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RESEARCH PAPER

Nociceptive and pro-inflammatory effects of dimethylallyl pyrophosphate via TRPV4 activation

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BACKGROUND AND PURPOSE

Sensory neuronal and epidermal transient receptor potential ion channels (TRPs) serve an important role as pain sensor molecules. While many natural and synthetic ligands for sensory TRPs have been identified, little is known about the endogenous activator for TRPV4. Recently, we reported that endogenous metabolites produced by the mevalonate pathway regulate the activities of sensory neuronal TRPs. Here, we show that dimethylallyl pyrophosphate (DMAPP), a substance produced by the same pathway is an activator of TRPV4.

EXPERIMENTAL APPROACH

We examined the effects of DMAPP on sensory TRPs using Ca^{2+} imaging and whole-cell electrophysiology experiments with a heterologous expression system (HEK293T cells transfected with individual TRP channels), cultured sensory neurons and keratinocytes. We then evaluated nociceptive behavioural and inflammatory changes upon DMAPP administration in mice *in vivo*.

KEY RESULTS

In the HEK cell heterologous expression system, cultured sensory neurons and keratinocytes, μM concentrations of DMAPP activated TRPV4. Agonistic and antagonistic potencies of DMAPP for other sensory TRP channels were examined and activation of TRPV3 by camphor was found to be inhibited by DMAPP. *In vivo* assays, intraplantar injection of DMAPP acutely elicited nociceptive flinches that were prevented by pretreatment with TRPV4 blockers, indicating that DMAPP is a novel pain-producing molecule through TRPV4 activation. Further, DMAPP induced acute inflammation and noxious mechanical hypersensitivities in a TRPV4-dependent manner.

CONCLUSIONS AND IMPLICATIONS

Overall, we found a novel sensory TRP acting metabolite and suggest that its use may help to elucidate the physiological role of TRPV4 in nociception and associated inflammation.

Abbreviations

4αPDD, 4-α-phorbol 12,13-didecanoate; DMAPP, dimethylallyl pyrophosphate; DRG, dorsal root ganglion; FPP, farnesyl pyrophosphate; GPP, geranyl pyrophosphate; HC067047, 2-methyl-1-[3-(4-morpholinyl)propyl]-5-phenyl-*N*-[3-(trifluoromethyl)phenyl]-1*H*-pyrrole-3-carboxamide; IPP, isopentenyl pyrophosphate; RN-1734, 1-alkoxycarbonylalkylidenetriphenylarsoranes; RR, ruthenium red; siRNA, small interfering RNA; TRP, transient receptor potential ion channel

Introduction

Members of the transient receptor potential ion channel (TRP) superfamily carry out diverse physiological and pathological functions in the body (Nilius *et al.*, 2007). The vanilloid subtype 4 (TRPV4) was initially found to be a channel activated by extracellular hypotonicity (Liedtke *et al.*, 2000; Strotmann *et al.*, 2000). To date, accumulating evidence has

shown that TRPV4 has a polymodal nature and is not only sensitive to abnormal osmolality, but also to heat, shear stress and chemicals (see review: Plant and Strotmann, 2007). A synthetic ligand 4-α-phorbol 12,13-didecanoate (4αPDD) (Watanabe *et al.*, 2002), endogenous epoxyeicosatrienoic acids (Watanabe *et al.*, 2003) and a natural compound bisandrographolide (Smith *et al.*, 2006) are examples of specific chemical activators of TRPV4.

Such polymodal sensitivity of TRPV4 strongly implicates it has a variety of biological roles. Indeed, growing evidence from *in vitro* and transgenic animal studies supports the idea that TRPV4 is involved in body osmoregulation (Liedtke and Friedman, 2003; Mizuno *et al.*, 2003), temperature preference (Lee *et al.*, 2005), noxious mechanical and thermal sensation (Liedtke and Friedman, 2003; Suzuki *et al.*, 2003), and vasodilatation (Hartmannsgruber *et al.*, 2007). Among these, nociceptive thermal and mechanical phenotypes are likely to be caused by activation, sensitization or overexpression of TRPV4 present in the peripheral sensory neurons and keratinocytes. As well as transducers for such physical stimuli, other members of the TRPs that are also expressed in sensory neurons such as TRPV1, TRPA1 and TRPM8, have been well studied in terms of chemical nociception through activation by their specific ligands (Bandell *et al.*, 2007). In contrast, nociception induced by chemical activation of TRPV4 has been rarely observed (Grant *et al.*, 2007; Alessandri-Haber *et al.*, 2009).

Recently, we reported that endogenous intermediates produced by the mevalonate pathway are able to modulate certain types of sensory TRP members leading to altered nociception (Bang *et al.*, 2010a; 2011). Here we found a novel TRPV4 activator in the same metabolic pathway. We examined the potency and specificity of this newly identified compound by using *in vitro* Ca^{2+} imaging and electrophysiology, and also determined whether it causes behavioural nociception or inflammation. The results of the present study suggest that this compound may contribute to further elucidation of TRPV4-related pain physiology and pharmacology.

Methods

Cell cultures

Cell cultures were performed as previously described (Bang *et al.*, 2007a,b). Briefly, HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were transiently transfected with 3 μg of individual TRP channel plasmid DNA (mTRPA1, rTRPV2, rTRPV1 or mTRPV4 in pcDNA3.1; mTRPA1, hTRPV3 or mTRPM8 in PCDNA5/FRT) per 35 mm dish using Fugene HD (Roche Diagnostics, Indianapolis, IN, USA). Dorsal root ganglia (DRGs) were dissected out of adult ICR mice and treated with 1.5 $mg \cdot mL^{-1}$ collagenase/dispase in DMEM containing 0.1% penicillin/streptomycin at 37°C for 45 min and then treated with 0.25% trypsin for 15 min. The tissue was then triturated and the dissociated cells were plated onto poly-L-lysine-coated cover slips in DMEM/F12 containing 10% FBS, 1% penicillin/streptomycin and 5 $ng \cdot mL^{-1}$ 2.5S NGF (Invitrogen). The cells were used for experiments 48–72 h after plating. The human keratinocyte cell lines (HaCaT) were maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin. All the experiments were conducted using keratinocytes passed 3–5 times. All cells were grown at 37°C and 5% CO_2.

Ca^{2+} imaging experiments

HEK293T cells were used for Fluo-3 Ca^{2+} imaging 16–48 h after plated in poly-L-lysine-coated glass cover slips (Kim *et al.*, 2008). The bath solution consisted of (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, titrated to pH 7.4 with NaOH. Cells were loaded with Fluo-3 AM (5 μM, at 37°C for 1 h) in a bath solution containing 0.02% pleuronic acid (Invitrogen). The imaging experiments were performed with a confocal microscope (LSM5 Pascal, Carl Zeiss, Oberkochen, Germany) and images at 488 nm excitation/514 nm emission were collected every 3 s using Carl Zeiss ratio tool software. Mouse DRG neurons or human keratinocytes were loaded with 5 μM Fura-2AM for 30 min and the cells were resuspended in the bath solution. Images of Fura-2 loaded cells with excitation wavelength alternating between 340 nm and 380 nm were captured with a cooled CCD camera at every 3 s (Retiga-SRV, Q-imaging Corp., Burnaby, BC, Canada). The ratio of fluorescence intensity of the two wavelengths (340/380 nm) in each experiment was analysed using MetaFluor (Molecular Devices, Sunnyvale, CA, USA). Values from different experiments were normalized to a baseline ratio of 340/380 nm.

Patch-clamp electrophysiology

Whole-cell voltage clamp recordings were performed as follows. The same bath solution was used as in the Ca^{2+} imaging experiments. The pipette solution contained (in mM) 140 CsCl, 5 EGTA, 10 HEPES, 2 MgATP, 0.2 NaGTP titrated to pH 7.2 with CsOH. The holding potential was –60 mV. Ramps (from –80 to +80 mV over 325 ms) were continuously repeated every 825 ms.

Nociceptive behavioural studies

The study was performed in accordance with protocols approved by the University Committee on Laboratory Animals. Male ICR mice (6 weeks old) were acclimated for 1 h to the test environment prior to the experiments. For conventional TRPV4-mediated flinch assays, we used intraplantar injection with 10 μL deionized water (for hypotonic stimulation) 30 min after priming with PGE_2 (intraplantar pretreatment with 10 μL saline containing 100 ng PGE_2) (Bang *et al.*, 2011). TRPV4-mediated nociceptive behaviours were observed by counting the number of hind paw flinches for 10 min immediately after the hypotonic stimulus. For dimethylallyl pyrophosphate (DMAPP) assays, DMAPP in 10 μL vehicle (PBS containing 0.5% Tween 80) was used instead of de-ionized water. Hargreaves (Plantar Analgesia meter, for thermal hyperalgesia), von Frey (Dynamic Plantar Aesthesiometer, for mechanical allodynia) and Randall–Selitto apparatus (Analgesy-meter, for nociceptive flexion reflex) were from UGO Basile (Comerio VA, Italy). Assays for changes in mechanical or thermal behaviours were performed as described previously (Bang *et al.*, 2010b; 2011;

2012). Baseline responses were measured 5 min before DMAPP administration. Drugs were injected in 10 μL vehicle into hind paws i.d. at the doses stated in the results. For small interfering RNA (siRNA) or scrambled RNA (scRNA) administration, 4.8 μg of siRNA in 20 μL saline with 20 μL EzWay™ transfection reagent (Komabiotech, Seoul, Korea) were injected into a hind paw 48 or 144 h before the DMAPP injection.

Hind paw inflammation studies

Paw oedema formation was assessed by measuring the dorsoventral diameter of the hind paw foot pads, after intraplantar DMAPP or vehicle injection, using calipers (Cravatt *et al.*, 2004). Tissue myeloperoxidase (MPO) enzyme activity was measured 2 h after intraplantar injection with DMAPP. Plantar tissue samples were homogenized using a motor-driven homogenizer in 0.5% hexadecyltrimethylammonium bromide phosphate buffered solution (pH 6.0). The homogenate was then centrifuged and the supernatants were used for MPO assay in a solution of 3, 3′-dimethoxybenzidine and 1% hydrogen peroxide. The enzymatic activity was assessed as optical density at 450 nm using a microplate reader. The results are expressed in U of enzyme mg^{-1} tissue protein compared with pure enzyme.

Data analyses and compounds

Data were analysed using Student's two-tailed *t*-test (***P < 0.001, **P < 0.01, *P < 0.05) and expressed as means ± SEM. For the comparison of the accumulating flinching numbers or paw diameter changes, one-way ANOVA with Bonferroni *post hoc* test was performed.

All chemicals were purchased from Sigma-Aldrich unless stated otherwise. DMAPP was purchased from Echelon Research Laboratories (Salt Lake City, UT, USA). RN-1734 and HC067047 were purchased from Menai Organics Ltd. (Gwynedd, UK) and Tocris Bioscience (Ellsville, MO, USA), respectively. mTRPV4-siRNA (target sequence: 5′-GAGUGA AAUCUACCAGUA-3′) and a non-target scrambled RNA (scRNA; target sequence: 5′-ACGTGACACGTTCGGAGAA-3′) were purchased from Bioneer Corporation (Daejeon, Korea) and Genolution Inc. (Seoul, Korea). Stock solutions were made using water or ethanol and were diluted with test solutions before use. All drug/molecular target nomenclature used here conforms to the *British Journal of Pharmacology*'s Guide to Receptors and Channels (Alexander *et al.*, 2011).

Results

DMAPP activates TRPV4 in HEK293T cells

In the Fluo-3 intracellular Ca^{2+} imaging experiments using TRPV4 expressing HEK293T cells, extracellular application of DMAPP increased intracellular Ca^{2+} levels, which was blocked by a broad TRP channel blocker ruthenium red (RR; Figure 1A). No such response was detected in untransfected HEK293T cells (n = 108, data not shown), indicating that the responses were mediated by TRPV4. The DMAPP response was reproduced in the whole-cell voltage clamp experiments. DMAPP 10 μM application rapidly elicited outwardly, rectifying current responses similar to those observed upon 4αPDD application in the TRPV4-HEK cells (Figure 1B). The DMAPP-elicited currents were readily blocked by co-application of 30 μM RN-1734, a TRPV4 channel blocker (n = 5). We next examined DMAPP specificity for the activation of six sensory TRP channels using HEK293T cells expressing individual TRPs (TRPV1, TRPV2, TRPV4, TRPA1 and TRPM8) in Fluo-3 Ca^{2+} imaging. Of the six TRP channels, only TRPV4 clearly exhibited a significant sensitivity to DMAPP (Figure 1C). We determined whether stimulation of a GPCR by DMAPP upstream of the TRPV4 was involved in this response. Gallein, a small molecule inhibitor of Gβγ, did not alter TRPV4 activation by DMAPP, indicating that GPCR signalling does not mediate this activation (data not shown) (Bang *et al.*, 2012; Wilson *et al.*, 2011). A dose–response curve for DMAPP was obtained from the TRPV4-mediated Ca^{2+} influx in the HEK cells, (Figure 1D). The EC_{50} of DMAPP was 2.5 μM and Hill coefficient was 2.0, indicating that μM concentrations of DMAPP activate TRPV4 and DMAPP binds to the channel in ~2:1 ratio. Interestingly, DMAPP was also found to have antagonistic efficacy on TRPV3 activation. DMAPP 30 μM suppressed agonist (camphor)-induced elevation of the intracellular Ca^{2+} levels of TRPV3-transfected HEK cells (Figure 2A). This effect was also repeated in the whole-cell voltage clamp experiments (Figure 2B) and was TRPV3-specific (Figure 2C). The IC_{50} of DMAPP for TRPV3 inhibition was 10.4 μM (Figure 2D).

DMAPP effect on TRPV4 was reproduced in native cells

Similar pharmacological effects of DMAPP were observed in the naturally TRP-expressing cells. We carried out Fura-2 Ca^{2+} imaging and whole-cell voltage clamp experiments using native cells expressing TRPV3 or TRPV4. From a subset of the mouse cultured DRG neurons, TRPV4-mediated 4αPDD responses were detected by Fura-2 Ca^{2+} imaging. DMAPP elicited a small but significant rise in the intracellular Ca^{2+} level of these, presumably, TRPV4-expressing neurons (Figure 3A). Similar outwardly rectifying current–voltage relationships were obtained from 4αPDD and DMAPP current responses in the whole-cell voltage clamp recording of sensory neurons (Figure 3B). Human cultured epidermal keratinocytes known to express TRPV3 and TRPV4 were also used to investigate native activities of the two channels. In a subset of keratinocytes, DMAPP significantly increased Ca^{2+} influx and these cells were also sensitive to 4αPDD, indicating that TRPV4-positive keratinocytes respond to DMAPP (Figure 3C). From the electrophysiological experiments, outwardly rectifying current influxes were also observed upon DMAPP application to keratinocytes (Figure 3D). However, in another subset keratinocytes, DMAPP attenuated the TRPV3-mediated camphor responses in both the Ca^{2+} imaging and electrophysiological experiments (Figure 3E,F). The percentage of DRG neurons that responded to 4αPDD and to DMAPP were 12% and 13%, respectively, and 89% of 4αPDD responders overlapped with 92% of DMAPP responders. For keratinocytes, 4αPDD and DMAPP responders were 34 and 35%, respectively. In this case 90% of 4αPDD responders overlapped with 92% of DMAPP responders. Overall, DMAPP was able to modify the two TRP activities both in heterologous expression systems and cultured native cells.

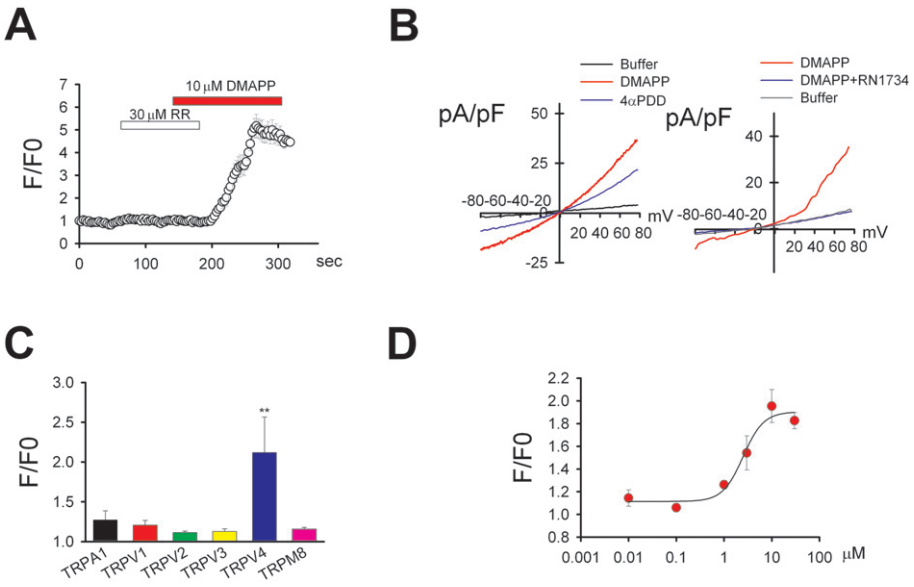

Figure 1

TRPV4 is activated by DMAPP. (A) 10 μM DMAPP elevated intracellular Ca^{2+} levels in TRPV4-transfected HEK293T cells in the Fluo-3 Ca^{2+} imaging experiments (n = 47). A non-specific TRP blocker RR blocked these intracellular Ca^{2+} elevations in the same cells. Responses during Ca^{2+} imaging experiments are displayed as means ± SEM. (B) Left: current–voltage curves obtained from 10 μM DMAPP-evoked or 4αPDD-evoked increase in the outwardly rectifying current of TRPV4-HEK cells in the whole-cell voltage clamp study (n = 6 and n = 5 respectively). Right: the DMAPP current was inhibited by co-application of a TRPV4-specific blocker, RN-1734 (30 μM; n = 5). Buffer indicates the curve without drug. (C) Summary of the intracellular Ca^{2+} elevation, assessed by Fluo-3 Ca^{2+} imaging, observed in cells transfected with TRPV4 or other sensory TRP channels after treatment with 10 μM DMAPP. DMAPP elevated intracellular Ca^{2+} levels only in the TRPV4-transfected HEK cells. DMAPP did not increase intracellular Ca^{2+} in untransfected cells (data not shown). More than 33 cells were used to test each TRP for sensory activity (n = 33–108). The threshold for intracellular Ca^{2+} increase was defined as 20% increase above the basal Ca^{2+} level (Story *et al.*, 2003; Ryu *et al.*, 2010). (D) Dose–response curve for DMAPP on TRPV4, assessed as increase in Ca^{2+} levels by Fluo-3 Ca^{2+} imaging. The curve was fitted by the Hill equation (EC_{50} = 2.5 μM and Hill coefficient = 2.0). Symbols represent mean values of the responses of Ca^{2+} influx to different concentrations of DMAPP (n = 21–38 for each point).

DMAPP elicited nociception

We hypothesized that DMAPP administration to animals causes TRPV4-activation-specific behavioural outcomes, because TRPV3 antagonism mimics TRPV3 knock-out; TRPV3 knock-out animals were indistinguishable from wild-type in that their phenotypes were the same in the absence of additional test stimulation (Moqrich *et al.*, 2005). Hypotonicity-evoked acute flinching behaviours have been reported in mice and rats primed with a subplantar PGE_2 injection and these behavioural responses are mediated by TRPV4 activation (Alessandri-Haber *et al.*, 2003; 2005; Bang *et al.*, 2010b). We also observed the same behavioural responses. As expected, similar flinching responses occurred in mice intraplantarly treated with 3 mM DMAPP into the hind paw pads (Figure 4A,B). Mechanical and thermal thresholds were obtained in the absence of PGE_2 priming . The von Frey mechanical threshold was decreased upon treatment with 3 mM DMAPP (n = 5) or with 3 mM 4αPDD (n = 5) (Figure 4C). DMAPP treatment also decreased the Randall–Selitto mechanical threshold (Figure 4D) and both heightened mechanical sensitivities in von Frey and Randall–Selitto tests were blocked by the TRPV4-specific agonists RN-1734 and HC067047, indicating that the TRPV4 activation by DMAPP underlies the occurrence of mechanical nociceptive behaviours. On the other hand, heat withdrawal latency was not affected by DMAPP (Figure 4E), indicating that acute heat sensation does not involve TRPV4 and that DMAPP does not cause non-selective nociceptive hypersensitivity, although 1 h after DMAPP treatment there was a significant increase in paw diameter (Figure 5A). Intradermal TRPV4 siRNA pretreatment for 48 h failed to prevent these acute nociception responses, which indicates that sensory neuronal TRPV4 is the major contributor to DMAPP nociception rather than epidermal TRPV4 (Supporting Information Figure S1B–D). We further confirmed that neuronal TRPV4 is involved in the nociception by using the same siRNA but with longer pretreatment (144 h), which was reported to enable DRG uptake and knockdown (Tan *et al.*, 2010). One hundred forty-four hours after the i.d. injection with siRNA, both DRG neuronal TRPV4 transcription and nociceptive behavioural responses were attenuated (Supporting Information Figure S1A–D).

PGE_2 is synthesized and released from keratinocytes and positively affects sensory neuronal excitation (Huang *et al.*, 2008). The COX inhibitor indomethacin, injected i.p., failed to prevent flinches despite reducing paw oedema formation, which further confirmed that acute nociception by DMAPP mainly involves direct neuronal TRPV4 activation (Supporting Information Figure S1F,G). Taken together, the behavioural data suggest that DMAPP activates TRPV4 in *in vivo* systems, which results in receptor-specific nociception.

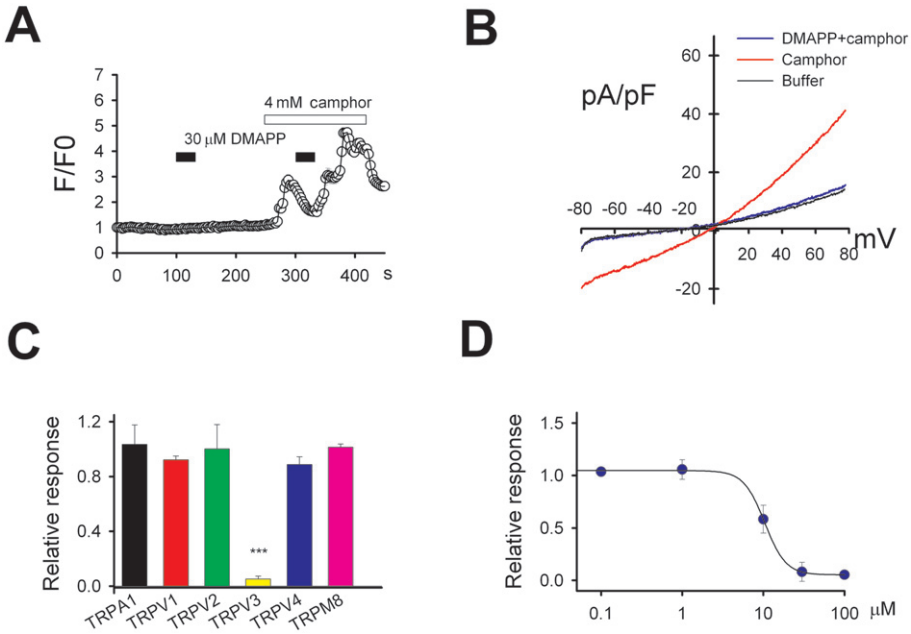

Figure 2

DMAPP inhibits TRPV3 activation. (A) DMAPP 30 μM suppressed the increase in intracellular Ca^{2+} induced by 4 mM camphor in hTRPV3-transfected HEK293T cells (n = 36) in the Fluo-3 Ca^{2+} imaging experiments. (B) Current–voltage curves obtained from 4 mM camphor-evoked outwardly rectifying current increases in the whole-cell voltage clamp study using the TRPV3-HEK cells (n = 5). The camphor-evoked current was inhibited by co-application with DMAPP (30 μM) (n = 5). (C) Summary of the suppression of agonist-induced increase in intracellular Ca^{2+} by DMAPP in cells transfected with TRPV3 or other sensory TRP channels (assessed by Fluo-3 Ca^{2+} imaging). Of the HEK cells transfected with TRP channels, DMAPP only reduced the agonist-induced increase in intracellular Ca^{2+} in the TRPV3-expressing HEK cells (agonists used: 0.1 μM capsaicin for TRPV1; 100 μM probenecid for TRPV2; 4 mM camphor for TRPV3; 3 μM 4α-PDD for TRPV4; 300 μM menthol for TRPM8; 300 μM cinnamaldehyde for TRPA1). More than 48 cells were used for the tests of each TRP activity (n = 28–76). (D) Dose–response curve for inhibitory effect of DMAPP on TRPV3, as assessed by Fluo-3 Ca^{2+} imaging. The curve was fitted by Hill equation (EC_{50} = 10.4 μM and Hill coefficient = 3.3). Symbols represent mean values of responses of Ca^{2+} influx to TRPV3 activation by 4 mM camphor in the presence of different DMAPP concentrations (n = 19–31 for each point).

DMAPP elicited inflammation

A possible involvement of neuronal or epithelial TRPV4 activation has been implicated in the development of inflammation (Reiter *et al.*, 2006; Vergnolle *et al.*, 2010). We examined whether DMAPP elicits inflammation. Two hours after an intraplantar injection of DMAPP, oedema was observed in the ipsilateral hind paw, measured as an increase in hind paw diameter (Figure 5). The time course of diameter changes showed that the peak was reached 2 h after injection (Figure 5A). Both pretreatment and post-treatment (1 h after DMAPP injection) with RN-1734 prevented oedema formation (Figure 5B,C). HC067047 further confirmed these effects were mediated by TRPV4 (Supporting Information Figure S2A,B). The contribution of neuronal TRPV4 to inflammation was shown previously (Vergnolle *et al.*, 2010). We also demonstrated that DMAPP induced the release of calcitonin gene-related peptide and substance P from cultured DRG neurons and that HC067047 prevented this effect of DMAPP (Supporting Information Figure S2C,D). As TRPV4 channels are also expressed in the surrounding tissues near the sensory nerve termini (Figure 3C,D; Watanabe *et al.*, 2003; Moqrich *et al.*, 2005), we checked whether these non-neuronal TRPV4 are involved in the inflammatory response by pretreatment with i.d. siRNA as in the nociception experiments. Compared with that with scrambled RNA, 48 h treatment with TRPV4 siRNA partly, but not completely, prevented oedema formation, which indicates non-neuronal and peripherally expressed TRPV4 are also involved in DMAPP-induced inflammation (Supporting Information Figure S1E). Further, we measured tissue MPO activity, another critical parameter for inflammation. Similar to oedema formation, DMAPP administration significantly elevated MPO levels and both pre- and post-treatment with RN-1734 were effective at reversing this effect of DMAPP (Figure 5D,E). These data suggest that DMAPP is able to provoke inflammation in a TRPV4-dependent manner. Altogether, our data demonstrate that DMAPP activates sensory neuronal and non-neuronal TRPV4 leading to acute nociception and inflammation.

Discussion

The present study shows that DMAPP activates TRPV4. DMAPP is an intermediate molecule in the mevalonate pathway in living organisms: Isopentenyl pyrophosphate (IPP) isomerase generates DMAPP from IPP. Condensation of DMAPP with IPP results in the formation of geranyl pyrophosphate (GPP), in turn used for farnesyl pyrophosphate (FPP) and cholesterol syntheses (Supporting Information Figure S2). Therefore, endogenously produced as well as

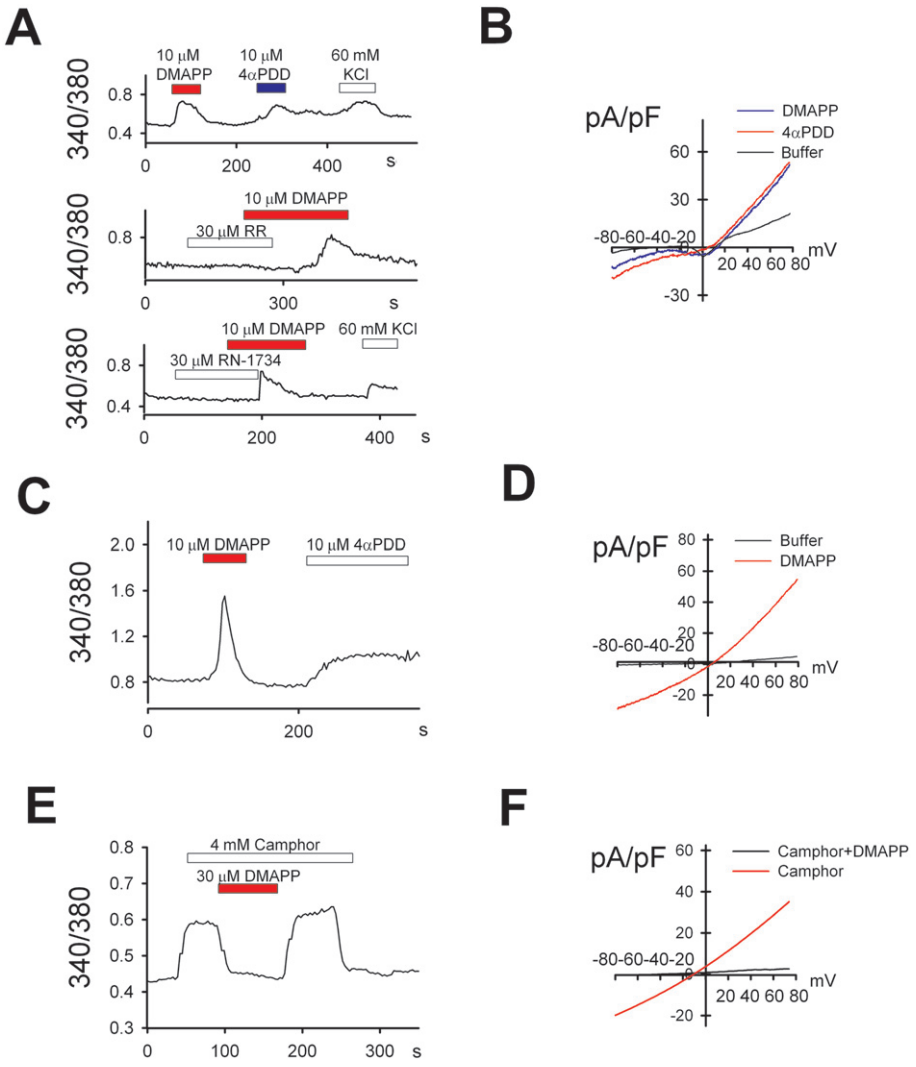

Figure 3

DMAPP activates TRPV4 and inhibits TRPV3 in native cells. (A) DMAPP 10 μM elevated intracellular Ca^{2+} levels in primarily cultured mouse DRG neurons (n = 8) in the Fura-2 Ca^{2+} imaging experiments. 4α-PDD also induced responses in the same cells. The finding that high KCl (60 mM) induced a response (via activation of voltage-gated channels) indicates that the cultured neurons retain their excitability (upper). Treatments with RR or RN-1734 blocked the increase in intracellular Ca^{2+} induced by DMAPP (middle and lower). (B) Current–voltage curves for the increases in outwardly rectifying current evoked by 10 μM DMAPP or 4αPDD in the whole-cell voltage clamp experiments with the cultured DRG neurons (n = 5 for both). The humps on the curves at ~0 mV were caused by activation of voltage-gated channels by the depolarizing ramp, indicating that the cultured neurons retained their excitability under our culture conditions (Bessac *et al.*, 2008). (C) DMAPP 10 μM elevated intracellular Ca^{2+} levels in human epidermal keratinocytes in the Fura-2 Ca^{2+} imaging experiments (n = 67). 4αPDD also induced a response in the same cells. (D) Current–voltage curves obtained for the increases in the outwardly rectifying current evoked by 10 μM DMAPP in the whole-cell voltage clamp experiments using keratinocytes (n = 5). (E) DMAPP 30 μM blocked the increase in intracellular Ca^{2+} induced by 4 mM camphor in human epidermal keratinocytes (n = 51) in the Ca^{2+} imaging experiments. (F) Inhibitory effects of 30 μM DMAPP on outwardly rectifying current responses to camphor in the whole-cell voltage clamp study using keratinocytes (n = 5).

extraneously injected DMAPP may affect TRPV4 activity. The tissue DMAPP concentration has not been thoroughly studied. An analytical study using a plant species and microorganisms once predicted 30–100 μM of cellular DMAPP (Fisher *et al.*, 2001). The human plasma level of FPP was reported to be in nM range (Saisho *et al.*, 1997) and higher concentrations of precursor molecules for FPP were expected (Bruenger and Rilling, 1988). Thus, it is possible that DMAPP may be present at around nM and μM concentrations in the plasma and cytosol. TRPV4 activation by DMAPP was observed at μM concentrations in the present study, therefore, certain pathological conditions in which cytosolic DMAPP can be released, for instance, cellular damage of adjacent cells may be critical to TRPV4 activation and subsequent nociception. In addition, it is possible that exogenous DMAPP from other organisms may also stimulate TRPV4, for example, the trigeminal TRPV4 in the oral cavity is exposed to some bacterial or vegetable isoprene after the consumption

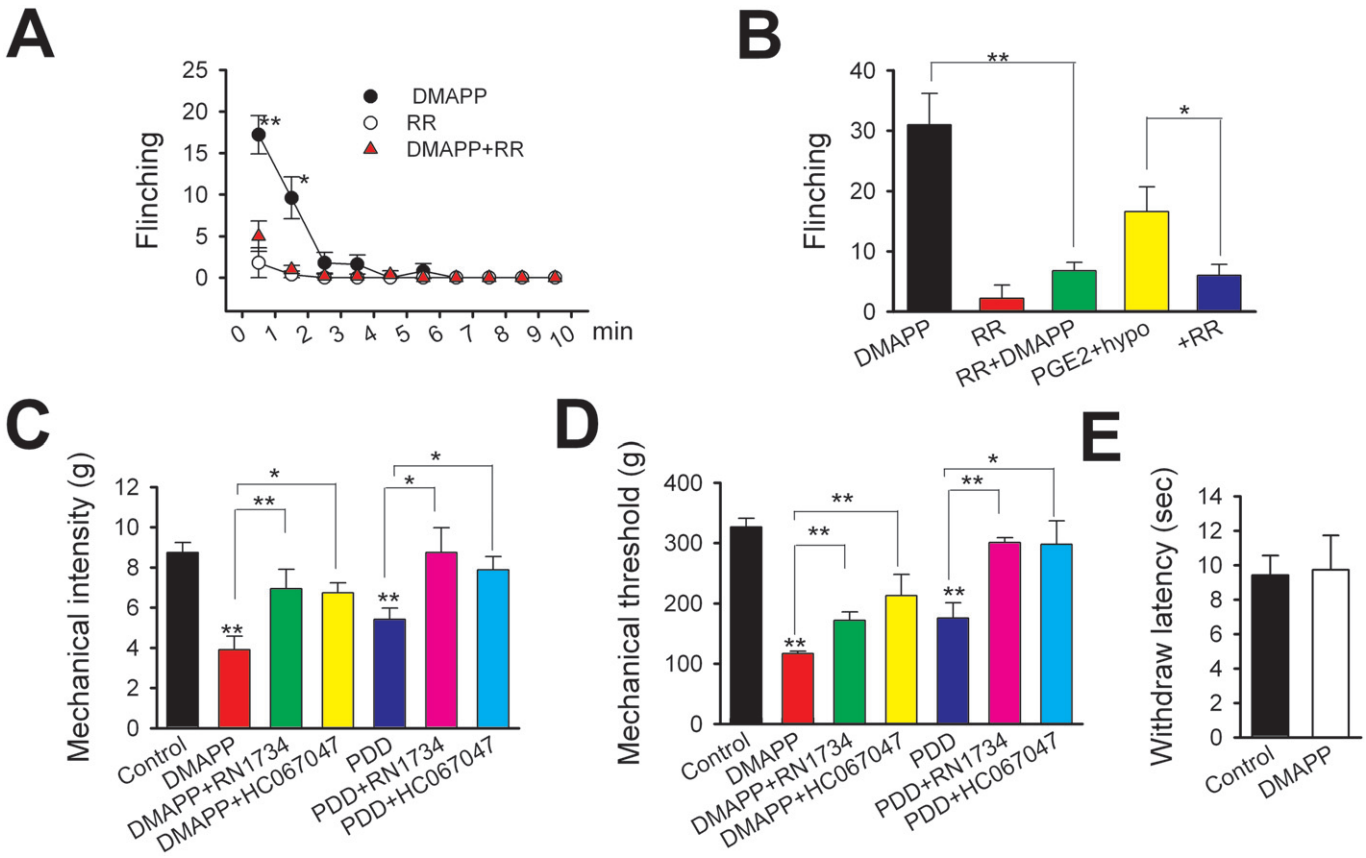

Figure 4

DMAPP induces acute nociception and lowers the mechanical threshold. (A) Summary of the time course of the flinching behaviours in mice injected intraplantarly with 3 mM DMAPP or 3 mM 4αPDD (in 10 μL vehicle) for a 10 min period immediately after the injection. The hindpaws for the test were primed with 100 ng PGE_2 5 min before the DMAPP injection ($n = 5$). The animals primed with PGE_2 without the DMAPP injection or injected with DMAPP without PGE_2 priming showed no flinching responses for 30 min (data not shown). Also when vehicle alone was injected into the primed hind paw, no flinch occurred in the mice for 10 min (data not shown). Mice pretreated with RR, injected into hind paw, showed no flinches from either DMAPP injected or non-injected hind paws ($n = 5$). (B) Summary of the total number of flinch responses from (A). The mean values of the accumulated flinch responses during the recording period (10 min) are displayed. (C) Summary of the changes in the mechanical thresholds induced by intraplantar DMAPP administration from von Frey tests. The average decreased ratio of the von Frey thresholds induced by DMAPP was 55.7 ± 6.6% ($n = 6$) and for 4αPDD was 37.2 ± 7.4% ($n = 5$). Immediately after intradermal pretreatment of the hind paw with RN-1734 (10 mM in 10 μL) or with HC067047 (1 mM in 10 μL) these threshold decreases were reversed ($n = 6$ for RN-1734; $n = 5$ for HC067047). (D) Summary of the changes in the mechanical thresholds induced by intraplantar DMAPP from Randall–Selitto tests. The average decreased ratio of the mechanical threshold induced by intraplantar administration of DMAPP (3 mM in 10 μL) was 64.2 ± 1.6% ($n = 6$). Immediately after intradermal pretreatment of the hind paw with RN-1734 (10 mM in 10 μL) or with HC067047 (1 mM in 10 μL) the threshold decreases induced by DMAPP were reversed ($n = 5$ for RN-1734; $n = 5$ for HC067047). (E) Summary of changes in heat thresholds from Hargreaves tests induced by intraplantar DMAPP ($n = 5$). Injection of vehicle alone did not affect von Frey or Randall–Selitto thresholds (data not shown). Hypo, hypotonic deionized water; PDD, 4αPDD; ND, no significance detected.

of food. Little is known about other targets of DMAPP in the body. DMAPP has been reported to be a non-peptide phosphor-antigen recognized by T cell receptors in γ9δ2-bearing T lymphocytes (Bürk *et al.*, 1995; Tanaka *et al.*, 1995). T cell receptors are not expressed in the sensory system and rapid cellular reactions between T cells and sensory neurons are unlikely, therefore, T cells are unlikely to involved in the mechanism of acute nociception induced by locally injected DMAPP in the present study.

We recently found that FPP, a downstream metabolite of DMAPP, is an endogenous TRPV3 activator (Bang *et al.*, 2010a). Nitrogen-containing bisphosphonates inhibit FPP synthase, which produces FPP by condensation of DMAPP and GPP (Supporting Information Figure S2). Certain types of bone cancer pain and neuropathic pain were shown to be alleviated by bisphosphonate treatment (Sharma *et al.*, 2006; von Moos *et al.*, 2008). Hence, it is possible that inhibition of FPP synthase by bisphosphonate contributes to analgesic mechanisms by preventing FPP-evoked TRPV3 nociception. In the same respect, IPP isomerase inhibition could be a comparably strategic target because inhibition may block the formation of not only FPP but also DMAPP, which, according to our results (Figure 4), also appears to be a putative nociceptive molecule. Furthermore, preventing this isomerase inhibition could cause the accumulation of a substrate of this enzyme, IPP, which is capable of attenuating multiple TRP

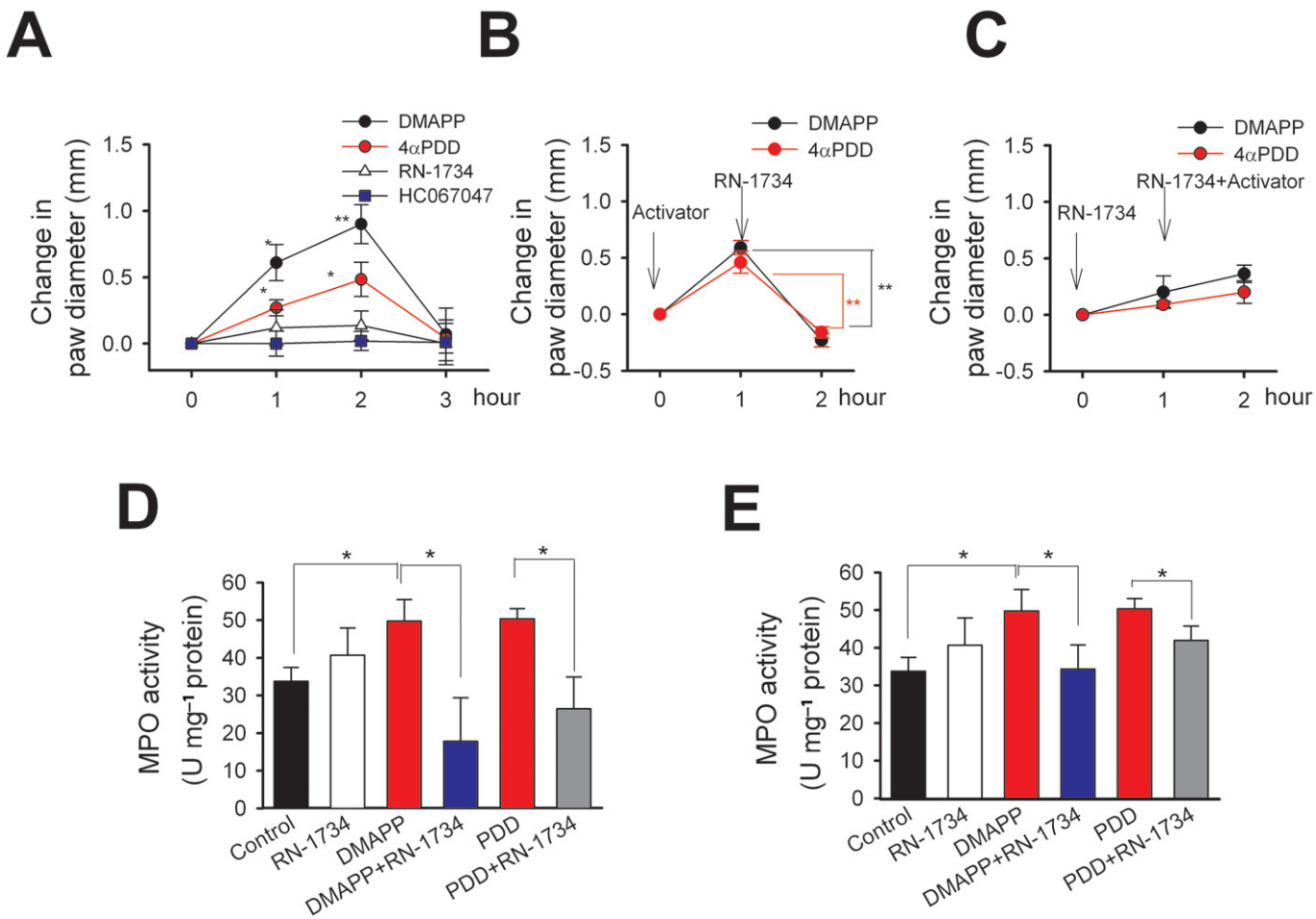

Figure 5

DMAPP induces acute inflammation. (A) Summary of the changes in ipsilateral paw diameter of mice injected intraplantarly with 3 mM DMAPP, 3 mM 4αPDD, 10 mM RN-1734 or 1 mM HC067047 in 10 μL vehicle. Five animals were tested for each drug. (B) Summary of the changes in ipsilateral paw diameter of mice injected intraplantarly with 3 mM DMAPP or 3 mM 4αPDD in 10 μL vehicle when 10 mM RN-1734 were additionally injected 1 h after the DMAPP or 4αPDD injection. Five animals were tested for each drug treatment. (C) Summary of the changes in ipsilateral paw diameter of mice injected intraplantarly with 3 mM DMAPP or 3 mM 4αPDD in 10 μL vehicle when 10 mM RN-1734 were injected before the DMAPP or 4αPDD injection. Five animals were tested for each drug treatment. (D) Tissue MPO activities of the animals in (B). (E) Tissue MPO activities of the animals in (C). Student's *t*-test was used to analyse results for (A)–(C) and ANOVA followed by Bonferroni *post hoc* test for (D) and (E).

activities (Bang *et al.*, 2011), and may also induce analgesia. Further evaluation of how much the mevalonate pathway contributes to pain development will be the critical step to determine the feasibility of these overall hypotheses.

Knowledge on TRPV4 agonists and antagonist is now increasing (for reviews: Broad *et al.*, 2009; Vriens *et al.*, 2009). Although TRPV4 was initially isolated from sensory neurons, only a synthetic phorbol ester agonist 4αPDD has been tested in terms of peripheral pain sensation (Grant *et al.*, 2007; Alessandri-Haber *et al.*, 2009). Here we demonstrated that DMAPP is also able to exert its nociceptive effect in behavioural assays. Moreover, RN-1734, a recently developed synthetic TRPV4 antagonist, was also shown to have receptor-specific anti-nociceptive effects in the present study, confirming that TRPV4 contributes to pain mediation. In fact, in addition to pain physiology, it has now become clear that TRPV4 is involved in a variety of pathological events, which include hypertension, altered bladder voiding, acoustic injuries, bone abnormalities and autosomal dominant brachyolmia. (Watanabe *et al.*, 2003; Tabuchi *et al.*, 2005; Gevaert *et al.*, 2007; Masuyama *et al.*, 2008; Rock *et al.*, 2008). It would be of interesting to see whether endogenously-generated DMAPP and its metabolites are also involved in these disease states via TRPV4 stimulation.

In this study we also demonstrated that DMAPP induces acute inflammation via TRPV4 activation. This result confirms a recent report that TRPV4 activation by its relevant stimuli, such as 4αPDD or hypotonicity, can provoke acute inflammation (Vergnolle *et al.*, 2010). In their study, Vergnolle *et al.* focused on a neurogenic mechanism via sensory neuronal TRPV4 activation but they also suggested that epidermal or endothelial TRPV4 might be involved. In contrast, we determined whether non-neuronal TRPV4 activation contributes to the development of inflammation.

From our observations, short-term (48 h) treatment with i.d. siRNA significantly inhibited, but did not completely suppress, oedema formation, whereas an additional 96 h treatment with the siRNA, which mitigates neuronal expression, completely blocked oedema formation. Thus, it appears that activation of both neuronal and non-neuronal TRPV4 are important for the development of inflammation. However, which of these mechanisms, ie epidermal TRPV4 or vascular endothelial TRPV4 (Watanabe *et al.*, 2003), is dominant for this anti-inflammatory effect remains to be elucidated. Interestingly, acute nociceptive behaviours in response to DMAPP were largely resistant to the 48 h TRPV4 knockdown, indicating that sensory neuronal TRPV4 is more important for chemical nociception than non-neuronal TRPV4. Different time courses of changes in paw diameters by DMAPP were observed compared with those of Vergnolle *et al.* (2010); this could have been due to differences in experimental conditions or species used.

It was interesting to find that DMAPP also affected TRPV3 activity. Although its antagonistic potency for TRPV3 was 10-fold lower than that of IPP, DMAPP exhibited better specificity for TRPV3. Therefore, at a restricted point of inhibition, DMAPP is a specific endogenous inhibitor for TRPV3. In tissues, if any, expressing TRPV3 but not TRPV4, specific pharmacological effects could be achieved with the use of DMAPP. However, the tissue expression patterns of both TRPVs should be determined before predicting TRPV3-specific outcomes from DMAPP use. FPP activates TRPV3 whereas their precursors, IPP and DMAPP inhibit TRPV3. Thus, the length of the isoprenyl repeats appears to determine whether a compound is an activator or an inhibitor of TRPV3. Because FPP contains an additional isoprenyl unit, the last isoprenyl unit may possibly contact a putative gating moiety of TRPV3 and the rest of the molecule (two isoprene repeats with bisphophate) may be clasped in the putative binding pocket. If this hypothesis is true, the distance between the ligand binding pocket and the gating moiety may be relatively close in TRPV4, as DMAPP is shorter than FPP. Future structural analyses will answer this question.

Together with our previous findings, the results of this study stress again the importance of mevalonate metabolites as sensory TRP modulators. Moreover, such unpaired multi-to-multi interactions between receptor subspecies and metabolites on a signalling pathway appear to be nearly unprecedented in ion channel biology. Further quantitative explanations about the complex network will help us to understand the molecular basis for the TRP-related peripheral pain mechanism. As well as academically accounting for this novel signalling in the mediation of pain, exact enzymatic modulation or the development of potent synthetic analogues may be useful to define a new therapeutic strategy for pain control. With regard to TRPV4, a hurdle in this channel research is the lack of potent and specific agonists. A recently developed TRPV4 agonist GSK1016790A can also evoke Ca^{2+} influx via an unknown mechanism (Willette *et al.*, 2008). Here we found that DMAPP is more specific, though less potent than the GSK compound, for TRPV4 activation. Moreover, the TRPV4-mediated nociceptive and inflammatory phenotype was also detected after the administration of DMAPP. Hence, DMPP may be useful pharmacological tool, which can be used investigate TRPV4-related physiological effects.

Acknowledgement

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Conflict of interest

SB and SWH applied for a Korean patent regarding the uses of DMAPP as a TRPV4 agonist (Patent Application No.: 10-2011-0000310).

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Supporting information

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

Figure S1 TRPV4 siRNA effects on DMAPP-induced nociceptive responses and oedema formation. (A) Reverse-transcriptase PCR (RT-PCR) results of TRPV4 Control and SI indicates no treatment and TRPV4-siRNA treatment respectively. Upper: RT-PCR results of TRPV4 mRNA in the mouse hind paw epidermis. 48 h after siRNA pretreatment, the pieces (~0.2 cm^2) of plantar skin epidermal layers were isolated from sacrificed mice. Extraction of total RNA from the epidermal layers was carried out using RNA preparation kit (Axygen, Union City, CA, USA). RT-PCR was performed using a PCR thermal cycler (Takara, Japan). Reverse transcription was performed using the amfiRivert single-step RT-PCR kit (GenDEPOT, Barker, TX, USA) according to the manufacturer's protocol. PCR primers were as follows: mTRPV4, 5′-ACAACACCCGAGAAACACC-3′ and 5′-CCCAAACTTACG CCACTTGT-3′; The PCR products were electrophoresized on agarose gels and stained with ethidium bromide. SI2 siRNA were used for other experiments throughout the results. Lower: RT-PCR results of TRPV4 mRNA in the mouse DRGs. 48 h or 144 h after siRNA pretreatment, DRGs were dissected out from sacrificed mice and subsequent experiments followed the above protocol. (B) Summary of the total numbers of hind paw flinches in response to intraplantarly injected DMAPP (3 mM in 10 μL vehicle) for 10 min (n = 5). siRNA or scRNA was injected into a hind paw 48 h or 144 h prior to the DMAPP injection (n = 5 respectively). Control (n = 5) indicates the vehicle administration without DMAPP. (C) Summary of the changes in the mechanical thresholds from von Frey tests by intraplantar DMAPP administration (n = 5 respectively). siRNA or scRNA was injected as in (B) (n = 5 respectively). Control indicates the threshold values measured before DMAPP injection. (D) Summary of the changes in the mechanical thresholds from Randall–Selitto tests by intraplantar DMAPP administration (n = 5 respectively). siRNA or scRNA was injected as in (B) (n = 5 respectively). (E) Summary of the changes in ipsilateral paw diameter of mice injected intraplantarly with 3 mM DMAPP in 10 μL vehicle. siRNA or scRNA was injected into a hind paw 48 h or 144 h prior to the DMAPP injection (n = 5 respectively). (F) Summary of the total numbers of hind paw flinches in response to intraplantarly injected DMAPP (3 mM in 10 μL vehicle) for 10 min (n = 5). A COX inhibitor indomethacin was injected intraperitoneally 1 h prior to the DMAPP injection (n = 5 respectively). Control (n = 5) indicates the vehicle administration without DMAPP. (G) Summary of the changes in ipsilateral paw diameter of mice injected intraplantarly with 3 mM DMAPP in 10 μL vehicle. indomethacin was injected intraperitoneally (40 $mg \cdot kg^{-1}$) 30 min prior to the DMAPP injection (n = 5 respectively). Student's t-test was performed for (C) and (D) and ANOVA followed by Bonferroni *post hoc* test was performed for (B), (E), (F) and (G).

Figure S2 (A,B) DMAPP induces TRPV4-mediated acute oedema formation. Summary of the changes in ipsilateral paw diameter of mice injected intraplantarly with 3 mM DMAPP (A) or 3 mM 4αPDD (B) in 10 μL vehicle. Injection with vehicle alone did not elicit a significant change in paw diameters. When 1 mM HC047067 were injected before the DMAPP or 4αPDD injection, oedema formations were attenuated (n = 5 respectively). (C,D) Calcitonin gene-related peptide (CGRP) and substance P (SP) releases from cultured DRG neurons upon DMAPP application. 48 h-old primarily cultured DRG neurons were used. After 30 min of incubation with 100 μM DMAPP or 100 μM 4αPDD, culture media were collected. For blocking TRPV4, 1 μM HC067047 was co-incubated. Each collection was reconstituted with assay buffer (Phoenix Pharmaceuticals, Burlingame, CA, USA) and analysed by enzyme immunoassay kits for CGRP (Phoenix Pharmaceuticals) and SP (Enzo Life Science, Farmingdale, NY, USA). (C) CGRP was detected from culture media when neurons were treated with DMAPP or 4αPDD. HC067047 co-incubation attenuated the CGRP releases. (D) SP was detected from culture media when neurons were treated with DMAPP or 4αPDD. While HC067047 intrinsically induced SP releases, SP amounts released upon either DMAPP or 4αPDD incubation were significantly reduced by co-incubation with HC067047.

Figure S3 The mevalonate pathway.

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