BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2010.00909.x
www.brjpharmacol.org

RESEARCH PAPER

Resolvin D1 attenuates activation of sensory transient receptor potential channels leading to multiple anti-nociception

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Keywords
Resolvin D1; TRPA1; TRPV3; TRPV4; pain; sensory neurons; keratinocytes; nociceptive behaviours; inflammation

Received
11 October 2009
Revised
21 April 2010
Accepted
11 May 2010

BACKGROUND AND PURPOSE

Temperature-sensitive transient receptor potential ion channels (thermoTRPs) expressed in primary sensory neurons and skin keratinocytes play a crucial role as peripheral pain detectors. Many natural and synthetic ligands have been found to act on thermoTRPs, but little is known about endogenous compounds that inhibit these TRPs. Here, we asked whether resolvin D1 (RvD1), a naturally occurring anti-inflammatory and pro-resolving lipid molecule is able to affect the TRP channel activation.

EXPERIMENTAL APPROACH

We examined the effect of RvD1 on the six thermoTRPs using Ca^{2+} imaging and whole cell electrophysiology experiments using the HEK cell heterologous expression system, cultured sensory neurons and HaCaT keratinocytes. We also checked changes in agonist-specific acute licking/flicking or flinching behaviours and TRP-related mechanical and thermal pain behaviours using Hargreaves, Randall-Selitto and von Frey assay systems with or without inflammation.

KEY RESULTS

RvD1 inhibited the activities of TRPA1, TRPV3 and TRPV4 at nanomolar and micromolar levels. Consistent attenuations in agonist-specific acute pain behaviours by immediate peripheral administration with RvD1 were also observed. Furthermore, local pretreatment with RvD1 significantly reversed mechanical and thermal hypersensitivity in inflamed tissues.

CONCLUSIONS AND IMPLICATIONS

RvD1 was a novel endogenous inhibitor for several sensory TRPs. The results of our behavioural studies suggest that RvD1 has an analgesic potential via these TRP-related mechanisms.

Abbreviations

4α–PDD, 4-α-phorbol 12,13-didecanoate; DRG, dorsal root ganglion; FPR2/ALX, formyl peptide receptor 2; PBS, phosphate-buffered saline; RvD1, resolvin D1 (7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid); TRP, transient receptor potential

Introduction

Temperature-sensitive transient receptor potential ion channels (thermoTRPs) expressed in the sensory neurons and skin keratinocytes play roles of detecting internal or external environmental changes. One of these thermoTRPs is TRPA1 (channel nomenclature follows Alexander *et al.*, 2009) that senses multiple noxious signals such as cold temperatures, irritant substances and mechanical insults (Bang and Hwang, 2009; Caspani and Heppenstall, 2009; Kwan and Corey, 2009; Stucky *et al.*, 2009). Another member, TRPV3 is known to detect temperatures exceeding 33°C (Peier *et al.*, 2002; Smith

et al., 2002; Xu *et al.*, 2002). TRPV4 is also able to sense warm temperature and mechanical insults as well (Liedtke *et al.*, 2000; Strotmann *et al.*, 2000; Güler *et al.*, 2002; Watanabe *et al.*, 2002b). Studies with knockout or transgenic mice have shown that pain is elicited through the activation of peripheral TRPA1, TRPV3 or TRPV4 channels (Liedtke and Friedman, 2003; Suzuki *et al.*, 2003; Moqrich *et al.*, 2005; Bautista *et al.*, 2006; Kwan *et al.*, 2006; Huang *et al.*, 2008; Karashima *et al.*, 2009). Thus, pharmacological manipulation for activating or inhibiting these sensory TRP channels seems critical for pain modulation.

For TRPA1, many natural and synthetic compounds have been found to activate the channel: For example, cinnamaldehyde and mustard oil are commonly used as tools for the specific activation of TRPA1 in pharmacological experiments (Bandell *et al.*, 2004; Jordt *et al.*, 2004). A potent agonist, supercinnamaldehyde was synthesized recently (Macpherson *et al.*, 2007), in addition to endogenous agonist covalent ligands such as 4-hydroxynonenal, 4-oxononenal, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and acetaldehyde (Bang *et al.*, 2007a; Trevisani *et al.*, 2007; Andersson *et al.*, 2008; Taylor-Clark *et al.*, 2008a,b). There is active development of synthetic antagonists, such as HC030031 and AP18, and both of these compounds exhibit significant pain reduction, indicating that negative modulation of TRPA1 channels is a feasible strategy for analgesia (McNamara *et al.*, 2007; Petrus *et al.*, 2007). There is much less information on specific ligands for TRPV3 or TRPV4 than for TRPA1 channels. The phytochemical camphor and 4-α-phorbol 12,13-didecanoate (4α–PDD) are relatively specific for activation of TRPV3 and TRPV4 channels, respectively (Watanabe *et al.*, 2002a; Moqrich *et al.*, 2005), but reports on TRPV3 or TRPV4-specific inhibitory ligands are still few.

For TRPA1, TRPV3 or TRPV4 channels, there are no reports of any endogenous antagonist. Resolvins are lipid metabolites capable of resolving inflammation and are endogenously produced by the pathways of ω-3 fatty acid metabolism (Ariel and Serhan, 2007; Serhan and Chiang, 2008; Kohli and Levy, 2009). Resolvin D1 (7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid: RvD1) is one example, found in human and animal tissues (Serhan *et al.*, 2002) and its complete stereochemistry was recently established (Sun *et al.*, 2007). Here, we report that RvD1 is an endogenous inhibitor for the three sensory TRP channels. In addition, RvD1 suppressed the three TRP channels-mediated nociceptive animal behaviours.

Materials and methods

Animals

All animal care and experimental procedures were in accordance with protocols approved by the University Committee on Laboratory Animals.

Cell cultures

HEK293T cells were cultured as previously reported (Bang *et al.*, 2007a,b). Cells were maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin. The HEK293T cells were transfected transiently with 3 μg of individual TRP channel plasmid DNA (mTRPA1, rTRPV1, rTRPV2 or mTRPV4 in pcDNA3.1; hTRPV3 or mTRPM8 in PCDNA5/FRT) per 35 mm dish using Fugene HD (Roche Diagnostics Corp., Indianapolis, IN). The human keratinocytes (HaCaT) (a kind gift from Tae-Yoon Kim at the Catholic University of Korea) were maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin. Mouse dorsal root ganglion (DRG) neurons were cultured. Briefly, mouse dorsal root ganglia were dissected out of adult ICR mice in cold phosphate-buffered saline (PBS) and treated with 1.5 mg·mL^{-1} collagenase/dispase (Roche Diagnostics Corp.) at 37°C for 45 min and followed by treatment with 0.25% trypsin (Invitrogen Corp.) for 15 min. Neurons were then plated onto poly-L-ornithine-coated glass cover slips in DMEM/F12 containing 10% FBS, 1% penicillin/streptomycin and 5 ng·mL^{-1} 2.5S NGF (Merck KGaA, Darmstadt, Germany). Experiments with DRG neurons were performed 48–72 h after plating. All cells were grown at 37°C and 5% CO_2.

Ca^{2+} imaging experiments

Ca^{2+} imaging experiments were carried out as previously reported (Kim *et al.*, 2008). Briefly, cells were plated onto poly-L-lysine-coated 35-mm glass cover slips and used for Fura-2 Ca^{2+} imaging 16–48 h later. The cells were loaded with 5 μM Fura-2AM for 30 min and the cells were resuspended in (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, titrated to pH 7.4 with NaOH. Images of Fura-2 loaded cells with the excitation wavelength alternating between 340 nm and 380 nm were captured with a cooled CCD camera (Retiga-SRV, Q-imaging Corp., Burnaby, BC, Canada). The ratio of fluorescence intensity of the two wavelengths in each experiment was analysed using MetaFluor (Molecular Devices, Sunnyvale, CA). Values from each of the experiments were normalized to baseline of the ratio 340/380 nm.

Patch-clamp electrophysiology

Whole-cell voltage clamp recordings were performed as described previously (Bang *et al.*, 2007a).

Briefly, the bath solution contained (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, titrated to pH 7.4 with NaOH. The pipette solution contained (in mM) 140 CsCl, 5 EGTA, 10 HEPES, 2.0 MgATP, 0.2 NaGTP titrated to pH 7.2 with CsOH. The holding potential was –60 mV and for the current-voltage analysis, 800 ms voltage-ramp pulses from –80 to +80 mV were used.

Behavioural studies

Six-week old Male ICR mice, TRPA1-null mice and their heterozygotes (a generous gift from Heung Sik Na) were used. Animals were acclimatized for 1 h to the test environment prior to performing the experiments. Hind paw licking and flicking behaviour was quantitated every minute for 10 min (for formalin test, 45 min) as previously described (Moqrich *et al.*, 2005; Bang *et al.*, 2007a). Hargreaves (Plantar Analgesia meter, for heat hyperalgesia), von Frey (Dynamic Plantar Aesthesiometer, for mechanical allodynia) and Randall-Selitto apparatus (Analgesymeter, for blunt pressure-evoked nociceptive flexion reflex) were from UGO Basile (Italy). Assays for changes in thermal or mechanical behaviours under inflammation were performed as described previously (Moqrich *et al.*, 2005; Yoo *et al.*, 2009). The Hargreaves and von Frey test were performed under unconstrained conditions, and in the Randall-Selitto test mice were suspended in a restraining device. For inflammation, 50 µL complete Freund's adjuvant (CFA) was injected into a hind paw 24 h prior to the RvD1 injection. Baseline responses were then measured 5 min prior to RvD1 administration. Paw withdrawal latencies or thresholds of the contralateral hind paws were also measured both before and after drug injection.

Drugs were injected in 10 µL vehicle (PBS containing 0.5% Tween 80) into mice hind paws intradermally at the doses detailed in the results.

Data analysis

Data are shown as means ± SEM and were analysed using the two-tailed Student's *t*-test For the comparison of the accumulating licking/flicking time or flinch numbers, one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test was performed and for the comparison of the data at each time point, Student's *t*-test was performed.

Materials

All chemicals were purchased from Sigma-Aldrich unless otherwise described. Stock solutions were made using water or ethanol and were diluted with the bath solution prior to use unless otherwise described. RvD1 was purchased from Cayman Chemical Co. (Ann Arbor, MI). Cinnamaldehyde was purchased from MP Biomedicals (Solon, OH). Formyl receptor agonists were purchased from Peptron (Peptron Inc., South Korea). RvD1 was stored at –80°C. Immediately before use, RvD1 was directly diluted to test concentrations with the bath solution and briefly sonicated. Under such manipulations, no degradation of RvD1 was detected in mass/mass spectroscopy measurements (data not shown).

Results

RvD1 inhibits TRPA1, TRPV3 and TRPV4 channels in heterologous expression system

Fura-2 Ca^{2+} imaging experiments were performed with transiently transfected HEK293T cells to test if RvD1 inhibited TRPA1, TRPV3 and TRPV4 channels. During RvD1 application, no intracellular Ca^{2+} increase in the TRPA1-expressing HEK cells was detected in response to cinnamaldehyde (Figure 1A). When RvD1 was washed out, cinnamaldehyde evoked Ca^{2+} influx in the same cells, indicating that the Ca^{2+} influx through activated TRPA1 by cinnamaldehyde was reversibly suppressed by RvD1 (Figure 1A). The same suppression by RvD1 were observed for activation of TRPV3 channels by camphor and of TRPV4 channels by 4α–PDD in transfected cell experiments (Figure 1B and C). Next, whole cell voltage clamp experiments were carried out with HEK cells transfected with TRPA1, TRPV3 or TRPV4 channels. During cinnamaldehyde application, outwardly rectifying current responses were detected from TRPA1-transfected HEK cells (Figure 1D). The current in response to cinnamaldehyde was apparently blocked by the addition of RvD1 (Figure 1D). As observed with TRPA1 channels, the TRPV3 and TRPV4 current responses to camphor and to 4α–PDD were robustly attenuated by co-application of RvD1 (Figure 1E and F). No current response or Ca^{2+} influx was observed during the application of RvD1 alone in transfected HEK cells, indicating that RvD1 did not have a partial agonist activity on the three TRP channels (n = 5–7 for whole cell electrophysiology and n = 26–30 for Fura-2 Ca^{2+} imaging. Figure 1A–C).

We performed Fura-2 Ca^{2+} imaging to examine the specificity of RvD1 as an inhibitor of thermoTRP channels, using HEK293T cells expressing the individual thermoTRP channels. Of the six thermoTRP channels we tested, three TRP channels (TRPA1, TRPV3 and TRPV4) showed clear suppression of channel activation by RvD1 (Figure 2A). From the Ca^{2+} imaging experiments again, dose–response curves of RvD1 were obtained (Figure 2B). The IC_{50} value for TRPA1, TRPV3 and TRPV4 channels was

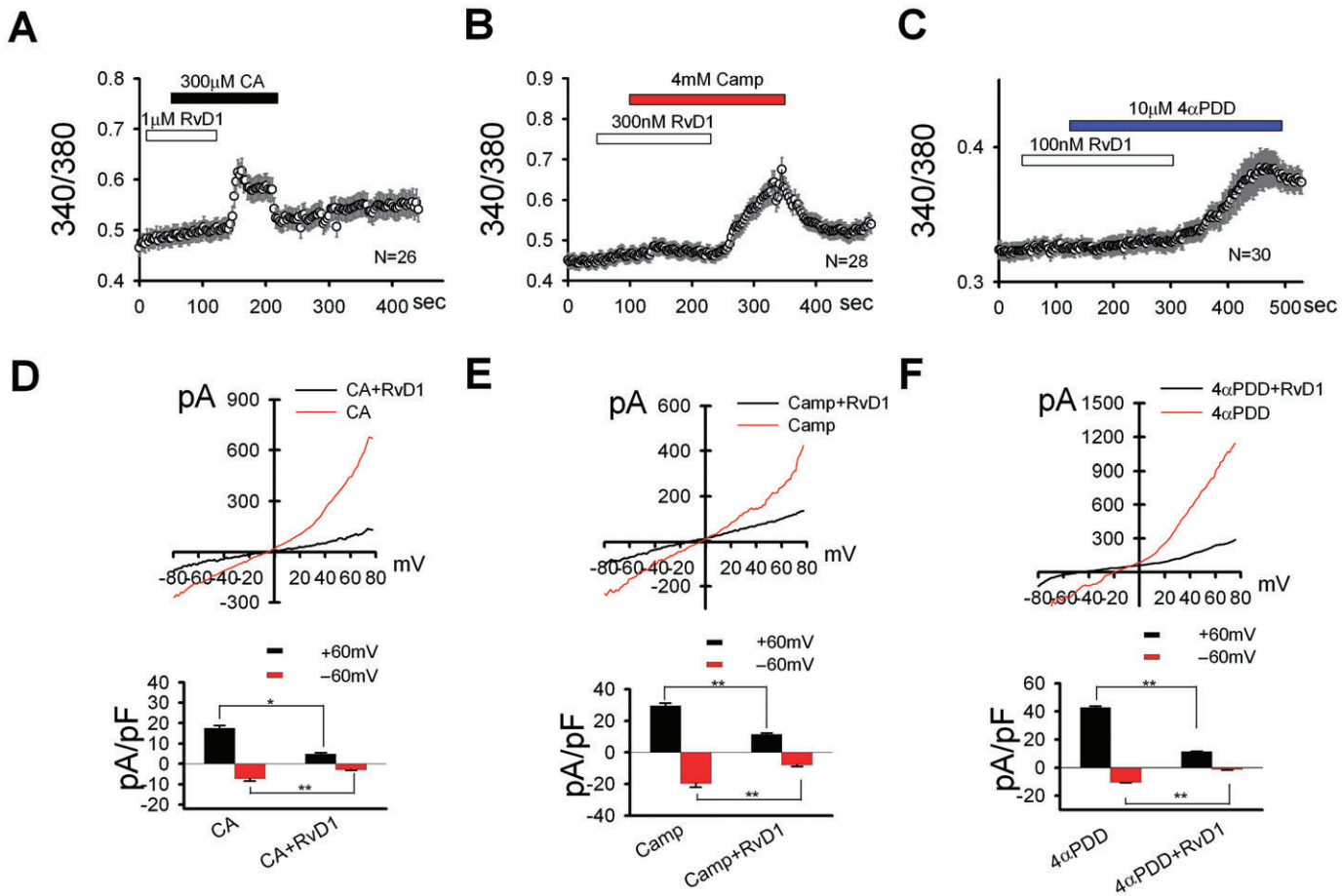

Figure 1

RvD1 inhibits thermoTRP channels in HEK293T cells. (A–C) 100–1000 nM RvD1 attenuated intracellular Ca^{2+} increases in response to TRP-specific agonists in Fura-2 Ca^{2+} imaging experiments using thermoTRP-expressing HEK cells. RvD1 attenuated TRPA1 (A), TRPV3 (B) and TRPV4 (C) activities in response to 300 μM cinammaldehyde (CA), 4 mM camphor (Camp) and 10 μM 4α–PDD respectively (n = 26, 28 and 30). (D–F) 100–1000 nM RvD1 attenuated current responses to TRP-specific agonists in whole cell voltage clamp experiments using thermoTRP-expressing HEK cells. Current-voltage curves from the responses to the agonist alone and the agonist plus 300 nM RvD1 were superimposed. RvD1 attenuated TRPA1 (D), TRPV3 (E) and TRPV4 (F) current responses upon applications with 300 μM cinammaldehyde, 4 mM camphor and 10 μM 4α-PDD respectively (n = 5, 7 and 6). Lower graphs in (D–F): average current density through each TRP channel. Current densities at ±60 mV for the agonist-induced activation of TRP channels and for agonist-induced TRP activation with RvD1 co-application. *P < 0.05, **P < 0.01; significantly different from responses with agonist alone. 4α–PDD, 4-α-phorbol 12,13-didecanoate; RvD1, resolvin D1 (7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid); TRP, transient receptor potential.

68.7 nM, 28.9 nM and 8.1 nM, respectively, indicating that RvD1 had a relatively greater potency against TRPV4 channels. Collectively, the data suggest that RvD1 is able to effectively and reversibly inhibit activities of TRPA1, TRPV3 and TRPV4 channels among thermoTRPs, in nanomolar and micromolar ranges. We asked whether such inhibitions of TRP channels by RvD1 were caused by activation of the formyl peptide receptor 2 (FPR2/ALX), a known RvD1 receptor (Krishnamoorthy *et al.*, 2010). Cathelicidin LL-37 and Trp-Lys-Tyr-Met-Val-Met (WKYMVM) are also activators of FPR2/ALX but these two compounds did not affect the three TRP channel activities, indicating that RvD1 did not use this metabotropic signalling pathway to block the TRP channels (Figure 2C and D).

RvD1 inhibits TRPA1, TRPV3 and TRPV4 channels expressed in native cells

We hypothesized that the similar inhibitory effect of RvD1 may be observed in naturally TRP-expressing cells. We carried out Fura-2 Ca^{2+} imaging and whole cell voltage clamp experiments using native cells expressing the three thermoTRPs. From cultured mouse DRG neurons, TRPA1-mediated cinnamaldehyde responses were detected in Ca^{2+} imaging in a population and addition of RvD1 robustly attenuated these responses (Figure 3A). For tests involving

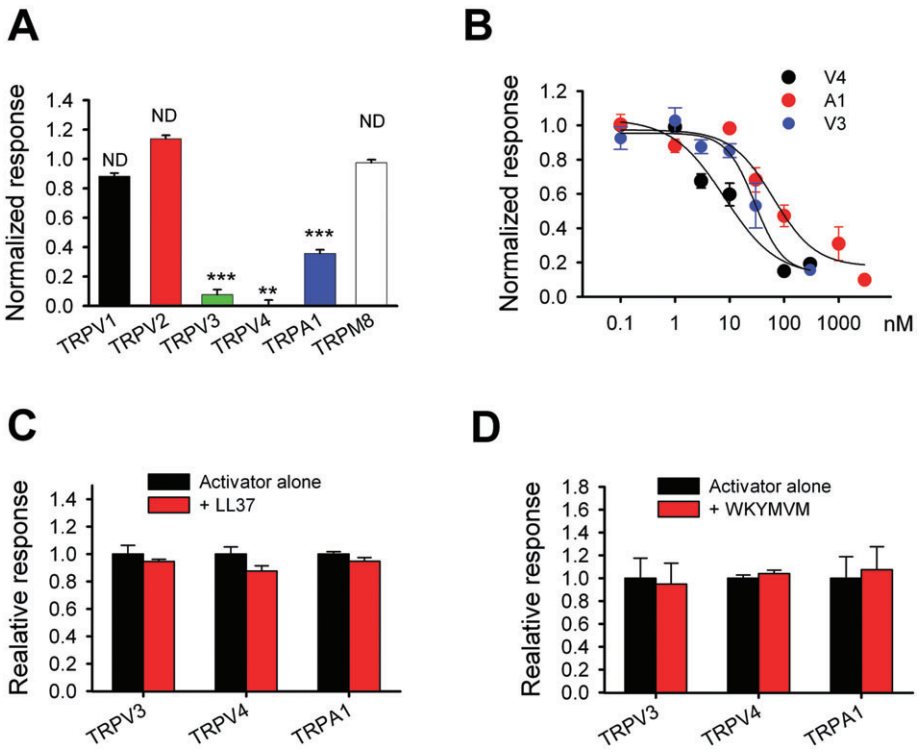

Figure 2

Specificity and potency of RvD1 on thermoTRP channels. (A) HEK293T cells transiently transfected with individual thermoTRP channels were used to determine 300 nM RvD1-induced reduction in the intracellular Ca^{2+} increases after appropriate agonists (0.2 μM capsaicin for TRPV1; 100 μM probenecid for TRPV2; 4 mM camphor for TRPV3; 10 μM 4α-PDD for TRPV4; 300 μM menthol for TRPM8; 300 μM cinnamaldehyde for TRPA1). Data, from Fura-2 Ca^{2+} imaging, were normalized to the averaged responses from each TRP channel after agonist alone and used for statistical comparisons ($n = 31–81$ for each TRP). $^{**}P < 0.01$, $^{***}P < 0.001$; significantly different from responses with agonist alone. (B) Dose–response curves for RvD1 on TRPA1 (A1), TRPV3 (V3) and TRPV4 (V4) responses in whole cell voltage clamp experiments. A total of 300 μM cinnamaldehyde for TRPA1, 4 mM for TRPV3, 10 μM 4α-PDD for TRPV4 were used as specific agonists. In total, 0.1–3000 nM of RvD1 were used for inhibition. The curves were fitted by Hill equation ($IC_{50} = 63.7$ nM; $n_H = 1.1$ for TRPA1, $IC_{50} = 28.9$ nM; $n_H = 1.6$ for TRPV3 and $IC_{50} = 8.1$ nM; $n_H = 0.9$ for TRPV4). Mean (±se mean) values of Ca^{2+} influx ($n = 5–6$ for each point) via TRPA1, TRPV3 and TRPV4 channels. (C–D) Lack of effects of FPR2/ALX agonists (10 μM LL-37 and 300 nM WKYMVM) on the intracellular Ca^{2+} increases after the appropriate agonists in Fura-2 Ca^{2+} imaging ($n = 17–61$ for each TRP). 4α–PDD, 4-α-phorbol 12,13-didecanoate; RvD1, resolvin D1 (7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid); TRP, transient receptor potential.

native TRPV3, channels we used HaCaT keratinocytes, as skin keratinocytes express many TRPV3 channels. Here also RvD1 attenuated the responses to camphor mediated by TRPV3 channels, in our Ca^{2+} imaging experiments (Figure 3B). DRG neurons responsive to 4α–PDD, assumed to express TRPV4 channels, also showed blockade of these responses on addition of RvD1 (Figure 3C). Furthermore, as in the HEK cell experiments, the same inhibitions were reproduced in our whole cell voltage clamp experiments (Figure 3D–F). Capsaicin induced Ca^{2+} influxes in a population of DRG neurons expressing TRPV1 channels. No inhibition of capsaicin responses in these mouse DRG neurons was detected after RvD1, confirming the resistance of TRPV1 channels to RvD1 (Figure 4A). High potassium-induced Ca^{2+} influx, which is mediated by native voltage-gated Ca^{2+} channels were readilyelicited in the sensory neurons and addition of RvD1 did not affect the 60 mM KCl responses, indicating that RvD1 does not activate or inhibit the voltage-gated channels (Figure 4B). In summary, RvD1 was able to suppress three thermoTRP channels, in heterologous expression systems and in cultured native cells.

RvD1 attenuates acute nociception in mice

Activation of thermoTRP channels is important to initiate pain sensation (Patapoutian *et al.*, 2009). To examine whether RvD1 is able to suppress receptor-specific, acute pain behaviours in mice, we used drug-induced hind paw licking/flicking or flinching assays for thermoTRP-mediated pain. When vehicle or RvD1 alone was intradermally injected into a hind paw, no such behaviours occurred in the mice (data not shown for vehicle; for RvD1, Figure 5A and B). Intradermal injection with a TRPA1-specific

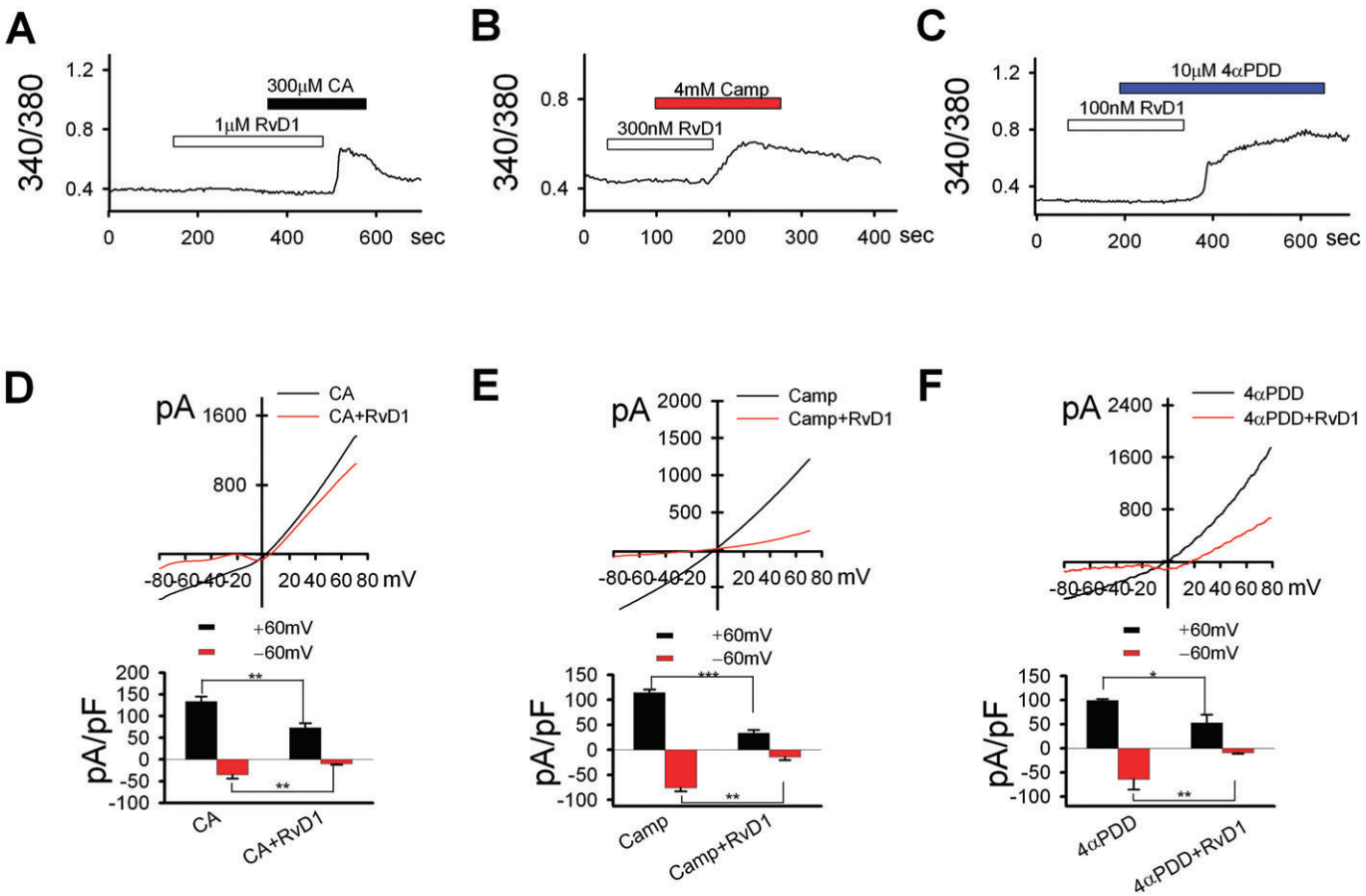

Figure 3

RvD1 inhibits thermoTRP channels in native cells. (A) 1000 nM RvD1 attenuated intracellular Ca^{2+} increases in response to 300 μM cinnamaldehyde (CA) in Fura-2 Ca^{2+} imaging experiments using cultured mouse DRG neurons (n = 21). (B) 300 nM RvD1 attenuated intracellular Ca^{2+} increases in response to 4 mM camphor (Camp) in Fura-2 Ca^{2+} imaging experiments using human HaCaT keratinocytes (n = 107). (C) 100 nM RvD1 attenuated intracellular Ca^{2+} increases in response to 10 μM 4α-PDD in Fura-2 Ca^{2+} imaging experiments using cultured mouse DRG neurons (n = 7). (D–F) 100–1000 nM RvD1 attenuated current responses to TRP-specific agonists in the whole cell voltage clamp experiments using native cells. Current-voltage curves from the responses to the agonist alone and the agonist plus RvD1 were superimposed (D–F). (D) 1000 nM RvD1 attenuated current responses to 300 μM cinnamaldehyde in cultured mouse DRG neurons (n = 7). (E) 300 nM RvD1 attenuated current responses to 4 mM camphor in HaCaT keratinocytes (n = 5). (F) 100 nM RvD1 attenuated current responses to 10 μM 4α-PDD in cultured mouse DRG neurons (n = 5). Lower graphs in (D–F): average current density through each TRP channel. Current densities at ±60 mV for the agonist-induced activation of TRP channels and for agonist-induced TRP activation with RvD1 co-application. *P < 0.05, **P < 0.01, ***P < 0.001; significantly different from responses with agonist alone. 4α–PDD, 4-α-phorbol 12,13-didecanoate; RvD1, resolvin D1 (7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid); TRP, transient receptor potential.

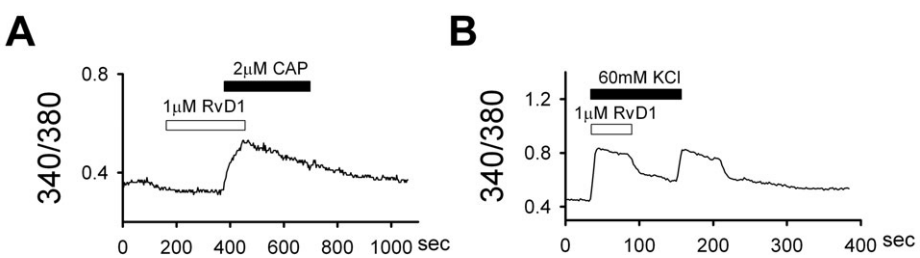

Figure 4

RvD1 did not affect TRPV1 or voltage-gated channel activities in sensory neurons. (A–B) 100 μM RvD1 did not block intracellular Ca^{2+} increases in response to 2 μM capsaicin (A) or 60 mM KCl (B) in Fura-2 Ca^{2+} imaging experiments, using cultured mouse DRG neurons (n = 27 and 17 respectively). 4α–PDD, 4-α-phorbol 12,13-didecanoate; DRG, dorsal root ganglion; TRP, transient receptor potential.

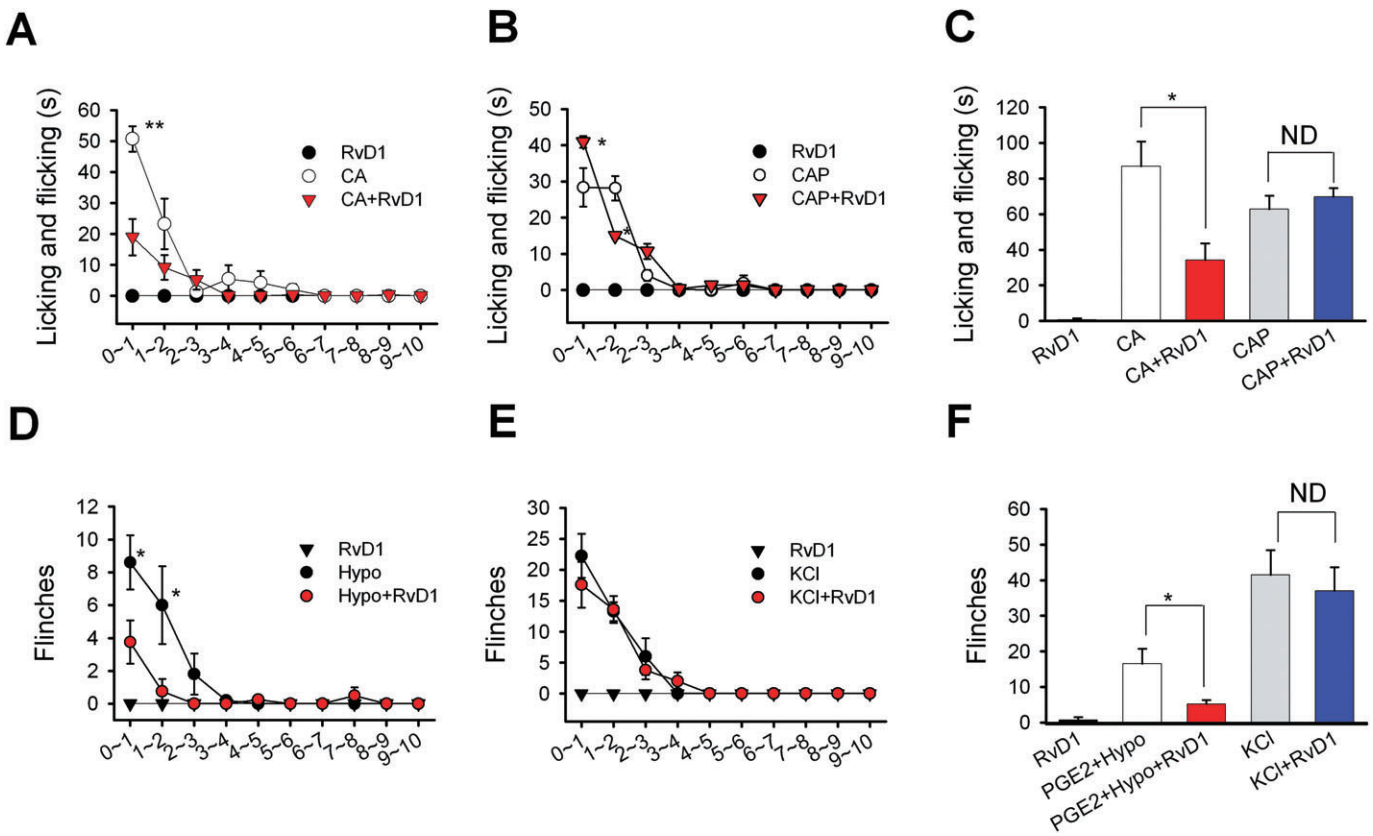

Figure 5

RvD1 suppresses thermoTRP-mediated acute nociception in mice. (A) Summary of the time course of licking/flicking behaviours in mice treated with cinnamaldehyde (CA; 13.2 μg in 10 μL) administered intradermally into hind paws for the 10 min period immediately following the injection ($n = 5$). Pretreatment with RvD1 (20 ng in 10 μL) 5 min prior to cinnamaldehyde prevented such behaviours ($n = 5$). Injection of one hind paw with RvD1 alone showed no licking/flicking responses from either injected or non-injected hind paws, comparable with the vehicle-injected controls ($n = 5$). (B) Summary of the time course of licking/flicking behaviours in mice treated with capsaicin (CAP; 20 ng in 10 μL) administered intradermally into hind paws, for the 10 min period immediately following the injection ($n = 5$). RvD1 (20 ng) was given 5 min before capsaicin ($n = 5$). (C) Summary of the accumulating licking/flicking time of (A) and (B). The mean ± se mean values of the sum of the licking/flicking time during the recording period (10 min) are shown. (D) Summary of the time course of the flinching behaviours in mice injected intraplantarly with 10 μL deionized water (Hypo) 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 injection of the deionized water and then mice showed flinching behaviours in response ($n = 5$). The animals primed with PGE_2 without the deionized water injection or animals injected with deionized water without PGE_2 priming showed no flinching behaviour responses for 30 min (data not shown). Averaged flinching data from the mice pretreated with RvD1 are shown ($n = 5$). Mice with a hind paw treated with RvD1 alone showed no flinches from either injected or non-injected hind paws ($n = 5$).). (E) Summary of the time course of the flinching behaviours in mice injected intraplantarly with 10 μL KCl solution (140 mM) for a 10 min period immediately after the injection ($n = 5$). Averaged flinching data from the mice pretreated with RvD1 are shown ($n = 5$). (F) Summary of the total flinching numbers of (D and E). The mean ± se mean values of the accumulated flinching numbers during the recording period (10 min) are shown.). $^*P < 0.05$ $^{**}P < 0.01$; significantly different from responses without RvD1. RvD1, resolvin D1 (7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid); TRP, transient receptor potential.

agonis,t cinnamaldehyde, in the hind paws immediately induced licking/flicking behaviours which lasted ~5 min, as reported previously (Bandell *et al.*, 2004; Bang *et al.*, 2007a). The behavioural effects of cinnamaldehyde were attenuated when the mice were intradermally pretreated with RvD1. Another TRPA1-specific activator, formalin, evokes acute inflammatory pain behaviours with two phases (Macpherson *et al.*, 2007). Intradermal RvD1 significantly suppressed both of the phases of this behavioural response (Figure 6A and B). These data indicated that RvD1 was able to alleviate TRPA1 channel-mediated acute nociception (Figure 5A and C).

TRPV4 channels are activated by a decrease in osmolarity (Liedtke *et al.*, 2000; Strotmann *et al.*, 2000). *In vivo*, nociceptive behaviours (flinches) were evoked by a subplantarly injected hypotonic solution when the paws had been acutely primed with an intradermal PGE_2 injection. Because the nociceptive behaviours are robustly suppressed in animals treated with TRPV4 antisense or in TRPV4

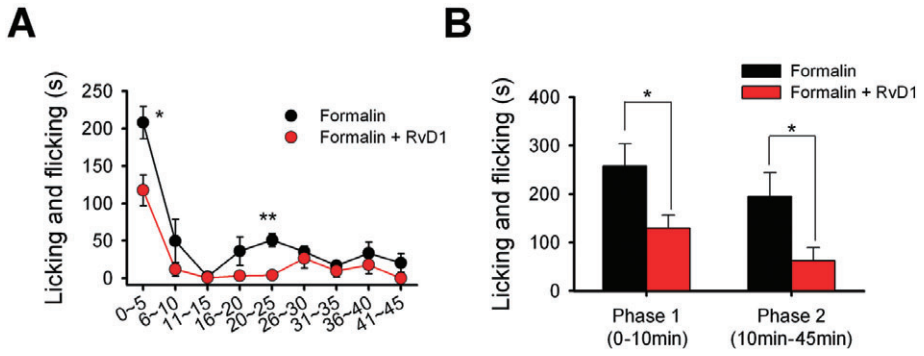

Figure 6

RvD1 suppresses formalin-induced pain. (A) Summary of the time course of licking/flicking behaviours in mice treated with formalin (4% in 20 μL) administered intradermally into hind paws, for the 45 min immediately following the injection (n = 5). RvD1 (20 ng) was given 5 min before formalin (n = 5). (B) Summary of the accumulating licking/flicking time of first and second phases.). *P < 0.05 **P < 0.01; significantly different from responses without RvD1. RvD1, resolvin D1 (7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid).

knockout mice, these behavioural responses appears to be mediated by TRPV4 channel activation (Alessandri-Haber *et al.*, 2003; 2005). We also observed the same behavioural responses and the responses were partly prevented by an intradermal pretreatment with RvD1 into the foot pads (Figure 5D and F). We also asked whether other receptor-specific nociceptive responses were affected by RvD1. Therefore, we carried out assays for licking/flicking induced by capsaicin to test for TRPV1-specific pain (Petrus *et al.*, 2007) and KCl-induced flinching assay for non-specific depolarization-induced pain. Distribution of licking/flicking occurrence elicited by local administration of capsaicin in RvD1-treated mice appeared to deviate slightly from the group without RvD1 treatment (Figure 5B), but the total licking/flicking time was not affected significantly (Figure 5C). Thus, in accordance with the *in vitro* results described above, RvD1 did not seem to act on TRPV1 channels *in vivo*. We had already demonstrated that KCl-induced sensory neuronal responses *in vitro* were not modified by RvD1 (Figure 4B) and consistent with these findings, the acute nociceptive response evoked *in vivo* by the KCl-induced depolarization was not prevented by RvD1 treatment (Figure 5D and F). Overall, the data suggest that RvD1 significantly attenuates behavioural nociception in a receptor-specific manner in acute pain behaviour tests.

RvD1 attenuates inflammatory hypersensitivity in mice

Earlier, it was shown that synthetic TRPA1 channel antagonists such as AP18 and HC-030031, blocked mechanical hypersensitivity in mice and rats with CFA-induced inflammation (Petrus *et al.*, 2007; Eid *et al.*, 2008). TRPV4 channels also mediate noxious mechanical sensation (Liedtke and Friedman, 2003; Suzuki *et al.*, 2003). We asked whether RvD1 would show analgesic activities in models of inflammatory mechanical hypersensitivity. RvD1 possibly has an anti-inflammatory activity that, irrespective of TRP-mediated action, may lead to decreased pain perception (Serhan and Chiang, 2008; Kohli and Levy, 2009; Xu *et al.*, 2010). Thus, we administered RvD1 locally and immediately before the tests, as in the above acute behavioural assays, to prevent the occurrence of the mixed mechanism and to focus on receptor-mediated actions. Injection with RvD1 alone did not affect the von Frey mechanical threshold or paw withdrawal latency of the Randall Selitto test in non-inflamed paws (Figure 7A and B). In the CFA-inflammation model, the von Frey threshold was significantly decreased and local treatment with AP18 partially reversed the fall in threshold (Figure 7A). Surprisingly, RvD1 exhibited a comparable analgesic effect to that of AP18 (Figure 7A). Furthermore, the analgesic profiles of RvD1 in the Randall Selitto assay were similar to those in the von Frey assays (Figure 7B). The TRPA1-mediated effects were further assessed with TRPA1 channel-knockout mice. While RvD1 retained its mechanical antinociceptive effects in the heterozygotes, these effects were abolished in the knockout animals (Figure 7D and E), suggesting that RvD1 was here also acting through TRPA1 channels, as reported previously (Petrus *et al.*, 2007). These data suggest that RvD1 exerted significant analgesic effects in models of inflammatory mechanical hypersensitivity, related to activation of TRPA1/TRPV4 channels.

Many reports have suggested that acute *in vivo* sensitivity to noxious heat does not seem to involve TRPV4 channels (Liedtke and Friedman, 2003;

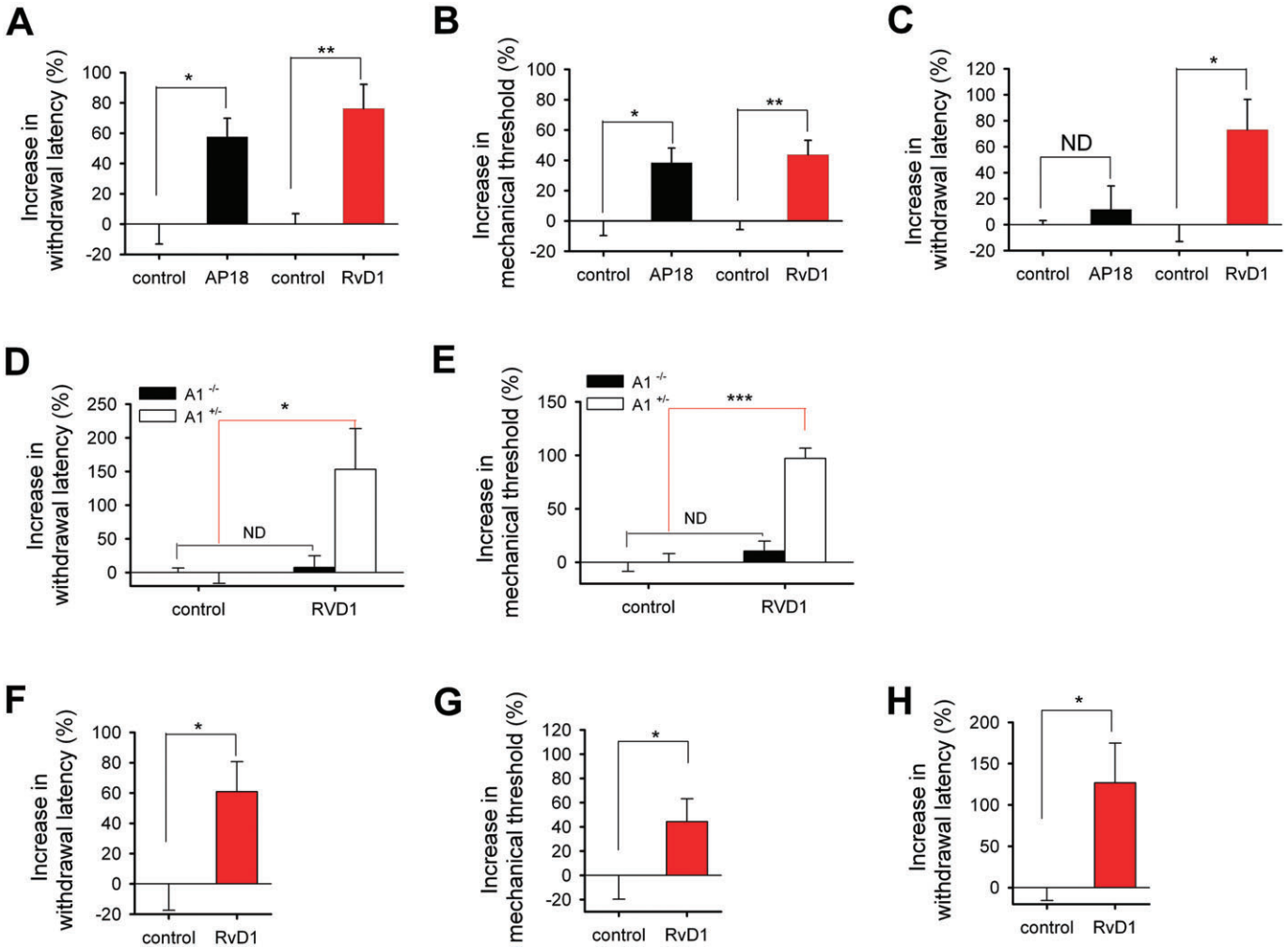

Figure 7

RvD1 suppresses inflammatory hypersensitivity. For CFA inflammation, CFA was injected in the hind paws 24 h prior to the experiments. (A) Summary of the changes in the mechanical thresholds from von Frey tests by AP18 or RvD1 treatment. Average decrease of the von Frey thresholds by CFA inflammation were 68 ± 4% (control for AP18, $n = 5$) and 68 ± 3% (control for RvD1, $n = 5$). Immediately after the hind paw intradermal administration of AP18 (2 μg in 10 μL) or RvD1 (20 ng), the threshold decreases were reversed ($n = 5$). (B) Summary of the changes in the paw withdrawal latencies from Randall-Selitto tests by AP18 or RvD1 treatment. Average decrease ratios of the withdrawal latencies by CFA inflammation were 57 ± 4% (control for AP18, $n = 5$) and 45 ± 3% (control for RvD1, $n = 5$). Immediately after the hind paw intradermal administration of AP18 (2 μg in 10 μL) or RvD1 (20 ng), the latency decreases were reversed ($n = 5$). (C) Summary of the changes in the paw withdrawal latencies from Hargreaves tests by AP18 or RvD1 treatment. Average decrease ratios of the Hargreaves latencies by CFA inflammation were 73 ± 1% (control for AP18, $n = 5$) and 64 ± 4% (control for RvD1, $n = 5$). Immediately after the hind paw intradermal administration of AP18 (2 μg in 10 μL) or RvD1 (20 ng), the paw withdrawal latency decreases were reversed by RvD1, but not by AP18 ($n = 5$) (D) Summary of the changes in the mechanical thresholds of TRPA1-null mice and their heterozygotes from von Frey tests by RvD1 treatment. Average decrease ratios of the von Frey thresholds by CFA inflammation were 50 ± 8% (control for TRPA1-null mice, $n = 5$) and 53 ± 9% (control for heterozygotes, $n = 5$). Immediately after the hind paw intradermal administration of RvD1 (20 ng) the threshold decreases were reversed only in heterozygote and not in the knockout mice ($n = 5$) (E) Summary of the changes in the mechanical thresholds of TRPA1-null mice and their heterozygotes from Randall-Selitto tests by RvD1 treatment. Average decrease ratios of the withdrawal latencies by CFA inflammation were 69 ± 6% (control for TRPA1-null mice, $n = 7$) and 70 ± 4% (control for heterozygotes, $n = 5$). Immediately after the hind paw intradermal administration of RvD1 (20 ng) the latency decreases were reversed only in heterozygotes and not in the knockout mice ($n = 5$). (F–H) Summary of the changes in the mechanical thresholds from von Frey tests (F), the paw withdrawal latencies from Randall-Selitto tests (G) and from Hargreaves tests (H) after intrathecal RvD1 treatments. Immediately after the intrathecal RvD1 injection (20 ng), the threshold and latency decreases were all reversed ($n = 5$, for each assay). *$P < 0.05$ **$P < 0.01$; significantly different from responses without RvD1. CFA, complete Freund's adjuvant; RvD1, resolvin D1 (7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid); TRP, transient receptor potential.

Suzuki *et al.*, 2003; Todaka *et al.*, 2004). On the other hand, thermal activation of TRPV3 channels contributes to pain behaviours (Moqrich *et al.*, 2005; Huang *et al.*, 2008). As RvD1 inhibits TRPV3 activity, we performed Hargreaves assays to determine if RvD1 is able to attenuate heat-induced pain behaviours (Moqrich *et al.*, 2005). RvD1 treatment did not change the heat threshold in normal animals (Figure 7C). In the CFA-inflammation model, heat thresholds were markedly reduced, indicating that thermal hypersensitivity had developed. Treatment with AP18 did not reverse this fall in heat threshold, as TRPA1 channels are not involved in heat hypersensitivity. In contrast, RvD1 significantly reversed the thermal hypersensitivity (Figure 7C). The data suggest that RvD1 can depress noxious heat sensation in inflamed tissues *in vivo* and that this maybe through inhibition of TRPV3 channels, rather than the other noxious heat sensor channel TRPV1. This suggestion was based on our *in vitro* analyses showing that only TRPV3 channel activity was inhibited by RvD1.

Central administration of RvD1 showed analgesic effects via altered synaptic transmission (Xu *et al.*, 2010). We confirmed these effects in our behavioural assay systems (Figure 7F–H), indicating that similar outcomes, despite different routes, can be obtained via an independent mechanism.

Overall, our results show that RvD1 has an acute antinociceptive effect in a wide spectrum of inflammatory pain, which may be due to inhibition of TRPA1, TRPV3 and TRPV4 channels.

Discussion

The present study shows that RvD1 inhibited TRPA1, TRPV3 and TRPV4 channel activity using intracellular Ca^{2+} imaging and whole cell electrophysiology experiments. The action of RvD1 on other sensory neuronal thermoTRPs or voltage-gated channels appeared to be negligible. The results were reproducible in the experiments using cell cultures of DRG neuron or skin keratinocyte. Furthermore, RvD1 suppressed pain behaviours (agonist-induced licking/flicking and flinching and thermal and mechanical pain behaviours) related to those three thermoTRP channels, in normal and under inflammatory conditions. The antinociceptive effect of RvD1 to mechanical nociception was comparable with that of AP18, a specific TRPA1 channel antagonist.

Endogenous antagonists for ion channels have been described before. For example, lipid metabolites inhibited some K^+ channels (Zou *et al.*, 1996; Li *et al.*, 1999; Kim and Pleumsamran, 2000) and kynurenic acid inhibited NMDA receptors and nicotinic acetylcholine receptors (Stone, 1993; Hilmas *et al.*, 2001). It would be interesting to test these endogenous antagonists in combination with with agonists or other stimulatory milieu, in view of the modulations of vascular tone and of pain states via the K^+ channels and NMDA receptors. In terms of the thermoTRP channels, only adenosine was reported as an endogenous TRPV1 channel inhibitor. It is possible that adenosine modulates TRPV1-dependent pain transmission as a negative feedback signal from activated afferents or as a counter to the pain-inducing molecule, ATP, as adenosine is one of the normal metabolites of ATP (Puntambekar *et al.*, 2004).

In the present study, RvD1 was found to be and endogenous molecule inhibiting several thermoTRP channels. Other lipid metabolites have been reported to activate or potentiate TRPA1, TRPV3 or TRPV4 channels. Thus, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 is a covalent ligand for TRPA1 channels (Andersson *et al.*, 2008; Taylor-Clark *et al.*, 2008a) and polyunsaturated fatty acids or cholesterol precursors directly potentiate or activate TRPV3 channels (Hu *et al.*, 2006; Bang *et al.*, 2010). Also, the epoxygenase metabolites of arachidonic acid are able to activate TRPV4 channels (Watanabe *et al.*, 2003). It is interesting that, in this study, an inhibitor was found among the lipid derived compounds. It may be relevant to note that there seems to be a switch of lipid mediators during inflammation, between ω-6 fatty acid derivatives as pro-inflammatory mediators in the early stages and ω-3 fatty acid derivatives as pro-resolvers in the later stages (Levy *et al.*, 2001; Serhan and Chiang, 2008). Coincidently, most of the above lipids that positively modulate TRP channels are the ω-6 species and another pain sensor TRP, TRPV1 channels are activated by 12(S)-hydroperoxyeicosatetraenoic acid which is also a ω-6 fatty acid-derived mediator (Hwang *et al.*, 2000; Shin *et al.*, 2002). On the other hand, RvD1 is a late stage lipid mediator, which mediates resolution of inflammation, and this could indicate that this switiching of lipid mediators may affect pain, as it affects inflammation. More data on the profile of lipid actions on the TRP channels are needed to assess this hypothesis.

Serhan's group were the first to report that the D-series of resolvins were generated by the 15- and 5-lipoxygenases from docosahexanoic acid and released from tissues (Serhan *et al.*, 2002; Hong *et al.*, 2003). The same group has described a range of anti-inflammatory, pro-resolving and analgesic actions of RvD1. For example, RvD1 interfered with transendothelial migration of human neutrophils

(Serhan *et al.*, 2002),suppressed IL-1β transcription induced by TNF-α in microglia and reduced polymorphonuclear leukocyte infiltration (Hong *et al.*, 2003). Spinal injection of RvD1, as we have also used, suppressed synaptic transmission, acutely attenuating inflammatory pain (Xu *et al.*, 2010). The actions on leukocytes would alleviate pain by improving the inflammatory conditions but these effects seem to require a relatively long-term period allowing intercellular interactions (Haworth *et al.*, 2008; Spite *et al.*, 2009a,b). We made our *in vivo* observations over a relatively short time scale and used localized injections to identify the peripheral, TRP-mediated, effects. The spinal effect appears to be mediated by activation of $G_{i/o}$-coupled metabotropic receptors. The data from our electrophysiology or from activators of metabotropic receptors confirm that RvD1 could inhibit TRP channels without metabotropic receptor signalling (Figure 2). Further, receptor-specific behavioural responses *in vivo* correlated with the *in vitro* TRP data (Figure 5). Therefore, peripheral RvD1 administration may reverse pain states in a TRP-dependent manner. Nonetheless, there is still a need to clarify whether other unrecognized RvD1 receptors are expressed in the periphery and are specifically and tightly coupled to the three TRP channels. It is surprising that different cellular targets of one molecule, such as the metabotropic receptors and the ion channels contribute, in parallel, to a common outcome. In this respect, RvD1 may be useful for developing novel analgesic strategies. It would also be interesting to investigate whether inhibition of thermoTRP channels by RvD1 leads to suppression of peripheral neurogenic mechanisms of inflammation.

The search for specific antagonists of TRPA1 channels appears to be active and, recently, two synthetic antagonists were reported (AP18 and HC-030031). Local treatment with AP18 reduced mechanical hyperalgesia in the CFA-induced and bradykinin-induced inflammatory pain models (Petrus *et al.*, 2007). AP18 also partially suppressed CFA-induced cold hyperalgesia (Petrus *et al.*, 2007). Systemic treatment with HC-030031 reduced mechanical hyperalgesia in CFA-induced inflammatory pain model and spinal nerve ligation-induced neuropathic pain models (Eid *et al.*, 2008). Such pharmacological evidence suggests that TRPA1 channels are a promising target for pain modulation. There are only a few reports of the pharmacological control of TRPV3-related pain, due to the limited availability of a potent and specific TRPV3 channel antagonist. However, TRPV3-knockout mice and TRPV3-overexpressing mice exhibited different nociceptive behaviours from those of wild type. Noxious heat sensitivity was blunted in the knockout mice and enhanced in the transgenic overexpressing mice (Moqrich *et al.*, 2005; Huang *et al.*, 2008). Therefore, TRPV3 channels are also likely to be important in thermal pain modulation. Our nociceptive behaviour data from the present study, using RvD1, appear to support conclusions from the genetically manipulated animals.

In terms of pain pharmacology, TRPV4 are similar to TRPV3 channels. Although TRPV4 channels are sensitive to both heat and mechanical stress, many reports have suggested that acute *in vivo* sensitivity to noxious heat was not related to TRPV4 action, but that these channels were more involved in the avoidance of hypotonic insult or noxious pressure (Liedtke and Friedman, 2003; Suzuki *et al.*, 2003; Todaka *et al.*, 2004). The hypotonicity-induced flinching behaviour test is the only reliable behavioural method for the measurement of TRPV4-specific pain responses (Alessandri-Haber *et al.*, 2003; 2005). Here, we reproduced TRPV4-mediated flinches and RvD1 was also able to protecting against these responses (Figure 5D). Noxious pressure-induced paw withdrawal behaviour in the Randall-Selitto test possibly reflects mixed actions of TRPA1 and TRPV4 channels (Liedtke and Friedman, 2003; Suzuki *et al.*, 2003; Eid *et al.*, 2008). In this test, RvD1 also showed anti-nociceptive effects in the inflamed paws (Figure 7B). Which of these two TRP channels is dominant in mediating RvD1 action and, more essentially, how the TRPA1 and TRPV4 channels cooperate in determining pressure sensitivity are still open questions, although RvD1 showed on-target effects in the TRPA1 channel knockout animals.

Many recent research results have indicated that TRPA1, TRPV3 and TRPV4 channels may be among critical peripheral analgesic targets. The present study shows that RvD1, which inhibits the activities of these TRPs, has local anti-nociceptive effects on various sensory modalities in animal inflammation models. Thus, our results suggest that RvD1 is a useful analgesic substance. Administration of RvD1 or improvement of its endogenous production might be a strategy to reverse the pain states involving TRP channels. Furthermore, RvD1 is being studied for its contribution to the resolution of inflammation itself. It is highly likely that such multiple beneficial functions of this substance may synergistically promote recovery from some injurious conditions and future studies will examine this possibility. Other molecules of the resolvin group of compounds could act by similar mechanisms. In addition, the present study may offer useful chemical suggestions for the design of synthetic antagonists that suppress the functions of multiple TRP channels.

Acknowledgements

This work was supported by the Korea Research Foundation Grant (code KRF-2008-331-E00457 and 2009-0076543), the Republic of Korea.

Conflict of interest

S. B and S. W. H. applied for Korean patents regarding the uses of RvD1 for analgesics (patent No. 10-2009-0087993, 10-2009-0087994 and 10-2009-0085737).

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