

Figure 7

Capsiate and capsaicin might activate hTRPV1 through the same binding sites. Representative whole-cell current traces upon application of capsaicin (left) or capsiate (right) in HEK293T cells expressing hTRPV1 mutants, Y511A (A) and S512Y (B). Membrane currents were recorded at –60 mV with voltage-ramp pulses (–100 to +100 mV in 40 ms) every 5 s. hTRPV1 activities were confirmed by application of acidic solution (pH 5).

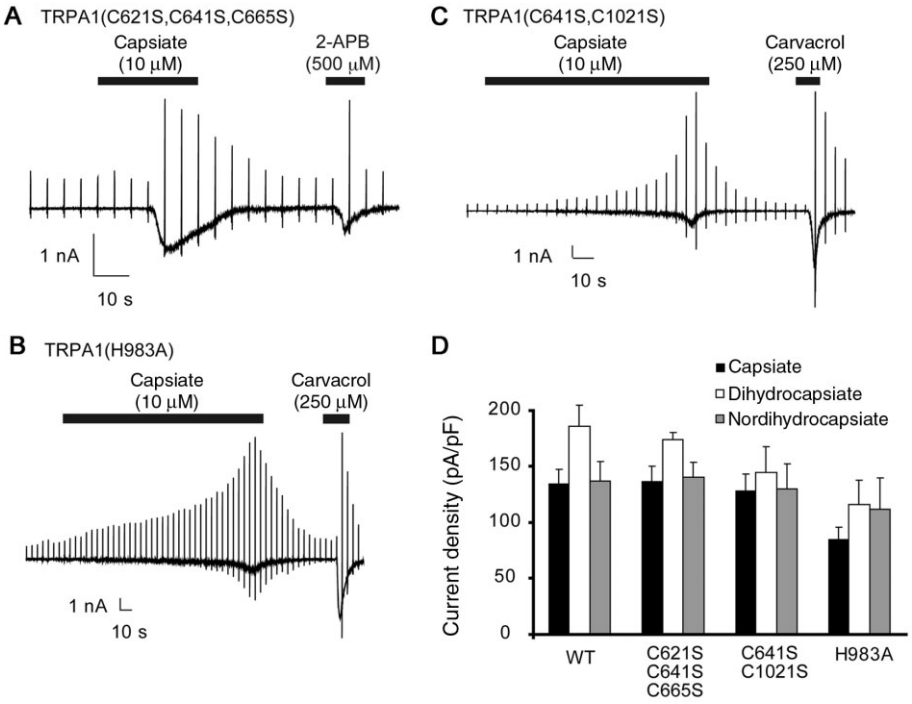

Figure 8

The hTRPA1 capsinoid binding site is distinguishable from those for AITC and zinc. Representative whole-cell current traces upon capsiate application in HEK293T cells expressing hTRPA1 mutants: C621S/C641S/C665S (A); C641S/C1021S (B); or H983A (C). Membrane currents were recorded at –60 mV with voltage-ramp pulses (–100 to +100 mV in 40 ms) every 5 s. hTRPA1 activities were confirmed by application of 2-APB (500 μM) or carvacrol (250 μM). (D) Current density evoked by 10 μM capsinoids (capsiate, dihydrocapsiate and nordihydrocapsiate) in HEK293T cells expressing WT hTRPA1 and mutants used in (A). Data are shown as the mean ± SEM of five to six cells.

unclear, we believe that the relevant structure could be one that is difficult for capsiate to reach. The fact that the known cysteine residues are not involved in capsiate hTRPA1 activation indicates that capsiate can be used as an agonist for hTRPA1 activation that does not involve covalent modification.

The results of this study showed that the three capsinoids produced similar increases in cytosolic Ca^{2+} concentrations and dose-response curves in patch-clamp experiments in HEK293T cells expressing hTRPA1 (Figures 1 and 2), indicating that the three capsinoids used have similar potencies for TRPA1 activation, probably because of their related structural formulas, which is consistent with the fact that the three capsinoids exhibit similar activation ability towards rTRPV1 (Sasahara *et al.*, 2010). Morita *et al.* (2006) reported that highly lipophilic capsinoids with carbons numbering 14 or more caused only a slight Ca^{2+} influx through plasma membrane rTRPV1, suggesting that there may be an optimal carbon chain length that could determine its lipophilicity. Thus, the conformation or bulkiness of the chemicals might strongly affect the TRP channel response to capsinoids. Jordt and Julius (2002) suggested that the capsaicin vanillyl moiety interacts with aromatic residues. Indeed, both capsaicin and capsiate have a vanillyl moiety that could activate hTRPV1. Furthermore, capsiate activated hTRPA1 whereas capsaicin and vanillyl alcohol, a capsinoid degradation product, did not. This suggests that an alkyl chain containing an ester bond could be important for capsinoid hTRPA1 activation. In this regard, there is an interesting report that compounds from Sichuan and Melegueta peppers activate TRPV1 through non-covalent binding while they induce TRPA1 activation through complex interactions involving covalent and non-covalent gating (Riera *et al.*, 2009). Further studies are needed to determine the structural basis for capsinoid hTRPA1 activation, although it appears that sites susceptible to covalent modification and zinc action are not involved.

Capsinoids are reported to have similar biological activities to capsaicin, including the ability to increase plasma catecholamine levels, enhance sympathetic nerve activity and increase energy expenditure following i.v. or oral administration to rodents and humans (Ohnuki *et al.*, 2001a,b; Masuda *et al.*, 2003; Kawabata *et al.*, 2006; Faraut *et al.*, 2007; Hachiya *et al.*, 2007; Inoue *et al.*, 2007; Sasahara *et al.*, 2010; Haramizu *et al.*, 2011). The potency of capsaicin for TRPV1 activation was reported to be more than 3–10 times greater than that of capsiate, although differences in efficacy were not as large (Iida *et al.*, 2003; Sasahara *et al.*, 2010). On the other hand, bio-potency, such as the ability to increase oxygen consumption, was similar between capsaicin and capsinoids (Ohnuki *et al.*, 2001a; Hachiya *et al.*, 2007; Sasahara *et al.*, 2010). These results suggest that there may be other capsiate targets in addition to TRPV1. As shown in this study, TRPA1 was indeed found to be another capsinoid target, which could explain, to an extent, the action of capsinoids on metabolism and is in good agreement with the fact that TRPA1-expressing sensory neurons also express TRPV1,

at least under naïve conditions (Story *et al.*, 2003). Thus, capsinoids could exert their effects by acting on several molecules, including TRPV1 and TRPA1.

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Conflict of interest

The authors declare that no conflict of interest exists.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Representative whole-cell current traces upon capsiate application to HEK293T cells expressing hTRPA1 (A) and mouse TRPA1 (mTRPA1, B). Membrane currents were recorded at –60 mV with voltage-ramp pulses (–100 to +100 mV in 40 ms) every 5 s.
Figure S2 (A) Representative whole-cell current traces upon AITC (20 μM) application to HEK293T cells expressing hTRPA1 in the presence (upper) or absence (lower) of capsiate (30 μM). Five more similar responses were observed. (B) Representative whole-cell current traces upon menthol (500 μM) application to HEK293T cells expressing rTRPM8 in the presence (upper) or absence (lower) of capsiate (30 μM). Four more similar responses were observed.
Figure S3 (A) Structural formulas of capsinoids: capsiate (top), dihydrocapsiate (middle) and nordihydrocapsiate (bottom) and their degradation products [vanillyl alcohol (6*E*)-8-methyl-6-nonenoicacid, 8-methylnonanoic acid and 7-methyloctanoic acid]. Capsinoids have an ester moiety in the middle of the carbon chain, which can be degraded by nucleophilic acyl substitution under aqueous conditions.
Figure S4 Representative whole-cell current traces upon application of dihydrocapsiate and nordihydrocapsiate degradation products, 8-methyl-6-nonanoic acid and 7-methyloctanoic acid, respectively, to hTRPA1-expressing HEK293T cells.

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