



TRPV4 mediates pain-related behavior induced by mild hypertonic stimuli in the presence of inflammatory mediator

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Abstract

The ligand-gated ion channel, TRPV4, functions as a transducer of hypotonic stimuli in primary afferent nociceptive neurons and contributes to inflammatory and neuropathic pain. Hypertonic saline also stimulates primary afferent nociceptors and the injection of mild hypertonic saline (2–5%) is widely used as an experimental model of pain in humans. Therefore, we tested whether TRPV4 participates in the transduction of hypertonic stimuli. Intradermal injection of 2% (607 mOsm) or 10% (3250 mOsm) saline solution in the hind paw of rats induced a concentration-dependent pain-related behavior, flinching. Sensitization with prostaglandin E₂ (PGE₂) caused a 7-fold increase in the number of flinches induced by 2% saline but failed to increase those caused by 10% saline. Spinal administration of antisense oligodeoxynucleotides to TRPV4 caused a 46% decrease in the number of flinches induced by 2% saline, but there was no change in flinching induced by 10% saline. Similarly, only the nociceptive behavior caused by 2% saline was reduced in TRPV4^{-/-} knockout mice. The TRPV4-mediated nociceptive behaviors induced by hyper- and hypotonic stimuli were dependent on Src tyrosine kinase. We suggest TRPV4 is a transducer in primary afferents that mediates nociceptive behavior induced by small increases or decreases in osmolarity. Such changes in osmolarity might contribute to pain in inflammatory and neuropathic states.

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1. Introduction

Injection of mildly hypertonic saline (2–5%) stimulates C-fiber afferents (Baraniuk et al., 1999; Bennell et al., 2004; Garland et al., 1995; Graven-Nielsen et al., 1997; Michaelis et al., 1997; Stohler et al., 2001; Weerakkody et al., 2003; Witonski and Wagrowska-Danielewicz, 1999) and is widely used as an experimental model of muscle or joint pain in humans (Arendt-Nielsen et al., 2000; Bennell et al., 2004; Graven-Nielsen et al., 1997; Jensen and Norup, 1992; Kellgren, 1937; Witonski and Wagrowska-Danielewicz, 1999). While the molecular mechanisms underlying the response to hypertonic stimuli in primary nociceptive afferents are still unknown, their elucidation

could have considerable clinical impact, especially since osmolarity disturbances are described in various diseases such as diabetes (Puliyel and Bhambhani, 2003), alcoholism (Vamvakas et al., 1998) and aquadynia (Misery et al., 2003). In addition, inhalation of ultrasonic nebulized distilled water and hypertonic saline is used as a provocative tests for the diagnosis of asthma (Allegra and Bianco, 1980; Baba et al., 1989; Choi et al., 2003; Fujimura et al., 1997; Liedtke and Simon, 2004; Smith and Anderson, 1986; Smith and Anderson, 1989) and osmolarity increases and pH decreases are believed to contribute to inflammatory pain (Hamamoto et al., 2000; Vakili et al., 1970).

The transient receptor potential vanilloid 4 (TRPV4; Liedtke et al., 2000; Strotmann et al., 2000) functions as a transducer of hypo-osmotic stimuli in primary nociceptive afferents, and hypotonic stimuli induce TRPV4-mediated

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nociceptive behaviors when nociceptive afferents are pre-sensitized by the inflammatory mediator prostaglandin E₂ (PGE₂, Alessandri-Haber et al., 2003) or during a neuropathic pain state (Alessandri-Haber et al., 2004). Therefore, we investigated whether TRPV4 may also participate in hypertonicity-induced nociceptive behavior.

Previous studies in heterologous expression systems failed to demonstrate activation of TRPV4 by hypertonic stimuli (Liedtke et al., 2000; Strotmann et al., 2000). However, two recent reports on TRPV4 gene-targeted mice implicate TRPV4 in hyper- as well as hypo-osmotic regulation in the central nervous system (Liedtke and Friedman, 2003; Mizuno et al., 2003). Specifically, Liedtke and Friedman demonstrated that knock out of TRPV4 attenuates the ADH-response and fluid-intake behavior evoked by hypertonic stimulation. In addition, Liedtke and co-workers (2003) demonstrated that mutation of the osmosensing TRPV gene, *Osm9*, in *C. elegans* causes ASH osmotic sensory neurons to become insensitive to hypertonicity, and these worms lose their hypertonicity avoidance behavior. Transgenic expression of mammalian TRPV4 in ASH neurons of *Osm-9* mutant worms restores the hyper-osmotic avoidance behavior. Therefore, we investigated if TRPV4 participates in the transduction of hypertonic stimuli in primary nociceptive afferents and if, as for hypotonic stimuli, its predominant contribution is in sensitized states associated with inflammation.

2. Material and methods

Experiments were performed on 180–200 g male Sprague-Dawley rats (Charles River, Hollister, CA) and on C57Bl6 mice lacking functional TRPV4 gene (TRPV4^{-/-} mice) and TRPV4 wild type littermates (TRPV4^{+/+} mice). The TRPV4^{-/-} mice were generated as described previously (Liedtke and Friedman, 2003). The experimental protocols were approved by the UCSF Committee on Animal Research and conformed to the NIH guidelines for the use of animals in research.

Dorsal root ganglia cell culture. L2–L6 dorsal root ganglia (DRG) were harvested from TRPV4^{+/+} and TRPV4^{-/-} mice and dissociated as described previously (Alessandri-Haber et al., 2003). Briefly, cells were maintained in culture for 3 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50–100 ng/ml nerve growth factor, 100 units/ml penicillin/streptomycin, MEM vitamins and 10% heat inactivated fetal calf serum (all culture components from Invitrogen Life Technologies, Carlsbad, CA). Dissociated cells were plated on cover slips treated with poly-DL-ornithine (0.1 mg/ml; Sigma, St Louis, MO) and laminin (5 µg/ml; Invitrogen Life Technologies) and incubated at 37 °C in 96.5% air, 3.5% CO₂.

Calcium imaging. Measurement of the intracellular concentration of free calcium ions ([Ca²⁺]_i) was performed by ratiometric imaging with an intensified charge-coupled device ICCD camera (Stanford photonics, Inc, Palo Alto, CA). Fluorescence was excited at 340 and 380 nm and the emitted light was long filtered at 520 nm. The fluorescence ratio, F340/F380, was calculated with METAFLUOR[®] software (Universal Imaging Corporation,

Downington, PA). Recordings were performed using the fluorescent calcium indicator fura 2-acetoxymethyl ester (fura-2AM), between 24 and 72 h after dissociation of DRG neurons as previously described (Alessandri-Haber et al., 2003). Briefly, neurons were loaded with 5 µM fura-2 AM for 20 min in isotonic solution (312 mOsm). Experiments were carried out at 20–23 °C with the perfusion at a flow rate of 1–2 ml/min. Cells were perfused with isotonic solution for 10 min before the beginning of the recording to allow complete removal of un-hydrolysed fura-ester. The vehicle for the fura-2 AM, dimethyl sulfoxide (DMSO) was tested on DRG neurons to verify that it did not alone induce a response.

Given the absence of specific blockers of TRPV4 and the ionic complexity of DRG neurons we have conservatively chosen to perform our experiments in conditions that were less likely to activate other ion channels (i.e. low sodium concentration and room temperature) but in which the response of TRPV4 may not be maximized. The standard isotonic solution (312 mOsm) contained (mM): 88 NaCl, 5 KCl, 1 MgCl₂, 2.4 CaCl₂, 110 D-mannitol, 10 HEPES buffered at pH 7.38 with NaOH. The hypotonic solution was adjusted to 212 mOsm (30% hypotonic) by lowering the amount of D-mannitol and hypertonic solutions were adjusted to 406, 552 and 646 mOsm by increasing the amount of D-mannitol. Osmolarity of each solution was measured before each experiment with a vapor pressure osmometer 5520 (Wescor, Logan, Utah).

During a 3 min hypo- or hypertonic stimulus, responsive neurons show an increase in [Ca²⁺]_i which reaches a plateau that is maintained until the perfusion is switched back to isotonic solution. To measure the response of DRG neurons to hyper- or hypotonicity, we averaged the value of the fluorescence ratio when the stimulus-induced increase in [Ca²⁺]_i reached a plateau. The fluorescence ratio at the plateau was then normalized: fluorescence ratio during 30% hyper- or hypotonic solution/fluorescence ratio during isotonic solution. For the experiments involving sensitization with PGE₂, neurons were challenged with a 30% hypertonic solution for 3 min, washed for 3 min with isotonic solution, perfused with an isotonic solution containing PGE₂ (2.5 µM) for 15 min and then challenged with 30% hypertonic solution containing PGE₂. Intracellular calcium increase was calculated as the fluorescence ratio during a 30% hypertonic solution containing PGE₂/fluorescence ratio during a 30% hypertonic solution. Excitability of neurons was confirmed by a short exposure to KCl 20 mM at the end of each experiment.

Oligodeoxynucleotides (ODN) preparation. The TRPV4 antisense ODN sequence 5'CATCACCAGGATCTGCCATACTG-3' (Invitrogen Life technologies), was directed against a unique region of the rat TRPV4 channel (GeneBank accession number AF263521). The mismatch ODN sequence was designed by mismatching 7 bases (denoted by bold face) of the TRPV4 antisense sequence, 5'-CAACAGGAGGTTTCAGGCAAACCTG-3' (Alessandri-Haber et al., 2004; Alessandri-Haber et al., 2003).

Intrathecal ODN treatment. ODNs were reconstituted in nuclease-free 0.9% NaCl (10 µg/l) and were intrathecally administered once daily at a dose of 40 µg for 3 days. As described previously (Alessandri-Haber et al., 2004; Alessandri-Haber et al., 2003), rats were anesthetized with 2.5% isoflurane inhalation anesthetic (97.5% O₂), a 30-gauge needle was inserted into the subarachnoid space on the midline between the L4 and L5 vertebrae and 20 µl ODN injected at 1 (µl/s), using a micro-syringe.

Nociceptive behavior in rat (flinch test): As described previously (Alessandri-Haber et al., 2004; Alessandri-Haber

et al., 2003), rats were acclimated in a transparent observation chamber for 30 min. They were then restrained while 10 μ l hypertonic (2% or 10% NaCl respectively 607 and 3250 mOsm), hypotonic (deionized water, 17 mOsm) or isotonic (0.9% NaCl, 283 mOsm) solution was administered intradermally on the dorsum of the hind paw using a 30-gauge needle connected to a 100 μ l Hamilton syringe by PE-10 polyethylene tubing. Nociceptive behavior (i.e. number of flinches) was recorded for a 5-min period starting immediately after the injection; (Alessandri-Haber et al., 2004; Alessandri-Haber et al., 2003; Houck et al., 2004; Khodorova et al., 2002; Sorkin et al., 2001; Zhang et al., 2003; Zheng and Chen, 2001). For experiments involving TRPV4 antisense treatment, the flinch test was performed on the fourth day following initiation of ODN treatment (18 h post-injection). PGE₂ (100 ng, 2.5 μ l) was injected intradermally 30 min prior to the injection of hypertonic solution at the same site on the dorsum of the paw. The number of flinches induced by hyper- or hypotonic stimulation was determined on a control group of rats which received an injection of 10 μ l hyper- or hypotonic solution in the hind paw while a test group received an injection of PGE₂ 30 min prior to the injection of hyper- or hypotonic solution at the same site.

Nociceptive behavior in mice: To investigate the effect of hypertonic injection in mice and compare it to the induced nociceptive behavior in the rat, we needed to have a comparative behavioral nociceptive test. These two species do not react similarly to an injection of hypo- or hyper-tonic solution; while a rat flinches its hind paw several times during the 5 min subsequent to the injection, a mouse shakes and licks its paw. The measurement of the time spent shaking, licking and biting the hind paw which received an injection is a well established nociceptive test usually used in the formalin test (Berrino et al., 2003; Hunskar et al., 1985; Mogil et al., 1999; Murray et al., 1988; Oliva et al., 2002; Piovezan et al., 2000). Mice were placed in a transparent plastic box on a metal mesh floor and allowed to explore for 15–20 min. The mice were then given an intraplantar injection in the hind paw of either hypotonic solution (deionized water, 5 μ l) or hypertonic solution (2% or 10% NaCl, 5 μ l). Mice were also observed for a 5-min period starting immediately after the injection and the amount of time during which the mice shook and licked the injected paw was recorded.

For experiments involving sensitization with PGE₂, a local intraplantar injection of PGE₂ (100 ng, 5 μ l) was administered 30 min prior to injection of the hyper- or hypotonic stimulus at the same injection site and for the experiments involving the tyrosine kinase inhibitors, an intraplantar co-injection of PGE₂ with either PP₁ or piceatannol was performed 30 min prior to the injection of hypo- or hypertonic solution at the same site.

Drugs. Stock solutions of PGE₂ (Sigma, St Louis, MO) were made in 10% ethanol and the tyrosine kinase inhibitors (Biomol, Plymouth Meeting, PA), PP₁ (Src family tyrosine kinase specific inhibitor, Hanke et al., 1996) and piceatannol (specific inhibitor of Syk, a related protein kinase not belonging to the Src family, IC₅₀ ~ 10 μ M Oliver et al., 1994) were made in 10% DMSO. Experimental concentrations were made daily from stock solutions in saline. The final concentrations of DMSO and ethanol were \leq 1%. As a negative control, we verified that the injection of 10% DMSO (unpublished data) or 1% ethanol (Khasar et al., 1994) in rat hind paw had no effect on paw threshold.

Data analysis. Calcium imaging and behavioral data are presented as mean \pm SEM and comparisons between groups were performed using Student's *t*-test or ANOVA and the *post hoc*

Scheffe test. A probability of $P < 0.05$ was considered significant. To determine if the percentage of DRG neurons responding to hypertonic stimulation in TRPV4^{+/+} mice differed significantly from that in TRPV4^{-/-} mice; a Chi square test with Yates correction was performed.

3. Results

3.1. Nociceptive behavior induced by 2% saline is enhanced by PGE₂

To investigate whether a hypertonic stimulus can induce TRPV4-mediated nociceptive behavior in rats, we injected 10 μ l of mild hypertonic solution (2% NaCl, 607 mOsm) intradermally in the rat hind paw. The injection of 2% NaCl induced a significant number of flinches during the 5 min following injection compared to the same volume of isotonic solution (Fig. 1, 0.9% NaCl, 283 mOsm, $n = 14$ for Iso and $n = 9$ for 2% Hyper, $P < 0.05$, unpaired Student's *t*-test). Of note, this is comparable to the number of flinches induced by the injection of 10 μ l hypotonic solution (Fig. 1; deionized water, 17 mOsm, Hypo, $n = 24$). We have previously shown that local injection of PGE₂ prior to intradermal injection of hypotonic solution enhances TRPV4-mediated nociceptive behavior (Alessandri-Haber et al., 2003). Therefore, we investigated whether injection of PGE₂ (100 ng, 2.5 μ l) would enhance the sensitivity to a hypertonic stimulus. Pre-sensitization with PGE₂, 30 min prior to hypertonic stimulation, at the same site, produced a 7-fold increase in the number of flinches elicited by mild hypertonicity (25 ± 4 , $n = 8$ after PGE₂ versus 3.5 ± 0.8 , $n = 9$ without PGE₂, $P < 0.05$, unpaired Student's *t*-test). As a control, we verified that both the injection of PGE₂ alone or the injection of PGE₂ 30 min prior to the injection of isotonic solution did not induce flinching (data not shown).

3.2. TRPV4 participates in the nociceptive behavior induced by 2% saline in the rat

To assess the contribution of TRPV4 to hypertonicity-induced flinches, rats were treated, by spinal intrathecal injection, with either antisense or mismatch ODN for TRPV4 for 3 days. On the fourth day, rats' hind paws were sensitized with an injection of PGE₂ administered 30 min prior to the hypertonic stimulus. Treatment with ODN antisense for TRPV4 caused a 46% decrease in the number of flinches induced by 2% NaCl compared to rats treated with mismatch ODN ($n = 10$ for antisense- and $n = 6$ for mismatch-treated rats, *post hoc* Scheffe test, $P = 0.02$).

3.3. Nociceptive behavior induced by 10% NaCl is not enhanced by PGE₂ and is not TRPV4-dependent

We next investigated if TRPV4 also participates in nociceptive behavior induced by a higher degree of saline

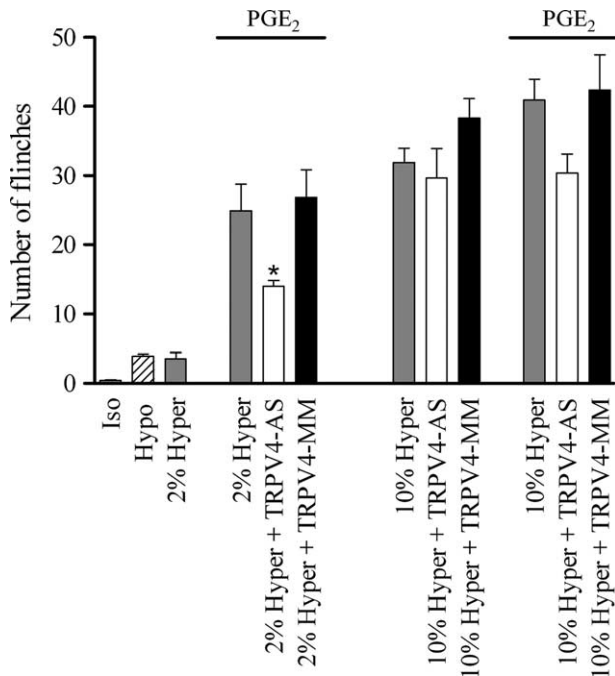


Fig. 1. Hypertonicity induces TRPV4-mediated nociceptive behavior in rat. Intradermal injection of 2% NaCl solution (2% Hyper) in the rat hind paw caused a significant number of flinches (3.5 ± 0.8 , $n=9$) compared to the same volume of isotonic solution (0.9% NaCl, Iso, 0.4 ± 0.1 , $n=14$, $P < 0.05$, unpaired Student's *t*-test). Pre-sensitization with PGE₂ induced a 7-fold increase in the number of flinches caused by the 2% NaCl solution (25 ± 4 , $n=8$ after PGE₂ versus 3.5 ± 0.8 , $n=9$ before, $P < 0.05$, unpaired Student's *t*-test). Spinal intrathecal injection of TRPV4 antisense ODN caused a 46% decrease in the number of flinches induced by 2% NaCl (14 ± 1 , $n=10$ for antisense-, 27 ± 4 , $n=6$ for mismatch- and 25 ± 4 , $n=8$ for non ODN-treated rats, $P < 0.05$ ANOVA). Intradermal injection of 10% NaCl solution (10% Hyper) induced a 9-fold increase in the number of flinches compared to the 2% NaCl solution (32 ± 2 , $n=6$ for 10% Hyper versus 3.5 ± 0.8 , $n=9$ for 2% Hyper, $P < 0.05$, unpaired Student's *t*-test) but treatment with antisense ODN for TRPV4 did not attenuate the number of flinches induced by 10% NaCl (30 ± 3 , $n=6$ for antisense-, 38 ± 3 , $n=6$ mismatch- and 32 ± 2 , $n=6$ for non-ODN-treated rats, $P=0.166$, ANOVA). Pre-sensitization with PGE₂ only slightly increased the number of flinches induced by 10% NaCl (41 ± 3 , $n=11$ after PGE₂ versus 32 ± 2 , $n=6$, $P < 0.05$, unpaired Student's *t*-test) and, even in the presence of PGE₂, antisense ODN for TRPV4 did not attenuate the number of flinches caused by 10% NaCl (41 ± 3 , $n=11$ for non-ODN-treated, 30 ± 3 , $n=5$ for antisense-treated and 42 ± 5 , $n=5$ mismatch-treated rats, $P=0.109$, ANOVA).

solution. Intradermal injection of 10% NaCl (10% Hyper, 3250 mOsm) induced a 9-fold increase in the number of flinches compared to a 2% NaCl solution (Fig. 1). However, treatment with antisense ODN for TRPV4 did not significantly reduce the number of flinches induced by 10% NaCl compared to mismatch- or non-ODN-treated rats ($n=6$ for each group of rats, $P=0.166$, ANOVA). Pre-sensitization with PGE₂ only produced a 1.25-fold increase in the number of flinches caused by 10% NaCl (41 ± 3 , $n=11$ after PGE₂ versus 32 ± 2 , $n=6$ without PGE₂, $P < 0.05$, unpaired Student's *t*-test). Treatment with antisense ODN for TRPV4 did not significantly decrease the number of flinches induced by 10% NaCl ($n=5$ for antisense-, $n=5$ for mismatch- and $n=11$ for non-ODN-treated rats,

$P=0.109$, ANOVA). To confirm that the hypertonicity-induced nociceptive behavior had not reached a maximum with the injection of 10% Hyper, we injected 10 μl of 15% NaCl solution in the rat hind paw. The number of flinches induced by 15% NaCl was significantly greater compared to 10% (38 ± 2 , $n=11$ for 15% hypertonicity and 32 ± 2 , $n=6$ for 10% hypertonicity, $P < 0.05$, unpaired Student's *t*-test). Of note, we have previously demonstrated the efficiency of the TRPV4 antisense ODN treatment used in this experiment by Western blot analysis (Alessandri-Haber et al., 2004; Alessandri-Haber et al., 2003).

3.4. TRPV4 mediates only nociceptive behavior induced by mild hypertonicity

To further confirm the role of TRPV4 in the transduction of nociceptive behavior induced by mild hypertonicity, an intraplantar injection of 5 μl of either 2% or 10% NaCl solution was performed in the hind paw of C57Bl6 mice lacking functional TRPV4 (TRPV4^{-/-}, Liedtke and Friedman, 2003) and wild type littermates (TRPV4^{+/+}). The injection of 2% and 10% NaCl solution both induced nociceptive behavior (Fig. 2A). The time of licking and shaking of the paw following the injection of 2% NaCl was significantly attenuated in TRPV4^{-/-} mice ($n=6$ for each genotype, $P < 0.05$, unpaired Student's *t*-test). In contrast, the nociceptive behavior following the injection of 10% NaCl solution in TRPV4^{+/+} mice was similar to that in TRPV4^{-/-} mice ($n=6$ for each genotype, $P > 0.05$, unpaired student's *t*-test). Thus, TRPV4 plays a role in the transduction of nociceptive behaviors induced by mild hypertonic solution whereas the nociceptive effect of a higher degree of hypertonicity involves other mechanisms.

3.5. TRPV4-dependent response to mild hypertonicity is enhanced by PGE₂

We have previously demonstrated that intradermal injection of hypotonic solution, in PGE₂ pre-sensitized hind paw, induces a significant number of TRPV4-mediated nociceptive flinches in the rat (Alessandri-Haber et al., 2003). In the present study, we confirm these results in the mouse; while intraplantar injection of 5 μl hypotonic solution (deionized water, 17 mOsm) in TRPV4^{+/+} or TRPV4^{-/-} mice did not induce nociceptive behavior (Fig. 2B), the injection of hypotonic solution 30 min after intraplantar injection of PGE₂ (100 ng, 5 μl), caused significant nociceptive behavior in TRPV4^{+/+} mice (Fig. 2B, 120 ± 5 , $n=10$ for TRPV4^{+/+} versus 2 ± 1 , $n=6$ for TRPV4^{-/-} mice).

We then tested whether injection of PGE₂ 30 min prior to the injection of 2 or 10% NaCl solution would enhance the TRPV4-mediated nociceptive behavior in the mouse. As shown in Fig. 2C, injection of PGE₂ prior to the injection of 2% NaCl caused a 12-fold increase in nociceptive behavior in TRPV4^{+/+} mice ($n=12$ after PGE₂ and $n=6$ without

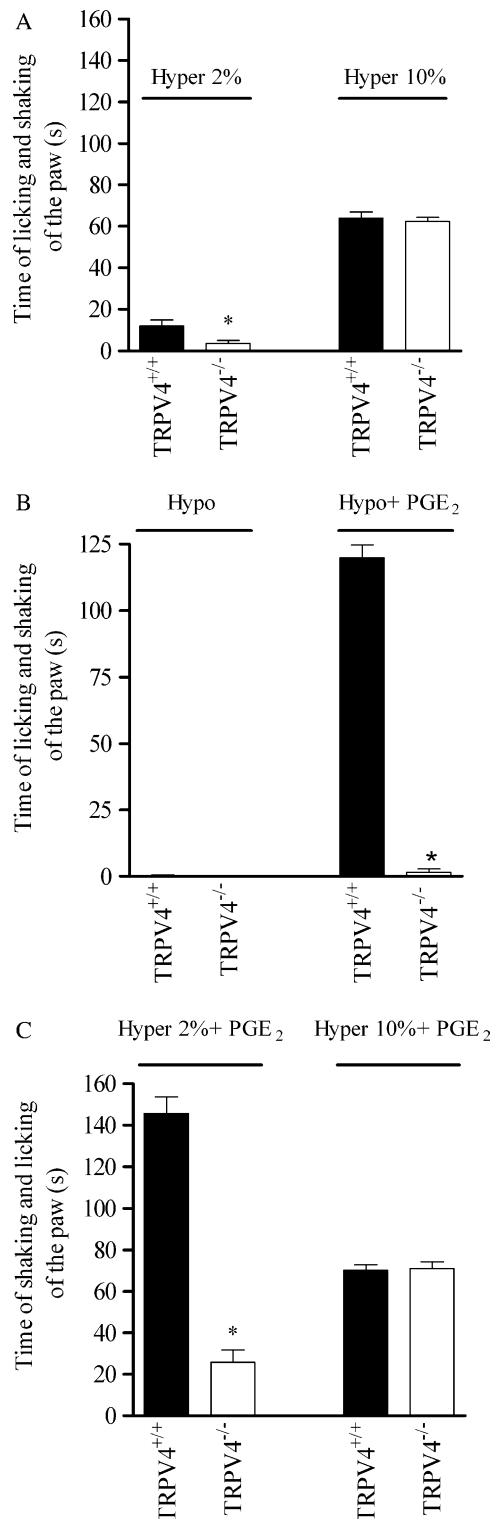


Fig. 2. TRPV4-mediated nociceptive behavior caused by 2% NaCl is enhanced by PGE₂ in mouse. A, Intraplantar injection of 2 or 10% NaCl solution in the hind paw of TRPV4^{+/+} and TRPV4^{-/-} mice induced nociceptive behavior. Nociceptive behavior caused by the injection of 2% NaCl was significantly attenuated in the TRPV4^{-/-} mice (12 ± 3 s, n = 6 for TRPV4^{+/+} mice versus, 4 ± 1 s, n = 6 for TRPV4^{-/-}, P < 0.05, unpaired Student's t-test). In contrast, the nociceptive behavior caused by the injection of 10% NaCl in TRPV4^{-/-} was similar to that in TRPV4^{+/+} mice (64 ± 3 s, n = 6 for TRPV4^{+/+} versus 62 ± 2 s for TRPV4^{-/-}, P >

PGE₂, P < 0.05, unpaired Student's t-test) whereas it caused only a small, but statistically significant, increase in nociceptive behavior in TRPV4^{-/-} mice (n = 12 after PGE₂ and n = 6 without PGE₂, P < 0.05, unpaired Student's t-test). However, the injection of PGE₂ prior to the injection of 10% NaCl did not significantly enhance the nociceptive behavior in TRPV4^{+/+} mice (n = 8 in presence of PGE₂ and n = 6 without, P > 0.05, unpaired Student's t-test) and the nociceptive behavior was not attenuated in TRPV4^{-/-} mice (70 ± 3 s for TRPV4^{+/+} mice versus 71 ± 1 s, n = 8 for TRPV4^{-/-} mice, unpaired Student's t-test). These results suggest that TRPV4 is essential for the transduction of small hypertonic changes in sensitized nociceptive afferents.

3.6. TRPV4-mediated nociceptive behaviors are dependent on a Src tyrosine kinase pathway

We recently demonstrated in a model of chemotherapy neuropathy induced by Taxol[®] that TRPV4-mediated nociceptive behaviors in rats partly depend on a Src tyrosine kinase pathway (Alessandri-Haber et al., 2004); injection of the Src family tyrosine kinase specific inhibitor PP₁ (1 µg/2.5 µl) in the rat hind paw significantly inhibited the number of hypotonicity-induced flinches and the mechanical hyperalgesia in Taxol[®]-treated rats whereas the injection of piceatannol (1 µg/2.5 µl) which inhibits Syk, a related protein kinase not belonging to the Src family had no effect. In addition, Xu and co-workers (2003) demonstrated that activation of TRPV4 by hypotonicity in vitro results in a rapid phosphorylation of TRPV4 that is inhibited by the Src family tyrosine kinase specific inhibitor PP₁ but not piceatannol. Therefore, we tested whether TRPV4-mediated hyper- and hypotonic-induced nociceptive behaviors, following PGE₂ sensitization, also depend on a Src tyrosine kinase phosphorylation. TRPV4^{+/+} and TRPV4^{-/-} mice received a co-injection of PGE₂ with either PP₁ (1 µg, 5 µl) or piceatannol (1 µg, 5 µl) 30 min prior to the injection of hyper- or hypotonic solution at the same site. *Hypertonicity*: in TRPV4^{+/+} mice, the injection of PP₁ caused a 79% reduction in nociceptive behavior induced by 2% hypertonicity whereas piceatannol had no significant effect

0.05, unpaired Student's t-test). B, Intraplantar injection of hypotonic solution did not induce nociceptive behavior in TRPV4^{+/+} or TRPV4^{-/-} mice. In contrast, after sensitization with PGE₂, the hypotonic solution caused a significant nociceptive behavior in the TRPV4^{+/+} mice (120 ± 5 s, n = 10 for TRPV4^{+/+} versus 2 ± 1 s, n = 6 for TRPV4^{-/-} mice, P < 0.05, unpaired Student's t-test). C, Pre-sensitization with PGE₂ induced a 12-fold increase in the nociceptive behavior caused by 2% NaCl solution in TRPV4^{+/+} mice (12 ± 3 s, n = 6 before versus 145 ± 8 s, n = 12 after PGE₂, P < 0.05, unpaired Student's t-test) whereas it only induced a small increase in nociceptive behavior in TRPV4^{-/-} mice (4 ± 1 s, n = 6 before versus 25 ± 6 s, n = 12, P < 0.05, unpaired Student's t-test). In contrast, the injection of PGE₂ prior to the injection of 10% NaCl solution did not significantly increase the nociceptive behavior in TRPV4^{+/+} mice (70 ± 3 s, n = 8, in presence of PGE₂ versus 64 ± 3, n = 6 without, P > 0.05, unpaired Student's t-test).

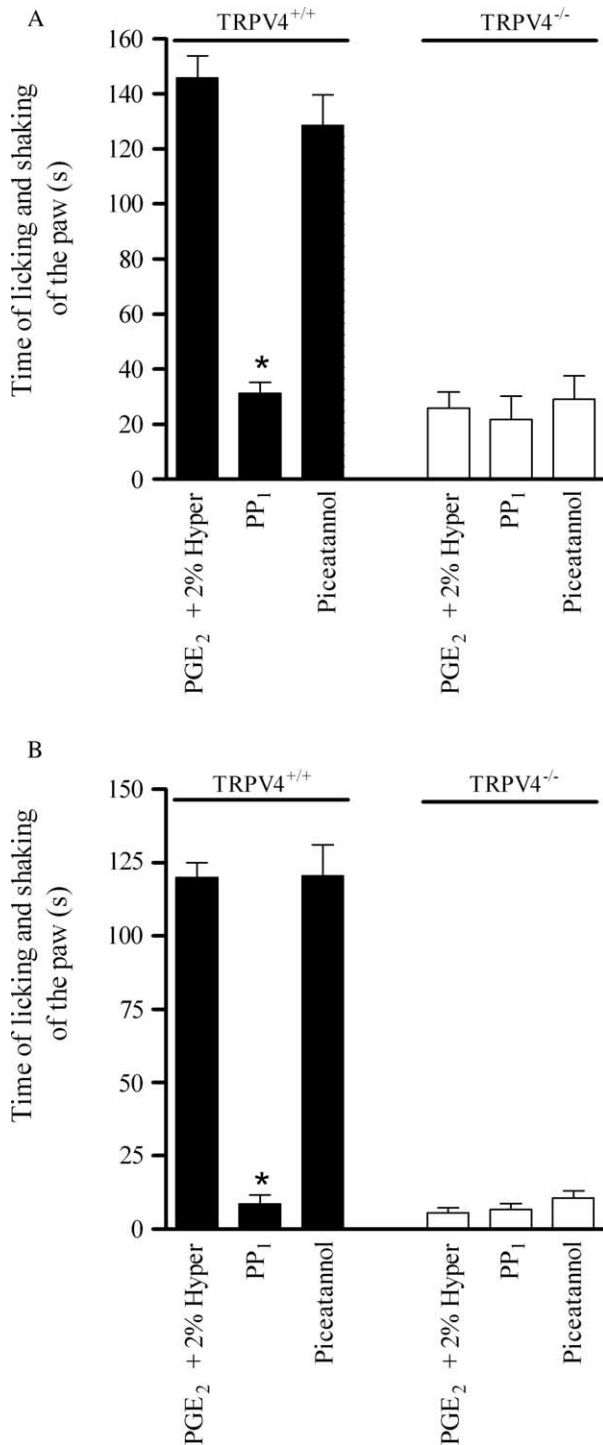


Fig. 3. TRPV4-mediated nociceptive behavior depends on a Src tyrosine kinase pathway. A, In TRPV4^{+/+} mice, the injection of PP₁ (specific inhibitor of Src family tyrosine kinase) caused a 79% reduction in nociceptive behavior induced by 2% NaCl (31 ± 4 s, n = 6 in presence versus 146 ± 8 s, n = 12 in absence of PP₁, P = 0.001, unpaired Student's *t*-test). In contrast, the injection of piceatannol (specific inhibitor of Syk, a related protein kinase) had no significant effect (129 ± 11 s, n = 7 in presence and 146 ± 12 s, n = 12 in absence of piceatannol, P > 0.05, unpaired Student's *t*-test). In contrast, in TRPV4^{-/-} mice, neither the injection of PP₁, nor piceatannol had an effect on the nociceptive behavior caused by 2% NaCl (22 ± 8 s, n = 6 in presence of PP₁, 29 ± 8 s, n = 6 in

(Fig. 3A). In TRPV4^{-/-} mice, neither PP₁ nor piceatannol have a significant effect on the nociceptive behavior induced by hypertonicity (Fig. 3A, n = 6 in presence of PP₁ or piceatannol and n = 12 in absence of blocker, P > 0.05, ANOVA).

Hypotonicity: the injection of PP₁ 30 min prior to a hypotonic stimulus prevented the hypotonicity-induced nociceptive behavior in TRPV4^{+/+} mice (Fig. 3B) whereas injection of piceatannol did not induce significant changes in the nociceptive behavior (Fig. 3B, n = 6 in presence of piceatannol and n = 10 in absence, P > 0.05, unpaired Student's *t*-test). In the TRPV4^{-/-} mice, the injection of PP₁ or piceatannol had an effect on the nociceptive behavior induced by hypotonicity (7 ± 2 s in presence of PP₁, 10 ± 3 s in presence of piceatannol, and 6 ± 2 s in absence of blocker, n = 6, P > 0.05, ANOVA).

3.7. Response of TRPV4^{+/+} and TRPV4^{-/-} DRG neurons to hypertonic stimuli

Calcium imaging recordings were performed to provide support for the suggestion that the hypertonicity-induced nociceptive behaviors were specifically attributable to the direct action of this stimulus on primary afferent neurons. Isolated small-diameter DRG neurons (≤ 25 μm) from TRPV4^{+/+} and TRPV4^{-/-} mice were recorded and neurons were identified as putative nociceptors if capsaicin (1 μM, 10 s) induced a significant increase in the fluorescence ratio. Hypertonic challenges below 406 mOsm (30% Hyper) failed to induce a detectable increase in the fluorescence ratio (data not shown), therefore neurons were challenged with a 30% hypertonic solution (cf Material and Methods, 30% Hyper, 406 mOsm; Fig. 4A, inset). There was no statistically significant difference between TRPV4^{+/+} and TRPV4^{-/-} mice in the percentage of DRG neurons that responded to 30% Hyper (15%, 32 out of 212 neurons for TRPV4^{+/+} versus 9.5%, 21 out of 219 neurons for TRPV4^{-/-}, P < 0.25, χ² test with Yates correction). Furthermore, there was no difference in the percentage of putative nociceptors that responded (19% for TRPV4^{+/+} and 12% for TRPV4^{-/-}, P < 0.25, χ² test with Yates correction). The mean of the hypertonicity-induced increase in fluorescence ratio was also similar in TRPV4^{+/+} mice and TRPV4^{-/-} mice (Fig. 4A, n = 28 for TRPV4^{+/+} and n = 23 for TRPV4^{-/-} mice, P > 0.05,

presence of piceatannol and 26 ± 6 s n = 12 in absence of blocker, P > 0.05, ANOVA). B, In TRPV4^{+/+} mice, the injection of PP₁ prevented 92% of the nociceptive behavior caused by hypotonicity (9 ± 3 s, n = 6 in presence versus 120 ± 5 s, n = 10 in absence of PP₁, P = 0.001, unpaired Student's *t*-test) whereas the injection of piceatannol did not induce any significant change (120 ± 10 s, n = 6 in presence and 120 ± 5 s, n = 10 in absence of piceatannol, P > 0.05 unpaired Student's *t*-test). In contrast, in TRPV4^{-/-} mice, the injection of neither PP₁ nor piceatannol induce significant change in the hypotonicity-induced nociceptive behavior (7 ± 2 s, n = 6 in presence of PP₁, 10 ± 3 s, n = 6 in presence of piceatannol and 6 ± 2 s in absence de blocker, n = 6, P > 0.05, ANOVA).

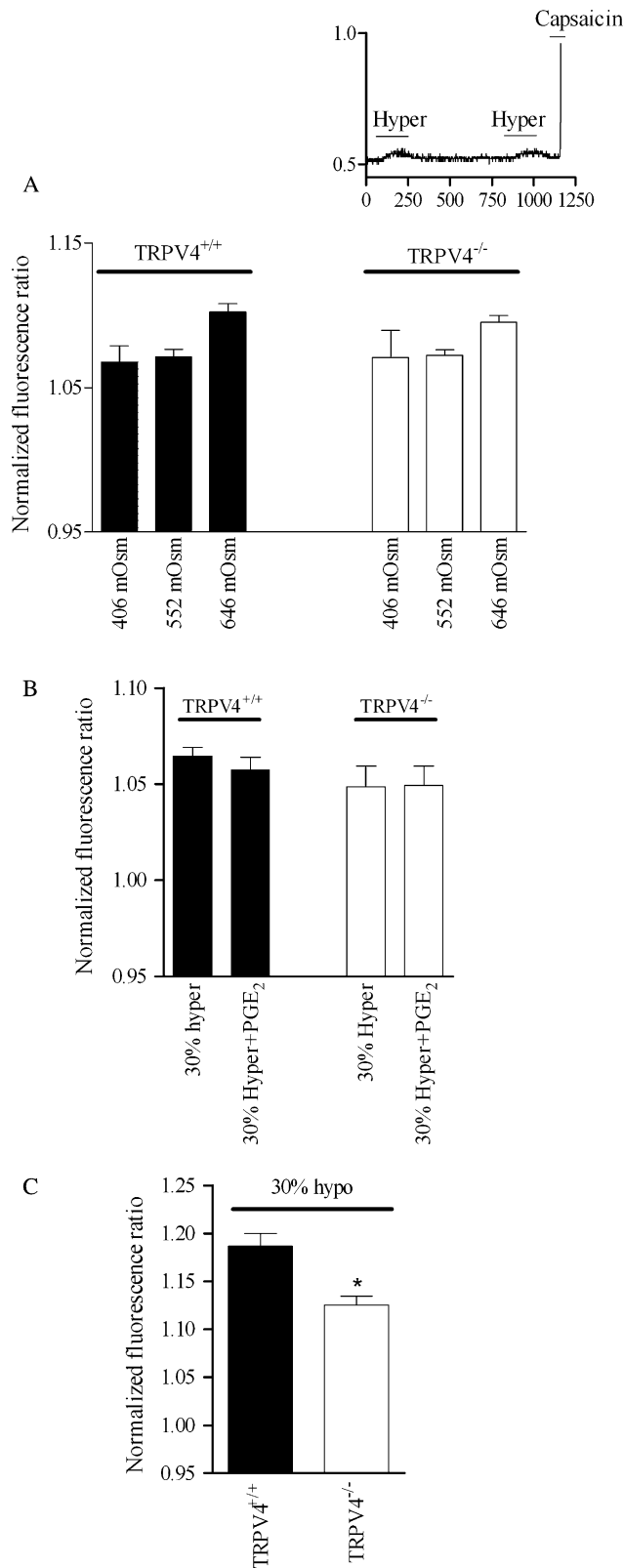


Fig. 4. Comparison of response of DRG neurons to hypertonicity in TRPV4^{+/+} and TRPV4^{-/-} mice. A, DRG neurons (diameter ≤25 μm) in culture were challenged with a 30% hypertonic (406 mOsm) for 3 min (inset). Neurons from TRPV4^{+/+} and TRPV4^{-/-} mice were stimulated with three degrees of hypertonicity (406, 552 and 646 mOsm). Data are

unpaired Student's *t*-test). Another set of nociceptors was tested with 2 increasing levels of hypertonicity (552 and 646 mOsm, Fig4A) similar to the osmolarity of the 2% NaCl solution (607 mOsm). In both TRPV4 genotypes, the mean response to hypertonic stimulus increased with degree of hypertonicity ($n=53$ for TRPV4^{+/+} and $n=33$ for TRPV4^{-/-}, $P < 0.001$, paired Student's *t*-test) but again neither the percentage of DRG neurons that responded to the hypertonic stimuli nor the mean of the hypertonicity-induced increase in fluorescence ratio were diminished in TRPV4^{-/-} mice. It was demonstrated that when temperature is increased to mammalian body temperature TRPV4 sensitivity to other stimuli is potentiated (Gao et al., 2003; Guler et al., 2002; Liedtke et al., 2000). Therefore, neurons were challenged with a 30% hypertonic solution at 33 °C. Again, the percentage of DRG neurons that responded to 30% Hyper at 33 °C (36 out of 54 neurons for TRPV4^{+/+} versus 39 out of 53 neurons for TRPV4^{-/-}) and the mean of the hypertonicity-induced increase in fluorescence ratio (1.086 ± 0.007 , $n=36$ in TRPV4^{+/+} and 1.075 ± 0.004 , $n=39$ in TRPV4^{-/-} mice, $P > 0.05$, unpaired Student's *t*-test) were similar in TRPV4^{+/+} and TRPV4^{-/-} mice.

Finally, because PGE₂ produced a 12-fold increase in the nociceptive behaviors induced by a 2% hypertonic stimulus in TRPV4^{+/+} mice, we investigated if PGE₂ could enhance the response of DRG neurons to a 30% hypertonic solution in vitro. However, the hypertonicity-induced increase in fluorescence ratio was similar in the presence or absence of PGE₂ in both TRPV4^{+/+} mice and TRPV4^{-/-} mice (Fig. 4B, $n=10$ for TRPV4^{+/+} and $n=9$ for trpv4^{-/-} mice, $P > 0.05$ paired Student's *t*-test).

We have previously shown that hypotonic solution induces a TRPV4-mediated increase in normalized fluorescence ratio in rat DRG nociceptors in vitro (Alessandri-Haber et al., 2003). In the present study, because we were unable to detect activation of TRPV4 by hypertonicity, to validate our model, we challenged DRG neurons from TRPV4^{+/+} and TRPV4^{-/-} mice with a 30% hypotonic solution (212 mOsm). As expected, the increase in the normalized fluorescence ratio induced by hypotonicity was significantly higher in TRPV4^{+/+} mice (Fig. 4C, 1.19 ± 0.01 , $n=66$ in TRPV4^{+/+} mice versus

← expressed as the fluorescence ratio amplitude during the hypertonic challenge, normalized to the fluorescence ratio obtained in isotonic solution (312 mOsm). B, DRG neurons were challenged with a 30% hypertonic solution for 3 min, rinsed for 3 min with isotonic solution, perfused with an isotonic solution containing PGE₂ (2.5 μM) for 15 min and then challenged with 30% hypertonic solution containing PGE₂. The response of DRG neurons to hypertonicity was unaffected by PGE₂ in TRPV4^{+/+} ($n=10$, $P > 0.05$ paired Student's *t*-test) and TRPV4^{-/-} mice ($n=9$, $P > 0.05$ paired Student's *t*-test). C, DRG neurons (diameter ≤25 μm) were challenged with a 30% hypotonic for 3 min. In contrast to the hypertonic stimulation, the hypotonicity-induced increase in the normalized fluorescence ratio was significantly higher in TRPV4^{+/+} mice (1.19 ± 0.01 , $n=66$ in TRPV4^{+/+} mice versus 1.13 ± 0.01 , $n=68$ in TRPV4^{-/-} mice).

1.13 ± 0.01 , $n = 68$ in TRPV4^{-/-} mice, $P < 0.05$, unpaired Student's *t*-test).

We conclude that whereas TRPV4 is activated by both hypo and hypertonicity in vivo, in our conditions, only hypotonic stimulation activates TRPV4 in DRG neurons in vitro.

4. Discussion

We investigated the role of TRPV4 in nociceptive behaviors induced by two degrees of hypertonicity; a 2% NaCl solution (607 mOsm) which is in the concentration range used in experimental models of muscle and joint pain in humans (Arendt-Nielsen et al., 2000; Bennell et al., 2004; Graven-Nielsen et al., 1997; Jensen and Norup, 1992; Kellgren, 1937; Witonski and Wagrowska-Danielewicz, 1999) and a higher concentration hypertonic stimulation (10% NaCl, 3250 mOsm). The two degrees of hypertonicity induced a concentration-dependent pain-related behavior. However, we demonstrate that TRPV4 participates in the transduction of nociceptive behavior induced by moderate increases in tonicity (2% saline) but that for greater increases in tonicity (10% saline) other mechanisms are involved.

This is the first report that provides evidence for the involvement of TRPV4 in the sensory transduction of hypertonicity. Recently, Liedtke and co-workers (2003) demonstrated that transgenic expression of mammalian TRPV4 in *C. elegans* mutant for the TRPV gene *Osm-9* restores both the activation of ASH osmotic sensory neurons by hypertonicity and worms avoidance behavior for hypertonicity. Interestingly, their findings also suggested that the mammalian TRPV4 channel confers its properties to the transgenic worm behavior; while wild type *C. elegans* worms respond weakly to osmolarities below 0.25 M glycerol (1725 mOsm), *Osm-9 ASH:TRPV4* worms exhibited a lower threshold to hypertonicity (0.08 M, 542 mOsm) and reached the maximal response at 0.125 M (862 mOsm, Liedtke et al., 2003). Our results demonstrate that TRPV4 in mammals has a low threshold to hypertonicity and plays an important role in the transduction of nociception induced by mildly hypertonic stimuli. However, for more strongly hypertonic stimuli (10% saline), we suggest that TRPV4 response, as in *C. elegans* mutants, reaches saturation and other mechanisms that do not depend on TRPV4 underlie the hypertonicity-induced nociceptive behavior. Based on our results we cannot exclude that TRPV4 is desensitized at higher hypertonicity, tachyphylaxis upon repeatedly applied hypotonic solution has been previously demonstrated (Guler et al., 2002) or that perhaps, the mechanisms activated by higher degree of hypertonicity actively inhibit TRPV4.

We were unable to demonstrate TRPV4 activation by hypertonic stimuli in vitro. Previous studies in heterologous expression systems or primary culture also failed to elicit TRPV4 activation by hypertonicity (Guler et al., 2002;

Liedtke et al., 2000; Liedtke et al., 2003; Strotmann et al., 2000). In addition, recent reports in HEK-293 cells transfected with TRPV4 illustrate the limitation of in vitro investigation of polymodal receptors such as TRPV4; while Xu and co-workers (2003) demonstrated that activation of TRPV4 by hypotonic stress depended on the Src tyrosine kinase pathway, Vriens and co-workers (2004) demonstrated that activation of TRPV4 depended on phospholipase A₂ activity but not on Src tyrosine kinase. It is likely that multiple mechanisms or signaling pathways underlie the regulation and gating of the polymodal TRPV4 channel and consequently, the effects of a hypertonic stimulus on cellular mechanisms in the soma of DRG neurons, in vitro, will differ from its effects in vivo at the nerve terminals. Perhaps, in contrast to hypotonicity, the transduction of hypertonicity by TRPV4 may depend on factors or molecules that are absent or not activated in the DRG nociceptor cell body in vitro. Our methods may also have reduced our ability to detect TRPV4 in vitro. Given the ionic complexity of DRG neurons and the lack of specific blockers for TRPV4, we conservatively chose to perform our experiments in conditions that were less likely to activate other ions channels (*cf.* Material and methods) but in which the response of TRPV4 was probably not maximized. Interestingly, Garland and co-workers (1995) demonstrated that hyperosmolar solutions could directly stimulate tachykinin release from isolated cultured DRG neurons that were phenotypically C-fibers. In their model, DRG neurons were maintained for a week in culture in presence of cytosine arabinoside (ganglionic fibroblasts suppressor) and were exposed to hyperosmolar stimulus for 30 min at 37 °C. However, we were unable to demonstrate a TRPV4-dependent increase in intracellular calcium following a 3 min hypertonic stimulus, at 23 or 33 °C, in isolated neurons cultured for a week (data not shown). Garland and co-workers (1995) stimulated DRG neurons with a longer hypertonic stimulation, which could suggest that, unlike hypotonicity that directly activates TRPV4, a hypertonic stimulation may indirectly activate TRPV4 through a yet unknown pathway.

Also, we cannot exclude the possibility that, in vivo, hypertonicity activates TRPV4 indirectly via other cells; perhaps upon a hypertonic stimulus, a factor could be released by epithelial cells and subsequently activate TRPV4 in the nerve terminals. However, in vivo studies performed in other tissues (i.e. gut mucosa, jugular ganglion and medial fat of the knee) suggest that mild hypertonic stimulation evoked a consistent sensory response due to activation of C-fiber afferents (Bennell et al., 2004; Drewes et al., 2003; Witonski and Wagrowska-Danielewicz, 1999) and more specifically substance-P containing C-fibers (Baraniuk et al., 1999; Bennell et al., 2004; Kummer et al., 1992; Ricco et al., 1996; Witonski and Wagrowska-Danielewicz, 1999). In addition, in vitro experiments demonstrate that hypertonic saline can directly activate C-afferents (Garland et al., 1995; Pedersen et al., 1998).

Our data suggest that while both hypo- and hypertonic stimulation of rodent hind paw induced nociceptive behavior mediated by TRPV4, only hypotonicity induces a significant TRPV4-dependent increase in intracellular calcium that is proportional to the degree of hypotonicity in sensory neurons in vitro (Alessandri-Haber et al., 2003). This discrepancy may suggest a more complex gating mechanism of activation of TRPV4 by osmolarity than what would be expected of an osmosensor per se.

We demonstrate in this study that hypo- and hyper-tonic-induced nociceptive behavior mediated by TRPV4 partly depend on the Src tyrosine kinase phosphorylation pathway. Moreover, we have shown that PP₁ also reduced nociceptive behaviors induced by hypotonic stimuli to a similar extent as did treatment with TRPV4-antisense in a model of chemotherapy neuropathy induced by the anti-cancer drug Taxol[®] (Alessandri-Haber et al., 2004). We conclude that Src tyrosine kinase phosphorylation is one of the pathways participating to the activation of TRPV4, in sensitized nociceptors, independent of the type of sensitizing agent (i.e. Taxol[®] and PGE₂) or the type of ‘mechanical’ stimulus (i.e. hypertonicity, hypotonicity or pressure stimulation). Interestingly, the Src tyrosine kinase pathway was also shown to be essential for the activation of another member of the TRP family, TRPC3 (Vazquez et al., 2004).

Of note, while PGE₂ enhanced the TRPV4-dependent nociceptive behavior induced by mild hypertonicity, it had no effect on those induced by the higher degree of hypertonicity (10%). We suggest that PGE₂ failed to enhance the nociceptive behavior induced by 10% NaCl not because the hypertonicity-induced nociceptive behavior had reached a maximum (injection of a 15% NaCl solution caused a significantly greater nociceptive behavior compared to 10% NaCl) but more likely because PGE₂ was specifically enhancing TRPV4’s function. Consistent with this finding, we have shown previously that PGE₂ specifically enhanced TRPV4’s function upon an hypotonic stimulus; single cutaneous C-fibers from saphenous nerve recorded in vivo responded to hypotonic stimulation and this response is enhanced by PGE₂ (Alessandri-Haber et al., 2003). However when rats were treated with antisense for TRPV4, PGE₂ failed to enhance the response of the fibers to hypotonicity (unpublished observations). Taken together, these data suggest that the role of TRPV4 in hyper- or hypotonicity-induced nociceptive behavior is crucial in the setting of inflammation.

In conclusion, we suggest that, as for hypotonic or mechanical stimuli (Alessandri-Haber et al., 2004; Alessandri-Haber et al., 2003), the predominant role of TRPV4 in the transduction of hypertonicity is during inflammatory and neuropathic states and we suggest that TRPV4 is a transducer mediating nociceptive behavior induced by small variations in osmolarity (i.e. hypo- and hypertonic). While osmolarity-induced pain is well documented, this is the first report to our knowledge showing the potential role of a specific receptor, TRPV4.

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