

RESEARCH PAPER

4 α -phorbol 12,13-didecanoate activates cultured mouse dorsal root ganglia neurons independently of TRPV4

R Alexander, A Kerby, AA Aubdool, AR Power, S Grover, C Gentry and AD Grant

Wolfson Centre for Age-Related Diseases, King's College London, London, UK

Correspondence

Andrew D. Grant, Wolfson Centre for Age-Related Diseases, King's College London, Strand, London WC2R 2LS, UK. E-mail: andrew.2.grant@kcl.ac.uk

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BACKGROUND AND PURPOSE

The Ca²⁺-permeable cation channel TRPV4 is activated by mechanical disturbance of the cell membrane and is implicated in mechanical hyperalgesia. Nerve growth factor (NGF) is increased during inflammation and causes mechanical hyperalgesia. 4 α -phorbol 12,13-didecanoate (4 α PDD) has been described as a selective TRPV4 agonist. We investigated NGF-induced hyperalgesia in TRPV4 wild-type (+/+) and knockout (–/–) mice, and the increases in [Ca²⁺]_i produced by 4 α PDD in cultured mouse dorsal root ganglia neurons following exposure to NGF.

EXPERIMENTAL APPROACH

Withdrawal thresholds to heat, von Frey hairs and pressure were measured in mice before and after systemic administration of NGF. Changes in intracellular Ca²⁺ concentration were measured by ratiometric imaging with Fura-2 in cultured DRG and trigeminal ganglia (TG) neurons during perfusion of TRPV4 agonists.

KEY RESULTS

Administration of NGF caused a significant sensitization to heat and von Frey stimuli in TRPV4 +/+ and –/– mice, but only TRPV4 +/+ mice showed sensitization to noxious pressure. 4 α PDD stimulated a dose-dependent increase in [Ca²⁺]_i in neurons from +/+ and –/– mice, with the proportion of responding neurons and magnitude of increase unaffected by the genotype. In contrast, the selective TRPV4 agonist GSK1016790A failed to stimulate an increase in intracellular Ca²⁺ in cultured neurons. Responses to 4 α PDD were unaffected by pretreatment with NGF.

CONCLUSIONS AND IMPLICATIONS

TRPV4 contributes to mechanosensation *in vivo*, but there is little evidence for functional TRPV4 in cultured DRG and TG neurons. We conclude that 4 α PDD activates these neurons independently of TRPV4, so it is not appropriate to refer to 4 α PDD as a selective TRPV4 agonist.

Abbreviations

4 α PDD, 4 α -phorbol 12,13-didecanoate; AITC, allylisothiocyanate; DRG, dorsal root ganglia; NGF, nerve growth factor; TG, trigeminal ganglia; TRPV4, transient receptor potential vanilloid 4

Introduction

The Ca²⁺-permeable cation channel transient receptor potential vanilloid 4 (TRPV4) was first cloned in 2000 (Strotmann

et al., 2000) and belongs to the TRP superfamily of channels. TRPV4 expression shows a broad tissue distribution, with expression identified in epithelia of the skin, lung and kidney, in vascular endothelium and smooth muscle and in

the brain by a combination of immunohistochemistry, *in situ* hybridization and functional studies (Strotmann *et al.*, 2000; Jia *et al.*, 2004; Cohen, 2005; Watanabe *et al.*, 2008).

Its physiological roles are equally diverse, with suggested roles in osmoregulation (Liedtke and Friedman, 2003), stretch sensation in the bladder (Gevaert *et al.*, 2007), flow sensation in kidney tubules (Wu *et al.*, 2007) and osteoclast maturation (Masuyama *et al.*, 2008), amongst others. Expression and activation of TRPV4 in the peripheral nervous system is implicated in mechanical nociception, particularly mechanical hypersensitivity following inflammation or neuropathy. It is less clear whether TRPV4 contributes to basal mechanosensation. Sensory deficits in response to noxious pressure (Suzuki *et al.*, 2003) and bladder stretch (Gevaert *et al.*, 2007) have been described in TRPV4 knockout mice. However, other studies have failed to identify basal differences in the response to mechanical stimuli between wild-type and knockout animals (e.g. Grant *et al.*, 2007; Alessandri-Haber *et al.*, 2008).

In common with other TRP channels, TRPV4 is activated by a wide range of physical and chemical stimuli. It was originally identified as a calcium channel responding to extracellular hypo-osmolarity and consequent cell swelling (Liedtke *et al.*, 2000). Application of positive or negative pressure to membranes of TRPV4-expressing HEK293 cells in cell-attached patch clamp experiments did not alter channel activity, which suggested that TRPV4 did not directly respond to swelling-induced membrane stretch (Strotmann *et al.*, 2000). An indirect mechanism of activation was proposed by Watanabe *et al.* (2003), who suggested swelling activates phospholipase A₂-mediated release of arachidonic acid, which is then metabolized to form a TRPV4 agonist 5',6'-epoxyeicosatrienoic acid (5',6'-EET). However, the results of two recent studies suggest that direct mechanical gating of TRPV4 by membrane stretch (Loukin *et al.*, 2010) or by force applied to the extracellular matrix (Matthews *et al.*, 2010) can activate the channel. The first synthetic TRPV4 agonist to be identified was 4 α -phorbol 12,13-didecanoate (4 α PDD), a non-PKC activating phorbol ester with an EC₅₀ around 200 nM at heterologously expressed human and murine TRPV4 (Watanabe *et al.*, 2002a). Direct interaction of 4 α PDD with a ligand-binding pocket formed by transmembrane regions 3 and 4 opens the channel (Vriens *et al.*, 2007). The most potent small molecule agonist at TRPV4 identified to date is GSK1016790A, with a structure distinct from that of the phorbol esters. It has an EC₅₀ around 18 nM at heterologously expressed murine TRPV4 (Thorneloe *et al.*, 2008; Willette *et al.*, 2008).

We recently identified TRPV4 expression in rat dorsal root ganglia neurons using RT-PCR and immunohistochemistry. In addition, we demonstrated that TRPV4 was sensitized by protease activated receptor 2 (PAR2) activation in cultured cells, and PAR2 activation *in vivo* produced a TRPV4-dependent mechanical hypersensitivity in mice (Grant *et al.*, 2007). The increase in intracellular calcium in response to hypotonic challenge was diminished in neurons from TRPV4 knockout mice and was not sensitized by inflammatory 'soup', in contrast to the response in wild-type neurons (Alessandri-Haber *et al.*, 2006). Inhibition of tetrodotoxin-resistant sodium currents by hypotonic challenge was reduced in trigeminal ganglia sensory neurons from TRPV4 knockout mice (Chen

et al., 2009). Small diameter osmosensitive hepatic neurons were absent in TRPV4 knockout mice. Neurons from these mice only showed a delayed, irreversible increase in intracellular calcium in response to 4 α PDD, whereas some wild-type neurons exhibited a rapid, reversible response. The authors interpreted the former response as a non-selective, toxic effect (Lechner *et al.*, 2011). The number of neurons that showed an increase in intracellular calcium after 4 α PDD treatment and the size of the increase were diminished in DRG neurons innervating the colon after administration of siRNA directed against TRPV4 (Cenac *et al.*, 2008).

Various laboratories have also carried out studies linking TRPV4 activation to the mechanical hyperalgesia observed in chemotherapeutic and diabetic neuropathies (Alessandri-Haber *et al.*, 2008), colonic inflammation (Sipe *et al.*, 2008), chronic constriction injury of sensory nerves (Zhang *et al.*, 2008) and pancreatitis (Ceppa *et al.*, 2010). Altered nerve growth factor (NGF) signalling is implicated as a causal mechanism of the mechanical hyperalgesia in many neuro-pathic and inflammatory pain models, via modulation of ion channel expression and activity in sensory neurons (Pezet and McMahon, 2006; Watson *et al.*, 2008). NGF increases the membrane presentation and activity of TRPV1 via phosphorylation at Y200, part of a phosphorylation sequence that is conserved in TRPV4 (Zhang *et al.*, 2005). Thus, it is possible that NGF treatment will similarly enhance TRPV4 activity.

To determine whether sensitization of TRPV4 in DRG neurons is a general mechanism underlying mechanical hyperalgesia, we investigated whether NGF sensitization of mechanosensation is dependent on TRPV4 and studied the TRPV4 agonist-induced changes in [Ca²⁺]_i in neurons from wild-type and TRPV4 knockout mice. We hypothesized that TRPV4 knockout mice would show reduced mechanical hyperalgesia after NGF treatment, and neurons from wild-type animals would show increased responses to TRPV4 activators in the presence of NGF, whilst TRPV4 knockout neurons would not respond to these stimuli.

Methods

Animals

TRPV4 wild-type and knockout C57BL/6 mice (Liedtke and Friedman, 2003) were a gift from Dr W Liedtke, Duke University, and were bred in house from pairs of heterozygote mice. Both male and female mice at least 8 weeks of age (20–30 g) were used in these studies. All mice were maintained on a normal diet and housed in a cage with a maximum of five animals, with access to food and water *ad libitum*, on a 12 h/12 h light/dark cycle in a climatically controlled environment. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986 and the rules of the King's College London animal ethics committee.

Collection of mouse dorsal root ganglia neurons, SDS-PAGE and Western blotting

Mice were killed by overdose of sodium pentobarbital (1 mg·g⁻¹), and the spinal column was removed. Thoracic and

lumbar dorsal root ganglia (DRG) were removed, combined and snap-frozen in liquid nitrogen. DRG were homogenized in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, pH 7.4) with protease inhibitors (Roche, Welwyn Garden City, UK). Lysates were separated by SDS-PAGE (8% acrylamide), transferred to nitrocellulose membranes and blocked in TBS with 0.1% Tween-20 and 5% powdered milk for 60 min. Membranes were incubated with rabbit anti-TRPV4 (1:250, Abcam, Cambridge, UK; Ab39260 or 1:500, Alomone, Jerusalem, Israel; #ACC-034) and mouse anti- β -tubulin (1:3000, Promega, Southampton, UK, #G7121) overnight at 4°C. Membranes were washed and then incubated with donkey anti-rabbit-IRDye 800 (1:15 000, LI-COR, Cambridge, UK) and goat anti-mouse-Alexa Fluor 680 (Molecular Probes, Paisley, UK) for 1 h at room temperature. Immunoreactive proteins were detected using an Odyssey Infrared Imaging System (LI-COR).

Dispersion and culture of mouse dorsal root ganglia and trigeminal ganglia neurons

Mice (both sexes, 3–9 months old) were killed by overdose of sodium pentobarbital, and DRG was collected as above. Trigeminal ganglia (TG) were collected from within the skull. Rather than snap-freezing, DRG and TG were incubated in F12 medium (Gibco, Paisley, UK) with collagenase (1.25%) for 60 min at 37°C. Individual neurons were dispersed by trituration and then pelleted by centrifugation for 6 min at 60 $\times$ g. Cells were resuspended in F12 basal medium supplemented with 1% penicillin/streptomycin, 30% BSA, 1% N2 neuronal growth supplement (PAA, Yeovil, UK) and plated on to sterile coverslips coated with poly-L-lysine (100 μ g·mL⁻¹, Sigma, Dorset, UK) and laminin (10 μ g·mL⁻¹; Sigma) in four-well plates.

Mouse NGF (2.5S; 100 ng·mL⁻¹; Alomone) or additional F12 medium (control) was added when required as per experiment protocol, and cells were left in culture overnight prior to calcium imaging experiments.

Immunostaining of cultured dorsal root ganglia neurons

Neurons were collected, dispersed and cultured as described above. After 24 h in culture, neurons were fixed in 4% paraformaldehyde for 30 min at 4°C. Coverslips were washed 3 times in PBS containing 0.1% Triton X-100 (PBS-T). Rabbit anti-TRPV4 (1:200, Abcam) was added to the cells and they were incubated overnight at 4°C. Coverslips were washed three times in PBS-T, then goat anti-rabbit-Alexa 488 (1:1000) was added to the cells, and they were incubated for 2 h at room temperature. Coverslips were washed three times in PBS-T then mounted on microscope slides with Vectashield with DAPI (Vector Labs, Peterborough, UK), and images were collected with an Axioplan 2 microscope (Zeiss, Cambridge, UK).

Behaviour

All behaviour experiments used age-matched male mice. They were acclimatized to the testing environment on 3 days prior to testing and were placed in test chambers 1 h prior to measurements. All measurements were made by experimenters blind to the genotype of the mice. Cutaneous mechanical sensitivity was measured by manual application of von Frey hairs (0.04–4 g) to the plantar surface of both hind paws.

Each hair was applied five times, and the frequency of withdrawal was recorded. The 50% withdrawal threshold was considered to be the force applied by the first hair to produce three or more out of five withdrawals. Cutaneous thermal sensitivity was measured by the Hargreaves's test. Infrared radiation was applied to the plantar surface of both hind paws, with intensity calibrated to give an average withdrawal response after 8–10 s. A cut-off time of 28 s was used to avoid any tissue damage. Averages of three applications to each side were recorded, with at least 5 min between applications. Sensitivity to pressure was measured by the Randall–Sellito test. The hind paw was placed on a flat plate, with increasing pressure applied by a probe on the dorsal surface until the paw was withdrawn. A cut-off pressure of 150 g was chosen to avoid tissue damage. Following baseline measurements, mice were injected with NGF (1 μ g·g⁻¹ in 100 μ L saline, i.p.), and measurements were repeated at 1 and 24 h after injection.

Measurement of intracellular [Ca²⁺] in mouse DRG neurons

Cultured DRG neurons were used for Ca²⁺ imaging experiments after 18–24 h in culture. Cells were incubated in buffer (HBSS with 10 mM glucose, 10 mM HEPES, pH 7.4) with Fura-2-AM (2 μ M) and probenecid (1 mM) for 60 min at 37°C. Coverslips were washed and mounted in an open chamber. Test compounds were diluted to required concentrations in buffer and applied to cells by continuous perfusion at 37°C. Hypotonic test solutions were generated by addition of deionized water to the standard buffer. The fluorescence of individual cells were measured at 340 and 380 nm excitation, and 510 nm emission using microscope-based imaging system (PTI, Ford, UK). Cells were challenged with KCl (50 mM) at the end of each experiment to activate voltage-gated Ca²⁺ channels and provide a maximal Ca²⁺ signal, against which to normalize other cellular responses. Neurons were identified morphologically and were excluded from analysis if they did not respond to KCl.

Measurement of intracellular [Ca²⁺] in HaCaT cells

The HaCaT human keratinocyte cell line was grown in defined keratinocyte serum-free medium (Invitrogen, Paisley, UK) at 37°C. Cells were plated in black-walled 96-well plates and grown to confluence for Ca²⁺ imaging experiments. Cells were loaded with Fura-2-AM (2 μ M) for 1 h in the presence of probenecid (1 mM) at 30°C in HBSS with 10 mM HEPES. Cells were washed, and the mean fluorescence across each well was measured at 340 and 380 nm excitation, and 520 nm emission using a Flexstation (Molecular Devices, Wokingham, UK). Each experimental run lasted 240 s, with fluorescence readings made at 6 s intervals. GSK1016790A was added at 20 s. In a further series of experiments, cells were pretreated for 15 min with the TRPV4 antagonist HC067047 before the start of Ca²⁺ imaging, and the antagonist remained in contact with the cells throughout.

Materials

4 α -phorbol 12,13-didecanoate, GSK1016790A, HC067047 and probenecid were obtained from Sigma. Mouse NGF (2.5S) was obtained from Alomone. Fura-2-AM was obtained from Molecular Probes (Invitrogen).

Statistical analysis

Comparisons between behaviour results were by one-way repeated-measures ANOVA, followed by Bonferroni's multiple comparisons test. Emission intensity ratios at 340 nm/380 nm excitation in neuronal imaging experiments were calculated using EasyRatioPro software (PTI, Ford, UK). Only neurons where the response to treatment was greater than 20% of the Δ ratio for 50 mM KCl were counted as responders. The response to GSK1016790A in the HaCaT experiments

was calculated as maximum Δ ratio in the presence of the agonist minus the mean baseline ratio.

Results

Expression of TRPV4 in dorsal root ganglia

Expression of TRPV4 protein was studied in DRG tissue lysates from wild-type and TRPV4 knockout mice by SDS-

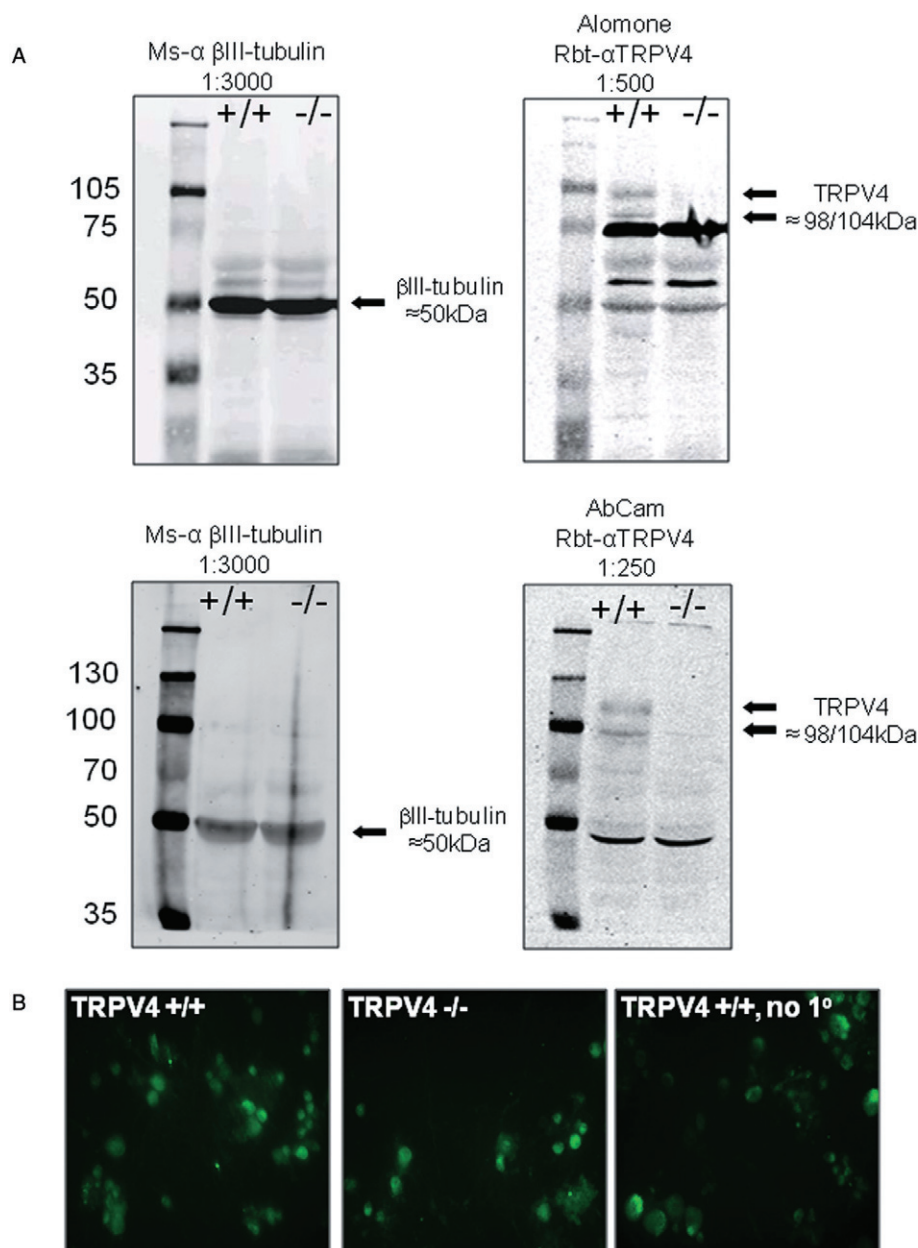


Figure 1

(A) Expression of TRPV4 in dorsal root ganglia lysate. Lysates (30 μ g of total protein) from both TRPV4 $+/+$ and $-/-$ mice showed expression of β III-tubulin (50 kDa, left column). Only lysates from TRPV4 $+/+$ mice showed the double band corresponding to TRPV4 (98 and 104 kDa, right column). (B) Immunostaining of cultured dorsal root ganglion neurons from TRPV4 $+/+$ and $-/-$ mice with rabbit anti-TRPV4 (1:200) and goat anti-rabbit-Alexa488 (1:1000) revealed no difference in staining intensity between $+/+$, $-/-$ and $+/+$ (no primary antibody) cells. Images are representative of data obtained from $n = 3$ independent experiments.

PAGE followed by Western blotting. Two different anti-TRPV4 antibodies were studied to allow comparison of their specificities. A strong band at 50 kDa, corresponding to the neuron-specific marker β III-tubulin, was seen in both sets of samples (Figure 1). A relatively weak double band (98 and 104 kDa) corresponding to TRPV4 was seen in the wild-type sample with both antibodies and in both cases was absent in the lysate from the knockout DRG. Both anti-TRPV4 antibodies also bound non-specifically to other proteins, as indicated by bands of lower molecular weight present in both lysates.

An attempt was made to immunostain cultured DRG neurons using the more selective of the antibodies (Abcam) tested in the Western blotting experiment. No difference was perceived in immunofluorescence between wild-type neurons, TRPV4 knockout neurons and wild-type neurons only exposed to secondary antibody.

Effect of TRPV4 deletion on thermal and mechanical responses

The latency of paw withdrawal to plantar application of radiant heat was the same at baseline in both TRPV4 $+/+$ and TRPV4 $-/-$ mice. The latency of withdrawal decreased significantly at 1 and 24 h after the injection of NGF ($1 \mu\text{g}\cdot\text{g}^{-1}$; i.p.) in both TRPV4 $+/+$ and $-/-$ mice (Figure 2A). The 50% withdrawal threshold to von Frey hairs of increasing thickness was the same at baseline in both TRPV4 $+/+$ and TRPV4 $-/-$ mice. Injection of NGF ($1 \mu\text{g}\cdot\text{g}^{-1}$, i.p.) produced a significant decrease in withdrawal threshold after 1 and 24 h (Figure 2B). The baseline withdrawal pressure was the same in both TRPV4 $+/+$ and TRPV4 $-/-$ mice. It was significantly reduced at 1 and 24 h in TRPV4 $+/+$ mice by systemic injection of NGF ($1 \mu\text{g}\cdot\text{g}^{-1}$, i.p.). However, NGF injection did not affect paw withdrawal to pressure in TRPV4 $-/-$ mice (Figure 2C).

Effect of TRPV4 activators on cultured sensory neurons

The normal blood osmolarity in both the wild-type and knockout mice was measured as 295 mOsm (Liedtke and Friedman, 2003). Exposure of cultured DRG neurons from wild-type and TRPV4 knockout mice to hypo-osmotic challenge [decrease from isotonic buffer (292 mOsm) to 264, 234 or 206 mOsm] produced an increase in $[\text{Ca}^{2+}]_i$ in a subset of neurons (Figure 3). The profile of the response to 206 mOsm buffer was the same in neurons from wild-type and TRPV4 knockout mice (Figure 3A, B). Decreasing the osmolarity increased the proportion of the neurons responding and the size of the change in $[\text{Ca}^{2+}]_i$ in responding neurons. The proportion of responding neurons and the size of the change in $[\text{Ca}^{2+}]_i$ in responding neurons was the same in neurons from wild-type and TRPV4 knockout mice (Figure 3C, D).

Exposure of cultured DRG neurons from wild-type and TRPV4 knockout mice to the TRPV4 agonist 4 α -PDD (1 – $10 \mu\text{M}$) produced an increase in $[\text{Ca}^{2+}]_i$ in a subset of neurons (Figure 4). The response profiles of these responding neurons can be divided into two main types: some neurons exhibited a transient increase in $[\text{Ca}^{2+}]_i$, which then returned to baseline, whereas others showed a prolonged elevation in $[\text{Ca}^{2+}]_i$, even after the removal of 4 α -PDD. Neurons of both genotypes showed both types of response (Figure 4A, B). After exposure to $10 \mu\text{M}$ 4 α -PDD, 64 of 145 responding wild-type

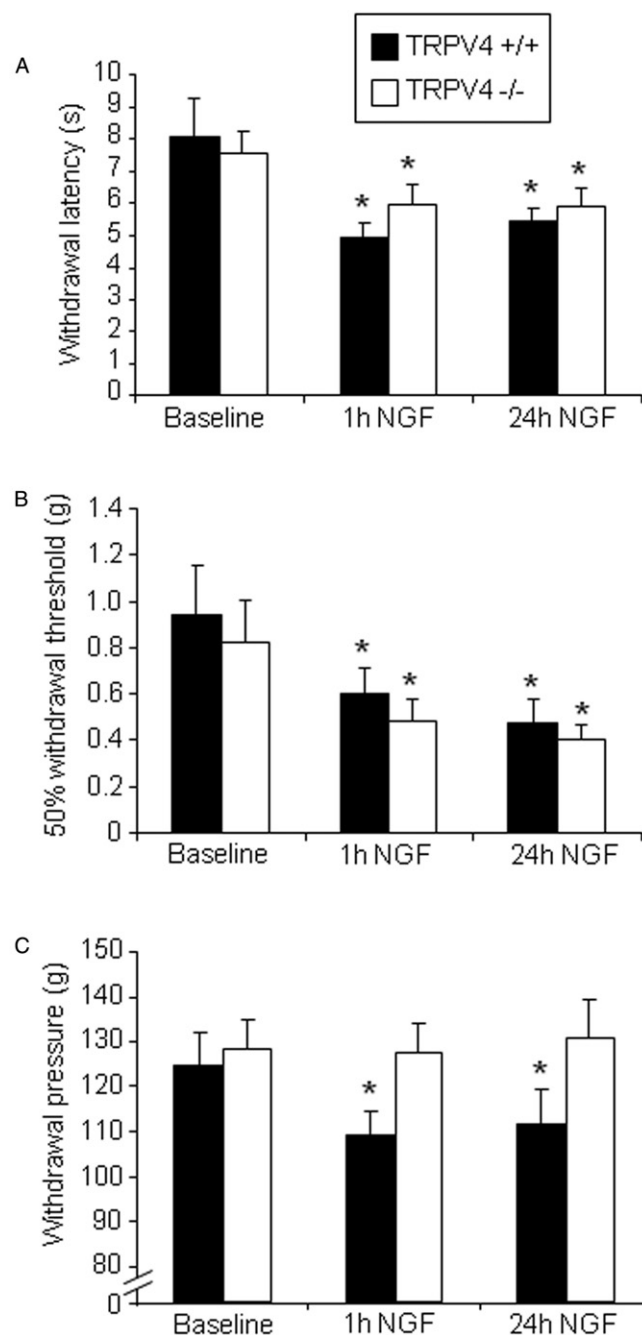


Figure 2

Effect of systemic NGF ($1 \mu\text{g}\cdot\text{g}^{-1}$; i.p.) treatment on thermal and mechanical nociceptive thresholds in TRPV4 $+/+$ and $-/-$ mice. (A). Latency of withdrawal to radiant heat, (B) 50% withdrawal threshold to application of von Frey hairs and (C) withdrawal pressure were measured at baseline and 1 and 24 h following NGF administration. * $P < 0.05$ compared with baseline, $n = 10$.

neurons (44.1%) showed a transient response, whilst 81 of 145 responding neurons (55.9%) showed a sustained response. Similarly, 98 of 222 knockout neurons that responded to $10 \mu\text{M}$ 4 α -PDD (44.1%) had a transient response, whereas 124 of 222 (55.9%) showed a sustained response. Increasing the concentration increased the propor-

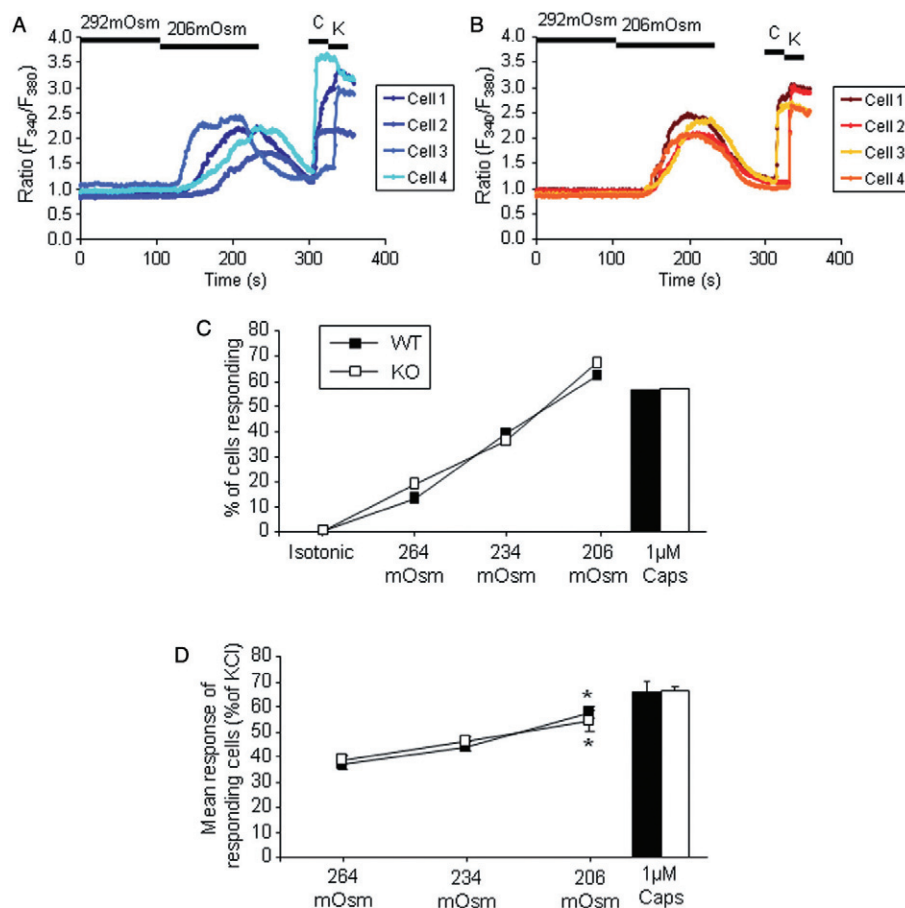


Figure 3

The effect of hypotonic challenge (264, 234 and 206 mOsm) on $[Ca^{2+}]_i$ in cultured mouse dorsal root ganglion neurons from TRPV4 $+/+$ and $-/-$ mice. Example response profiles of (A) TRPV4 $+/+$ and (B) TRPV4 $-/-$ neurons to 206 mOsm buffer, 1 μ M capsaicin (C) and 50 mM KCl (K). (C) The % of total neurons responding and (D) mean response of individual responding neurons relative to the response to KCl are also shown. $n = 6-9$ coverslips, with a total of at least 200 neurons from three mice of each genotype for each data point. $*P < 0.05$ compared with 264 mOsm, one-way ANOVA followed by Dunnett's test.

tion of the neurons responding and the size of the change in $[Ca^{2+}]_i$ in responding neurons. The proportion of responding neurons and the size of the change in $[Ca^{2+}]_i$ in responding neurons was the same in neurons from wild-type and TRPV4 knockout mice (Figure 4C, D).

In contrast, exposure of cultured DRG neurons from wild-type mice to the selective TRPV4 agonist GSK1016790A (100 nM–1 μ M) failed to increase $[Ca^{2+}]_i$ in a greater proportion of neurons than responded to the vehicle control (1% DMSO), with only a small minority of neurons responding (Figure 5A). The size of the change in $[Ca^{2+}]_i$ in responding neurons was the same in response to either DMSO or GSK1016790A (Figure 5B). To confirm the ability of GSK1016790A to activate TRPV4, a dose-response curve was generated in HaCaT keratinocyte cells. GSK1016790A (5–500 nM) induced a dose-dependent increase in $[Ca^{2+}]_i$ in these cells, with an EC_{50} of 43 ± 1 nM (Figure 5C). The TRPV4 antagonist HC067047 (5–2000 nM) dose-dependently inhibited the increase in $[Ca^{2+}]_i$ to 100 nM GSK1016790A, with an IC_{50} of 350 ± 1 nM (Figure 5D).

Few studies have directly examined TRPV4 function in sensory neurons. However, neuronal responses that were absent in cells from TRPV4 knockout animals have been reported in a subset of thoracic DRG neurons (Lechner *et al.*, 2011) and in trigeminal ganglia (TG) neurons (Chen *et al.*, 2009). An increase in $[Ca^{2+}]_i$ following exposure to 1 μ M GSK1016790A was seen in a small number of neurons cultured from DRG at spinal levels T6–T12. Significantly more wild-type neurons responded to GSK1016790A than knockout neurons (17 of 261 WT vs. 1 of 252 KO; $P < 0.001$, Chi-squared test). In contrast, 46.3% of wild-type and 44.4% of knockout neurons showed an increase in $[Ca^{2+}]_i$ following exposure to 10 μ M 4 α -PDD (Figure 6A). This was a transient, rather than sustained, increase in 34.7% of wild-type and 32.1% of knockout neurons. There was no significant difference in the size of the change in $[Ca^{2+}]_i$ following exposure to either agonist between the genotypes (Figure 6B).

Similarly, only a small number of cultured TG neurons of either genotype showed an increase in $[Ca^{2+}]_i$ following exposure to 1 μ M GSK1016790A, whereas greater numbers of both

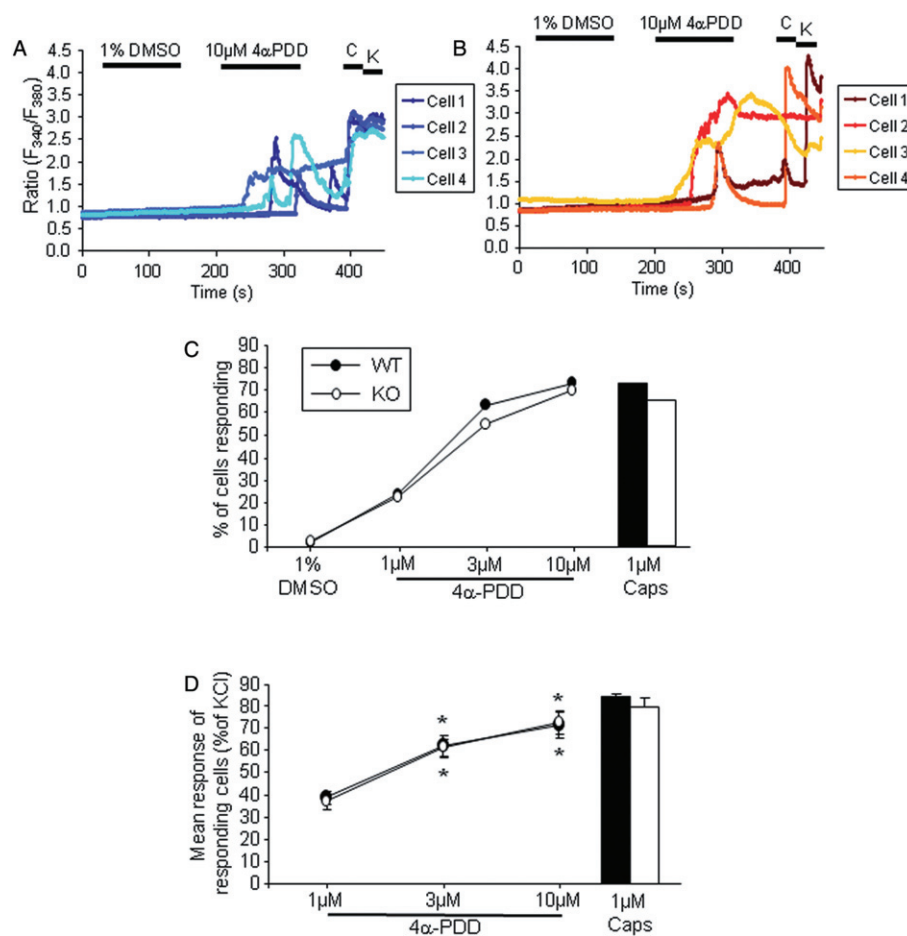


Figure 4

The effect of 4 α PDD (1–10 μ M) on $[Ca^{2+}]_i$ in cultured mouse dorsal root ganglion neurons from TRPV4 +/+ and -/- mice. Example response profiles of (A) TRPV4 +/+ and (B) TRPV4 -/- neurons to 10 μ M 4 α PDD, 1 μ M capsaicin (C) and 50 mM KCl (K). (C) The % of total neurons responding and (D) mean response of individual responding neurons relative to the response to KCl are also shown. $n = 6$ –9 coverslips, with a total of at least 200 neurons from three mice of each genotype for each data point. * $P < 0.05$ compared with 1 μ M 4 α PDD, one-way ANOVA followed by Dunnett's test.

wild-type and TRPV4 knockout neurons responded to 10 μ M 4 α -PDD (Figure 6A). Again, there was no significant difference between the size of the increase in $[Ca^{2+}]_i$ following exposure to these agonists in TG neurons of either genotype (Figure 6B).

Several agonists of TRP channels show poor selectivity and can activate multiple channels. TRPV1 and TRPA1 are both highly expressed in sensory neurons and are activated by numerous different chemical compounds. However, the proportion of neurons exhibiting an increase in $[Ca^{2+}]_i$ following exposure to 222 mOsm buffer or 3 μ M 4 α -PDD was similar in cultures from wild-type, TRPV1 knockout and TRPA1 knockout mice (Figure 6C). In contrast, the increase in $[Ca^{2+}]_i$ in response to the TRPV1 agonist capsaicin (1 μ M) was abolished in TRPV1 -/- neurons, and the increase in $[Ca^{2+}]_i$ in response to the TRPA1 agonist allyl isothiocyanate (AITC, 100 μ M) was almost entirely absent in TRPA1 -/- neurons (Figure 6C). No increase in $[Ca^{2+}]_i$ in response to 234 mOsm buffer or exposure to 3 μ M 4 α -PDD was observed in neurons from wild-type mice in the absence of external Ca^{2+} (data not shown).

Addition of NGF (100 ng·mL⁻¹) to the culture medium for the DRG neurons for the entire 18–24 h culture period had no effect on either the proportion of cells responding to submaximally stimulating hypotonic buffer (249 or 222 mOsm) or the mean response of these cells (Figure 7). Similarly, the number of cells showing an increase in $[Ca^{2+}]_i$ after exposure to 4 α -PDD (1 or 3 μ M) and the mean size of this change was unaffected by pre-exposure to NGF (Figure 7).

Discussion

These studies suggest that TRPV4 protein is expressed within dorsal root ganglia, and that deletion of the TRPV4 gene can affect mechanosensation in a whole animal. However, our experiments investigating TRPV4 activity in cultured DRG and TG neurons did not identify any differences in the responses of neurons collected from wild-type and TRPV4 knockout mice to various TRPV4 activators. Additionally,

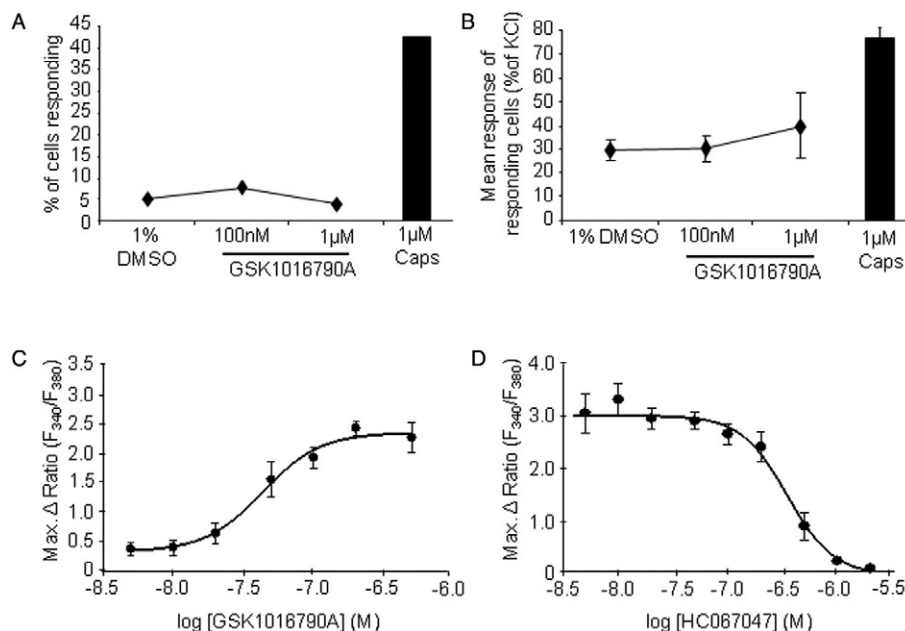


Figure 5

The effect of GSK1016790A (100 nM–1 μM) on $[Ca^{2+}]_i$ in cultured mouse dorsal root ganglion neurons from TRPV4 $+/+$ mice. (A) The % of total neurons responding and (B) mean response of individual responding neurons relative to the response to KCl are shown. $n = 8$ –9 coverslips, with a total of at least 300 neurons from three mice for each data point. (C) GSK1016790A (5 nM–500 nM) caused a dose-dependent increase in $[Ca^{2+}]_i$ in HaCaT keratinocytes. (D) The TRPV4 antagonist HC067047 caused a dose-dependent inhibition of the increase in $[Ca^{2+}]_i$ caused by application of 100 nM GSK1016790A to HaCaT keratinocytes.

these findings do not support previous studies that claim that 4α -PDD is a selective activator of TRPV4.

Our Western blots, carried out with two different TRPV4 antibodies, confirm the presence of TRPV4 protein in whole DRG lysates. A doublet band, with molecular weights of approximately 98 and 104 kDa was observed. It is likely that the doublet is due to glycosylation of the mature TRPV4 protein, as demonstrated by Xu *et al.* (2006). It was absent in lysates from TRPV4 $-/-$ mice, confirming its identity as TRPV4. However, it is noticeable that both antibodies also recognized bands at lower molecular weights in both wild-type and knockout lysates, suggesting that the antibodies are not truly selective for TRPV4. This has implications for using these antibodies in immunohistochemical study of its localization, as a positive reaction on tissue sections may occur in the absence of TRPV4.

TRPV4 wild-type and knockout mice showed no differences in their baseline sensitivity to radiant heat, and both showed a significant sensitization to a heat stimulus after treatment with NGF. TRPV4 does not play a major role in inflammatory thermal hyperalgesia (Huang *et al.*, 2011), so this result was as expected. NGF promotes increased expression and sensitization of thermosensitive TRPV1 (Ji *et al.*, 2002; Zhang *et al.*, 2005), which probably underlies the observed hyperalgesia. Baseline responses to punctate mechanical stimulation with von Frey hairs and to application of pressure to the paw didn't vary between genotypes. This is in keeping with the majority of previous studies that found no basal difference in somatic sensation in TRPV4 $-/-$ mice (e.g. Grant *et al.*, 2007; Alessandri-Haber *et al.*, 2008).

Interestingly, both wild-type and knockout mice showed increased sensitivity to punctate stimuli after NGF, whereas only wild-type mice showed an increase in sensitivity to noxious pressure. This suggests that von Frey hairs and noxious pressure activate different patterns of neuronal and non-neuronal mechanosensory cells in the paw, with TRPV4 activation only required during the detection of pressure. The reason why the sensitization of responses to von Frey hairs by NGF is unaffected by TRPV4 deletion, in contrast to previous studies in chronic pain models where von Frey sensitivity is reduced (e.g. Alessandri-Haber *et al.*, 2008), is unclear. Broadly, TRPV4 activation during mechanosensation seems to be more relevant to pathological conditions of hyperalgesia, such as during inflammation, rather than normal physiological sensation.

The ability to sense and respond to alterations in extracellular osmolarity is a fundamental homeostatic process, so redundancy in these pathways is unsurprising. Hypotonic challenge only produces an increase in $[Ca^{2+}]_i$ in HEK cells (Xu *et al.*, 2003; Grant *et al.*, 2007) and CHO cells (Liedtke *et al.*, 2000) after heterologous TRPV4 expression, confirming that decreased extracellular osmolarity can indeed activate the channel. In this study, our finding that hypotonic solutions increase $[Ca^{2+}]_i$ as effectively in neurons from TRPV4 knockout mice as in wild-type neurons confirms that mechanisms other than TRPV4 activation can detect extracellular hypotonicity. A recent study by Lechner *et al.* (2011) identified a subpopulation of osmosensory thoracic DRG neurons in wild-type mice that were absent in TRPV4 $-/-$ animals, suggesting that they require TRPV4 for their osmosensory

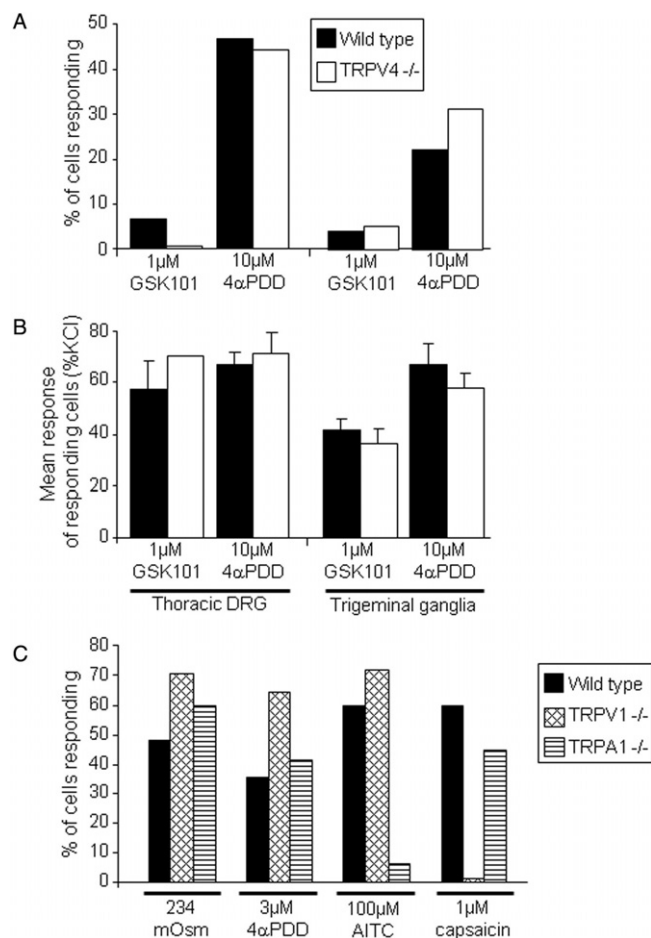


Figure 6

(A) The % of cultured thoracic DRG and trigeminal ganglion neurons responding to GSK1016790A (1 μ M) or 4 α -PDD (10 μ M) with an increase in $[Ca^{2+}]_i$ and (B) mean response of individual responding neurons relative to the response to KCl. $n = 8$ –11 coverslips, with a total of at least 250 (thoracic) or 180 (TG) neurons from three mice of each genotype for each data point. (C) The % of total neurons responding to 234 mOsm buffer, 4 α -PDD (3 μ M), AITC (100 μ M) and capsaicin (1 μ M) on $[Ca^{2+}]_i$ in cultured mouse dorsal root ganglion neurons from TRPV4 $+/+$, TRPV1 $-/-$ and TRPA1 $-/-$ mice. $n = 5$ –6 coverslips, with a total of at least 100 neurons from two mice for each data point.

properties. The vast majority of DRG neurons innervate somatic rather than visceral targets, so these specialized neurons may have also been present in our cultures, but not apparent amongst the far greater number of neurons that can apparently respond to extracellular hypo-osmolality in the absence of TRPV4. When we focused solely on thoracic neurons, significantly more wild-type (17 of 261) than knockout (1 of 252) neurons responded to the TRPV4 agonist GSK1016790A, which may represent some of these rare TRPV4 expressing neurons. Although some trigeminal ganglia neurons are suggested to respond to hypotonicity via TRPV4 activation (Chen *et al.*, 2009), we were unable to find any difference in the response to GSK1016790A or 4 α -PDD in cultured TG neurons to support a functional expression of TRPV4 in these neurons.

The activation of TRPV4 by 4 α -PDD was first described by Watanabe *et al.* (2002a). Exposure to 4 α -PDD only produced a significant increase in $[Ca^{2+}]_i$ in HEK cells and astrocytoma 1321N1 cells after transfection with TRPV4. We, and others, have also demonstrated a gain in responsiveness to 4 α -PDD after transfecting TRPV4 into HEK cells that supports its identification as a TRPV4 agonist (Xu *et al.*, 2003; Grant *et al.*, 2007). However, in this study, we have shown that the increase in $[Ca^{2+}]_i$ in cultured murine sensory neurons occurs independently of TRPV4, so it does not seem appropriate to describe 4 α -PDD as a selective agonist. Of the many previous studies using 4 α -PDD, only a few have confirmed the selectivity of its actions in cells or animals with deletion of TRPV4. Deletion of TRPV4 abolished the increase in cytoplasmic Ca^{2+} following application of 4 α -PDD to murine cochlear hair cells (Shen *et al.*, 2006), urothelial cells (Gevaert *et al.*, 2007), ciliated pulmonary epithelial cells (Lorenzo *et al.*, 2008), macrophages (Hamanaka *et al.*, 2010), chondrocytes (Clark *et al.*, 2010) and oesophageal keratinocytes (Mihara *et al.*, 2011), all of which are non-neuronal. Thus 4 α -PDD may selectively activate TRPV4 in certain cell types, but does not do so in DRG or TG neurons. In whole animal studies, the sensitization of viscera motor responses to colorectal distension by 4 α -PDD (Cenac *et al.*, 2008) and induction of paw oedema following intraplantar 4 α -PDD injection (Vergnolle *et al.*, 2010) were both abolished in TRPV4 knockout mice, and this was taken as evidence for a direct effect on sensory neuron TRPV4. However, an equally valid interpretation of these data is that the 4 α -PDD is activating a non-neuronal cell to initiate the biological response.

To our knowledge, only one previous study has studied responses to 4 α -PDD in sensory neurons from TRPV4 $+/+$ and $-/-$ mice. Lechner *et al.* (2011) identified two distinct responses to 10 μ M 4 α -PDD: a reversible increase in $[Ca^{2+}]_i$ and an irreversible increase, which they suggest is a non-specific toxic effect. The reversible increase was only observed in approximately 5% of TRPV4 $+/+$ neurons and was never seen in $-/-$ neurons. In contrast to this, we found that approximately 30% (44% of the 70% of neurons that responded) of both $+/+$ and $-/-$ neurons showed transient increases in $[Ca^{2+}]_i$ to 10 μ M 4 α -PDD. When we focused on thoracic DRG neurons, we identified the transient increase in 34.7% of responding wild-type and 32.1% of responding knockout neurons. Lechner and colleagues may have failed to identify TRPV4 knockout neurons that transiently respond to 4 α -PDD because of the relatively small number of neurons considered in their study. Overall, our data suggest that although 4 α -PDD is a TRPV4 agonist, it is not selective for TRPV4 on sensory neurons.

Another interesting observation is that not only are responses to hypotonic challenge and 4 α -PDD present in neurons from TRPV4 $-/-$ mice, but the proportion of cells that respond and mean size of the response are unchanged. This was surprising, as previous studies have proposed the existence of functional TRPV4 in sensory neurons (e.g. Chen *et al.*, 2009; Lechner *et al.*, 2011). Additionally, the Western blots we performed on whole DRG lysates suggested expression of TRPV4 protein, although we were unable to confirm protein expression in individual cultured neurons by immunohistochemical staining. It would be expected that, even if hypotonic challenge and 4 α -PDD do not selectively activate the

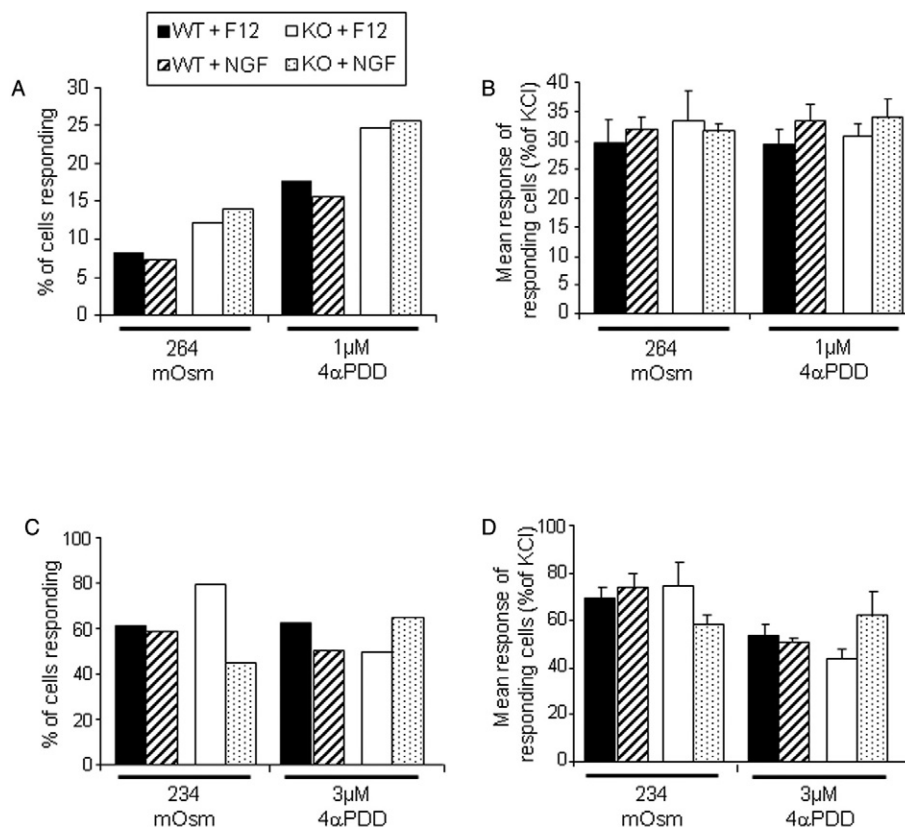


Figure 7

The effect of pre-treatment with NGF (100 ng·mL⁻¹; 24 h) on responses to hypotonic buffer (264 mOsm and 234 mOsm) or 4αPDD (1 and 3 μM) in cultured mouse dorsal root ganglion neurons from TRPV4 +/+ (WT) and TRPV4 -/- (KO) mice. (A and C) The % of total neurons responding and (B and D) mean response of individual responding neurons relative to the response to KCl are shown. *n* = 8–9 coverslips, with a total of at least 200 neurons from three mice for each data point.

channel, TRPV4 would contribute at least partially to the observed increase in [Ca²⁺]_i. If this were the case, then a decrease in the number of responding neurons and/or size of their response would be predicted. To investigate this further, we tested the effects of the recently described synthetic TRPV4 agonist GSK1016790A (Thorneloe *et al.*, 2008), on cultured neurons from wild-type mice. At concentrations that were maximally effective at stimulating an increase in [Ca²⁺]_i in a keratinocyte cell line, no changes in [Ca²⁺]_i greater than those in vehicle-treated neurons were observed. However, when we focused solely on neurons from thoracic DRG, a small group of responding neurons that were not present in knockout cells was observed. This suggests that functional TRPV4 is only present in a restricted subset of our cultured neurons, suggesting that the lack of immunostaining may represent a genuine absence of the protein.

One possible explanation for the TRPV4 expression in whole DRG lysates is that it is actually expression in the vasculature of the DRG or in other non-neuronal cells. TRPV4 expression and function has regularly been demonstrated in murine vascular endothelial cells (e.g. Watanabe *et al.*, 2002b; Hartmannsgruber *et al.*, 2007), so the entirety of the protein seen by Western blotting could be of vascular origin. Alternatively, neuronal TRPV4 expression could be lost under culture conditions. The cells were used for Ca²⁺ imaging and

immunostaining within 24 h of collection to minimize this possibility, well within the culture time used in previous studies (e.g. Lechner *et al.*, 2011). It has been suggested that exposure to NGF can increase cell surface presentation and activity of TRPV1, though phosphorylation of a tyrosine residue conserved in TRPV4 (Zhang *et al.*, 2005). To determine whether TRPV4 required NGF for full functional expression, we supplemented our culture medium with 100 ng·mL⁻¹ NGF. However, this had no significant effect on the responses to either hypotonic challenge or 4α-PDD, further supporting the conclusion that the cultured DRG neurons do not express functional TRPV4. Activating mutations in human TRPV4 have been linked to skeletal dysplasias such as brachyolmia (Rock *et al.*, 2008) and spondylometaphyseal dysplasia (Krakow *et al.*, 2009), and to motor neuropathies such as Charcot-Marie tooth disease type 2C (Landourey *et al.*, 2010). These diseases are not associated with sensory abnormalities, suggesting that human sensory nerves also have little, if any, expression of TRPV4.

The mechanisms by which hypotonic solutions and 4αPDD can induce an elevation in neuronal [Ca²⁺]_i independently of TRPV4 are not clear. The TRPV4 agonist 5',6-EET has recently been shown to modulate neuronal activity through activation of TRPA1, rather than TRPV4 (Sisignano *et al.*, 2012). However, we found that neurons lacking TRPA1 or

TRPV1 still responded to both hypotonic buffer and 4 α PDD, suggesting that these channels are not responsible. Neuronal expression of 5-HT₃ receptors (Linz and Veelken, 2002), TRPC1 (Staaf *et al.*, 2009) and TRPC5 (Gomis *et al.*, 2008) have all previously been suggested to provide sensitivity to hypo-osmotic challenge. However, identification of the precise mechanisms responsible for hypotonic and 4 α PDD-induced neuronal activation is beyond the scope of this study.

In conclusion, our data support the hypothesis that TRPV4 is involved in noxious mechanosensation, but this is not necessarily due to an activation of TRPV4 protein on sensory neurons. Indeed, we have failed to find any evidence for functional TRPV4 expression in the vast majority of DRG and TG neurons in culture. If this is also the case *in vivo*, the effects of TRPV4 agonists such as 4 α PDD on nociceptive behaviours should be interpreted based on a non-neuronal site of TRPV4 activity, such as in the Merkel cells (Boulais *et al.*, 2009) or keratinocytes of the skin (Chung *et al.*, 2003), or as an action on an alternative target.

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Conflicts of interest

None.

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