Hypotonicity Induces TRPV4-Mediated Nociception in Rat

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Summary

We hypothesized that TRPV4, a member of the transient receptor family of ion channels, functions as a sensory transducer for osmotic stimulus-induced nociception. We found that, as expected for a transducer molecule, TRPV4 protein is transported in sensory nerve distally toward the peripheral nerve endings. In vivo single-fiber recordings in rat showed that hypotonic solution activated 54% of C-fibers, an effect enhanced by the hyperalgesic inflammatory mediator prostaglandin E_2. This osmotransduction causes nociception, since administration of a small osmotic stimulus into skin sensitized by PGE_2 produced pain-related behavior. Antisense-induced decrease in expression of TRPV4 confirmed that the channel is required for hypotonic stimulus-induced nociception. Thus, we conclude that TRPV4 can function as an osmo-transducer in primary afferent nociceptive nerve fibers. Because this action is enhanced by an inflammatory mediator, TRPV4 may be important in pathological states and may be an attractive pharmacological target for the development of novel analgesics.

Introduction

Members of the transient receptor potential (TRP) family, such as the vanilloid receptor TRPV1 (Caterina et al., 1997), the vanilloid receptor-like protein TRPV2 (Caterina et al., 1999), and the TRPM8 receptor (Peier et al., 2002), are expressed in mammalian sensory neurons and are thought to function as transducers for thermal and chemical stimuli. Recently, the expression of a new TRP family member TRPV4 (Liedtke et al., 2000; Strotmann et al., 2000; Wissenbach et al., 2000; Delany et al., 2001) was also detected by in situ hybridization in trigeminal ganglion neurons (Liedtke et al., 2000) and by Northern blot analysis in dorsal root ganglia (Guler et al., 2002).

TRPV4 is a nonselective cation channel, first described as an osmosensor, activated by a decrease in osmolarity of as little as 30 mOsm (Liedtke et al., 2000; Strotmann et al., 2000). The opening of the TRPV4 channel by a hypotonic stimulus does not appear to depend on direct mechanotransduction since it is not affected by membrane stretching (Strotmann et al., 2000). Rather, it seems to result from Src family tyrosine kinase-dependent phosphorylation (Xu et al., 2003). TRPV4 gating

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seems tightly regulated by intracellular and extracellular calcium concentration (Strotmann et al., 2003; Watanabe et al., 2003).

Recent studies suggest that, like TRPV1, TRPV4 may be a polymodal receptor, since TRPV4 can be activated by heat (Guler et al., 2002; Watanabe et al., 2002b), low pH, and citrate (Suzuki et al., 2003) in addition to osmotic stimulation. TRPV4 is also activated by phorbol ester derivative 4α-PDD, which does not activate protein kinase C, suggesting the existence of an endogenous lipid ligand (Watanabe et al., 2002a).

Although literature reports describe the presence of TRPV4 mRNA in sensory neurons (Liedtke et al., 2000; Guler et al., 2002), most studies on TRPV4 activation and function have been performed in transfected cells or cell lines expressing TRPV4 (Liedtke et al., 2000; Strotmann et al., 2000; Wissenbach et al., 2000; Nilius et al., 2001; Voets et al., 2002; Watanabe et al., 2002a, 2002b). Watanabe et al. (2002b) showed the existence of an endogenous current in aortic endothelial cells with functional hallmarks of TRPV4, and they proposed TRPV4 to be a temperature-sensing channel in native endothelium. Recently, Suzuki et al. (2003) showed impaired acid and pressure sensation in mice lacking TRPV4, whereas hot and touch sensation was unaffected. TRPV4 has been detected in sensory tissues such as cochlear hair cells, vibrissal Merkel cells, and sensory ganglia (Liedtke et al., 2000; Guler et al., 2002), but Suzuki et al. (2003) were the first to report a contribution of TRPV4 to sensory function.

In the present study, we addressed the physiological role of TRPV4 in rat primary afferent neurons and specifically asked whether TRPV4 is involved in the detection of osmolarity in nociceptors and whether osmotransduction results in nociception.

Results

Hypotonicity Depolarizes Nociceptive DRG Neurons

To investigate the effect of hypotonic challenge on the membrane potential of cultured dorsal root ganglion (DRG) neurons, current clamp recordings were performed. In isotonic solution (312 mOsm), DRG neurons did not spontaneously fire action potentials. The perfusion of a 30% hypotonic solution (219 mOsm) induced a depolarization of the membrane in some neurons without eliciting any action potentials (data not shown). We then investigated the effect of a hypotonic challenge when neurons were already stimulated by two different electrical protocols: a long depolarizing pulse (1 s/100 pA) or a combination of a hyperpolarizing (500 ms/100 pA) followed by a depolarizing (500 ms/100 pA) current pulse. Under those conditions, perfusion of a 30% hypotonic solution elicited firing of multiple action potentials in 32% of neurons (11 of 34; Figure 1A). As shown in Figure 1B, in responsive neurons, perfusion of hypotonic solution led to membrane depolarization (from 54 ± 4 to 40 ± 5 mV, n = 11, p < 0.05) while in nonresponsive
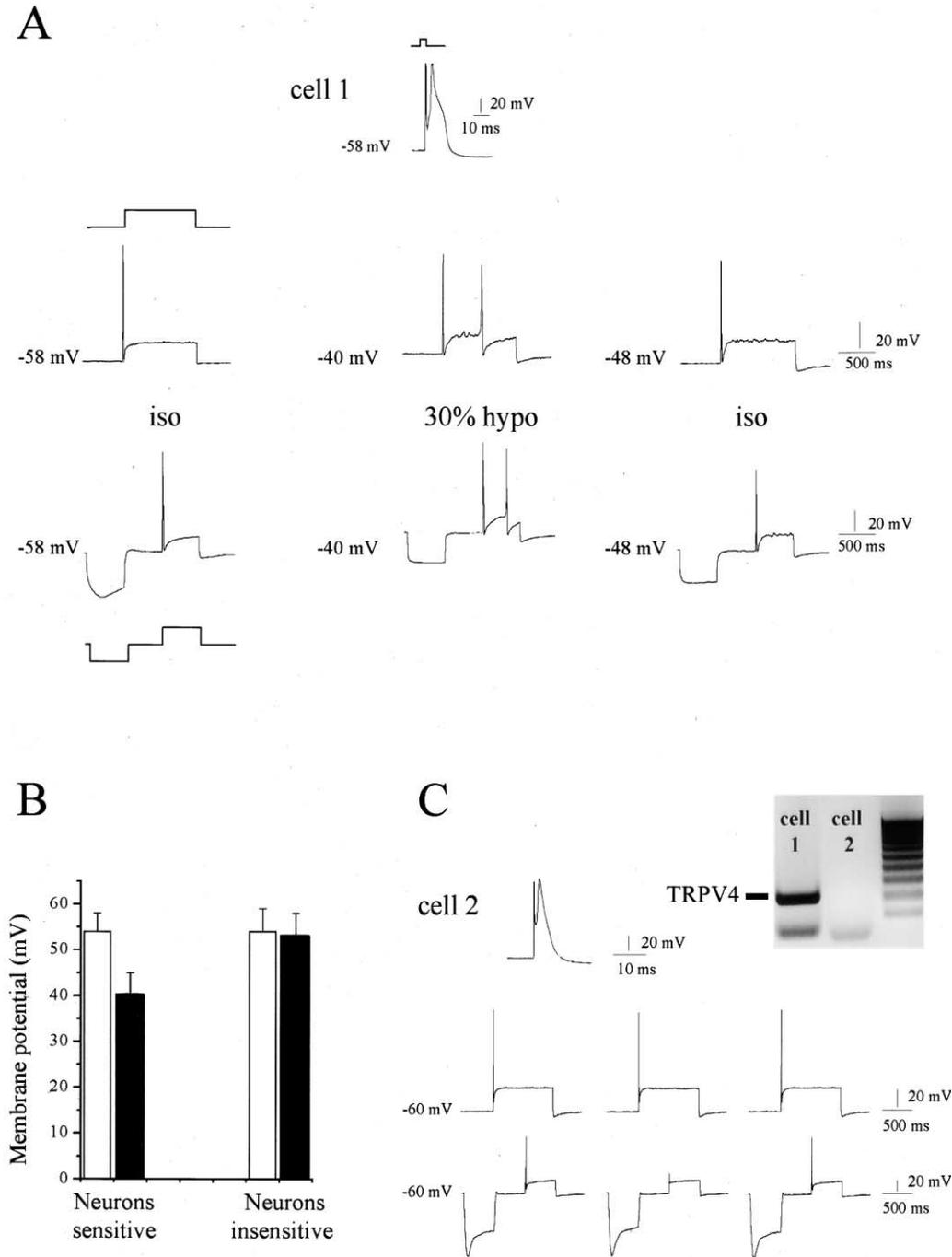
Figure 1. Hypotonic Solution Excites Neurons Expressing TRPV4

(A) Current clamp recording of DRG neurons. A short depolarizing (10 ms/100 pA) stimulus was used in isotonic solution (312 mOsm) to characterize the action potential waveform of each neuron. Two current stimulation protocols were applied to DRG neurons: a long depolarization (1 s/100 pA) and a depolarization (500 ms/100 pA) following a prehyperpolarizing current (500 ms/100 pA). The response to the two stimulation protocols were recorded under isotonic, 30% hypotonic (219 mOsm), and after return to isotonic solution.

(B) Mean ± SEM of the membrane potential value of sensitive (n = 11) or insensitive (n = 23) neurons in isotonic (white bars) and in 30% hypotonic (black bars) conditions.

(C) A cell responsive to the hypotonic challenge expressed TRPV4 mRNA (cell 1), whereas a nonresponsive cell (cell 2) did not.

neurons, the membrane potential remained unchanged (54 ± 5 to 53 ± 5 mV, n = 23). Cells that fired became quiescent a few seconds after replacing the bath with isotonic solution, and over a couple of minutes, the membrane potential returned to baseline.

In 31 neurons, immediately after electrophysiological recordings, the cytoplasm was harvested and single-cell RT-PCR analysis was performed. TRPV4 mRNA was expressed in the 11 neurons that fired action potentials in response to an electrical depolarization during a 30%

hypotonic stimulus, whereas the mRNA was undetectable in the 20 neurons that did not fire action potentials (Figure 1C).

Two types of DRG neurons have been described based on the shape of the falling phase of the action potential (Gorke and Pierau, 1980; Harper and Lawson, 1985). Nociceptive DRG neurons usually have a long-duration action potential with a shoulder on the falling phase of the action potential (Figure 1A), whereas non-nociceptive neurons have a shorter duration monophasic action potential. A short depolarizing (10 ms/100 pA) stimulus was used in isotonic solution to characterize the action potential waveform (Figure 1A) of each neuron recorded. In 9 of 11 neurons responsive to hypotonic stimuli, a shoulder occurred on the falling phase of the action potential, strongly suggesting that TRPV4 is involved in the response of nociceptors to hypotonicity.

Hypotonic Stimulus Increases Intracellular Ca^{2+} in Nociceptors

Calcium ions appear to play an integral role in TRPV4-mediated responses to hypotonicity (Liedtke et al., 2000; Strotmann et al., 2000; Wissenbach et al., 2000; Nilius et al., 2001). Using ratiometric calcium imaging, 35% of neurons tested (102/288) showed an increase in the intracellular concentration of free calcium ions ($[Ca^{2+}]_i$). As seen in Figure 2A, while 45% of small- to medium-sized neurons (diameter less than 40 μm) were responsive to a 30% hypotonic stimulus, only 17% of large neurons (diameter 41 to 80 μm) were responsive. This finding is consistent with responding cells being nociceptors, and we narrowed our subsequent studies of the hypotonicity-induced increase in $[Ca^{2+}]_i$ to neurons with diameters less than 40 μm.

To confirm that nociceptors respond to hypotonic stimulation, we determined whether the neurons that respond to hypotonic stimuli were capsaicin sensitive. Following hypotonic challenge, after $[Ca^{2+}]_i$ had recovered to control level, capsaicin (1 μM) was perfused for 10 s. As shown in Figure 2B, 77% (96/126) of DRG neurons, with a diameter less than 40 μm, responsive to hypotonicity were also capsaicin sensitive. Since our goal was to characterize TRPV4 function in nociceptors, the remainder of our studies focused on those capsaicin-sensitive neurons with diameters less than 40 μm.

Hypotonicity-Induced Increase in $[Ca^{2+}]_i$ Depends on Stimulus Intensity

In transfected cells, TRPV4 can be activated by a decrease in tonicity as small as 30 mOsm, and the hypotonicity-induced increase in $[Ca^{2+}]_i$ depends on the degree of hypotonicity (Liedtke et al., 2000; Strotmann et al., 2000). We investigated whether TRPV4 had similar properties in nociceptors. We challenged 86 neurons with a 15% hypotonic solution (265 mOsm) for 2 min followed by a 30% hypotonic solution (219 mOsm). Increasing the hypotonicity increased the number of cells responding to hypotonic stimulus, as well as the magnitude of the response in individual cells (Figure 2C). When challenged with the 15% hypotonic solution, 27 of 86 neurons showed a small increase in $[Ca^{2+}]_i$ (normalized fluorescence ratio, 1.09 ± 0.02, n = 11) and 34 neurons showed a larger increase (1.19 ± 0.03, n = 11) with the

30% hypotonic stimulus. Another set of nociceptors was tested with three increasing levels of hypotonicity (15%, 30%, and 40%), and in those cells the mean response to hypotonic stimulus also increased progressively with degree of hypotonicity (Figure 2D). Following calcium imaging, the cytoplasm of 13 cells responsive to an increasing level of hypotonic stimulus (15% and 30%) was harvested and RT-PCR was performed. As shown in Figure 2C (inset), TRPV4 expression was detectable in most (11/13) of the responding cells.

Increase in $[Ca^{2+}]_i$ Depends on Extracellular Calcium

Presence of calcium in the extracellular solution is essential for the hypotonic stimulus-induced increase in $[Ca^{2+}]_i$ via TRPV4 (Strotmann et al., 2000; Nilius et al., 2003). To confirm that an extracellular influx of Ca^{2+} ions is involved in the hypotonicity-induced response of nociceptors, we challenged nociceptors with a 30% hypotonic solution, perfused them with iso-osmotic Ca^{2+}-free solution for 3 min, and then challenged them with a 30% hypotonic Ca^{2+}-free solution. None of the hypotonic-responsive neurons showed an increase in $[Ca^{2+}]_i$ when challenged with a hypotonic Ca^{2+}-free solution (data not shown, n = 10). We then challenged 28 neurons by perfusing a 30% hypotonic solution (219 mOsm). When the $[Ca^{2+}]_i$ increase reached a plateau, the solution was switched to Ca^{2+}-free 30% hypotonic solution (218 mOsm). Switching to a Ca^{2+}-free hypotonic solution induced an immediate decrease in $[Ca^{2+}]_i$ (Figures 3A and 3B). Thus, the initiation, as well as the plateau, of the hypotonic stimulus-induced $[Ca^{2+}]_i$ increase in nociceptors depends on entry of extracellular Ca^{2+}, probably through TRPV4 channels.

Extracellular Ca^{2+} Influx Triggers Mobilization from Intracellular Calcium Stores

To test if intracellular calcium stores contribute to the hypotonicity-induced increase in $[Ca^{2+}]_i$, neurons were preincubated with different blockers for 20 min: SKF 96365 (20 μM, ryanodine receptor blocker), or SKF 96365 (20 μM) + thapsigargin (4 μM, Ca^{2+}-ATPase inhibitor), or SKF 96365 (20 μM) + CPA (20 μM, cyclopiazonic acid, endoplasmic reticulum calcium pumps blocker). After the preincubation, neurons were challenged with a hypotonic solution also containing the blockers. To measure the response, we averaged the value of the fluorescence ratio when the hypotonicity-induced increase in $[Ca^{2+}]_i$ reaches a plateau. In parallel to the cells incubated with the blockers, control cells were recorded the same day in the same conditions but without intracellular store blockers. The fluorescence ratio at the plateau was then normalized (fluorescence ratio during 30% hypotonic/fluorescence ratio during isotonic) and reported in Figure 3C. The intracellular store blockers did not prevent the hypotonic-induced $[Ca^{2+}]_i$ increase or diminish the number of cells responding to the stimulus, but the hypotonicity-induced increase in $[Ca^{2+}]_i$ consistently reached a lower fluorescence ratio in presence of blockers. The normalized fluorescence ratio in control cells was 1.17 ± 0.02 (n = 60) in hypotonic solution, whereas in neurons preincubated with blockers, the ratio was 1.07 ± 0.01 (n = 16) for hypotonic +
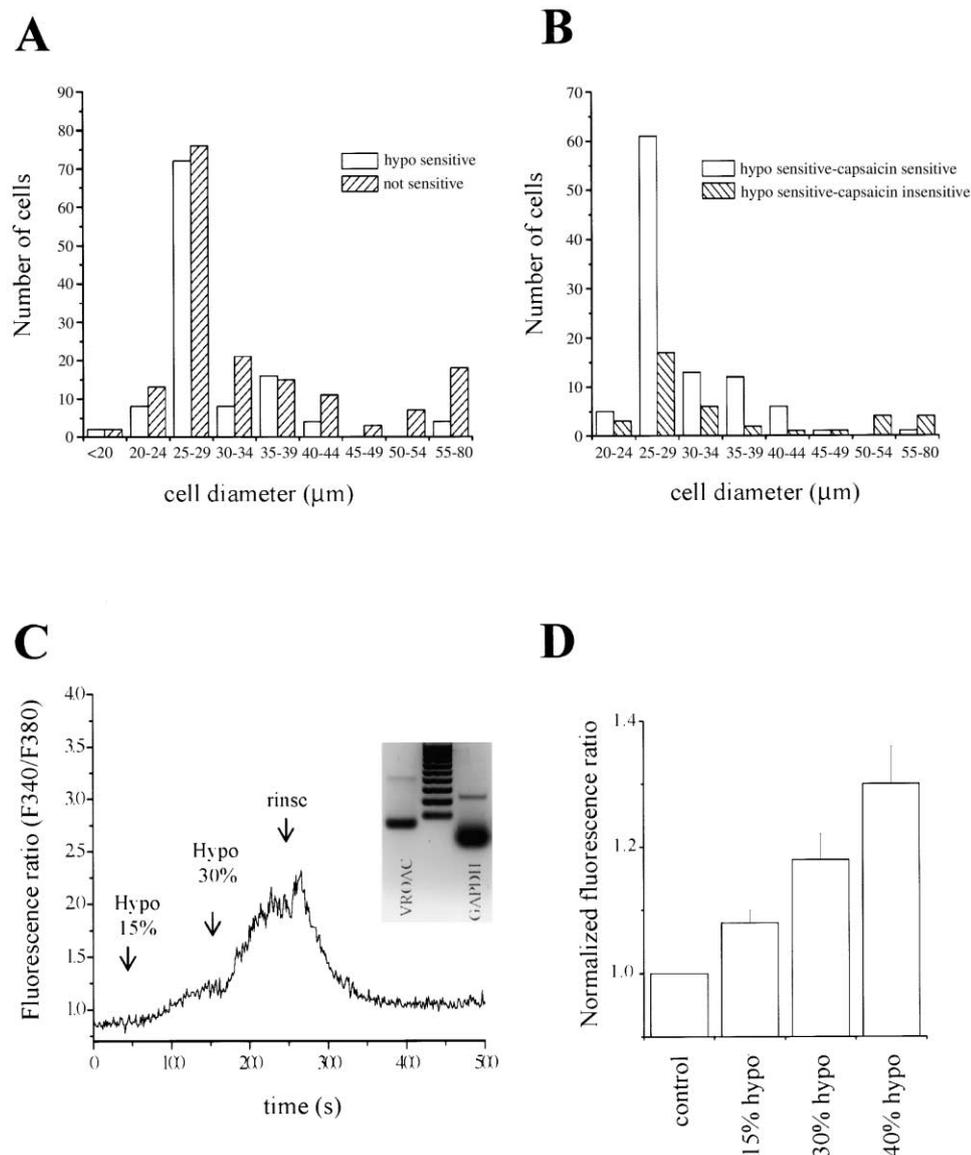
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Figure 2. Hypotonicity Increases [Ca^{2+}]$_i$

(A) Diameter distribution of neurons sensitive to a 30% hypotonic stimulus.

(B) Diameter distribution of hypotonic and capsaicin-responsive neurons.

(C) Example of a cell responding gradually to two degrees of hypotonicity (15% and 30%). This cell expressed mTRPV4, and the housekeeping gene GAPDH was chosen as a positive control.

(D) Peak increase in [Ca^{2+}]$_i$ (mean $\pm$ SEM) obtained for neurons stimulated by three degrees of hypotonicity (15%, 30%, and 40%). Only neurons responding to the three hypotonic concentrations were included in the graph (n = 10). Data are expressed as the fluorescence ratio amplitude of the effect of hypotonic stimulation, normalized to the fluorescence ratio obtained in isotonic solution (312 mOsm).

SKF 96365, 1.07 $\pm$ 0.02 (n = 8) for hypotonic + SKF 96365 + thapsigargin, and 1.07 $\pm$ 0.02 (n = 7) for hypotonic + SKF 96365 + CPA (Figure 3C). The decrease in the hypotonicity-induced increase in [Ca^{2+}]$_i$ was statistically significant in presence of SKF 96365 versus the control cells (p < 0.05, unpaired Student's t test), and adding thapsigargin or CPA did not induce further decrease. We conclude that intracellular stores were not essential for the response to hypotonicity in nociceptors but may play a role in amplification of the response.

Blockers of TRPV4 Attenuate the Hypotonic Stimulus-Induced [Ca^{2+}]$_i$ Increase in Nociceptors

Lanthanum (La^{3+}) and ruthenium red (RR) are known to reduce TRPV4-mediated response (Strotmann et al., 2000; Watanabe et al., 2002a). We tested each agent on stably transfected CHO-TRPV4 and CHO-MOCK cells in order to calibrate their effect on TRPV4-mediated [Ca^{2+}]$_i$ increases in nociceptors. The CHO-TRPV4 cells showed a 2.5-fold increase in the fluorescence ratio during a 30% hypotonic stimulus (mean $\pm$ SEM of the
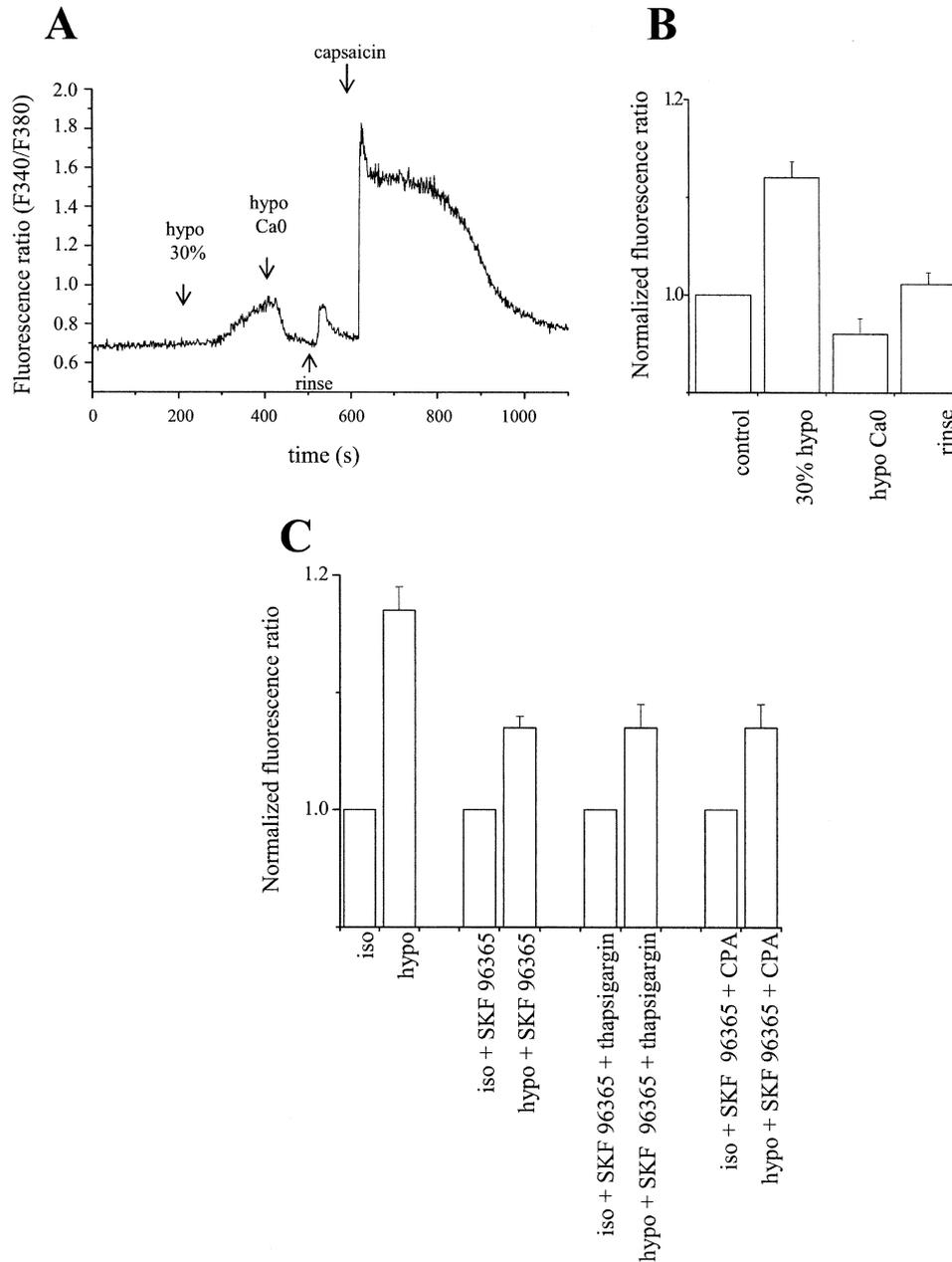
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Figure 3. Extracellular Calcium Contributes to the Hypotonicity-Induced Increase in [Ca^{2+}]$_i$

(A) Response of a DRG neuron as the 30% hypotonic solution (215 mOsm) was switched to a 30% hypotonic solution Ca^{2+}-free (hypoCa0) when the hypotonicity-induced increase in [Ca^{2+}]$_i$ reaches a plateau.

(B) Mean ± SEM of the mean of normalized fluorescence ratio when neurons are challenged with the protocol shown in (A), n = 28.

(C) Mean of normalized fluorescence ratio ± SEM for DRG stimulated by various extracellular solutions (n = 8 for each condition). Cells were preincubated for 20 min in isotonic solution (control cells) or isotonic containing either SKF 96365 (20 μM), or SKF 96365 (20 μM) + thapsigargin (4 μM), or SKF 96365 (20 μM) + CPA (20 μM). After preincubation, cells were challenged during 3 min with either 30% hypotonic, or 30% hypotonic + SKF 96365 (20 μM), or a 30% hypotonic solution + SKF96365 (20 μM) + thapsigargin (4 μM), or a 30% hypotonic solution + SKF 96365 (20 μM) + CPA (20 μM).

fluorescence ratio was 0.56 ± 0.01 in isotonic and 1.41 ± 0.05 in hypotonic solution; n = 40), while the control CHO-MOCK cells did not show any increase (the fluorescence ratio was 0.32 ± 0.01 in isotonic and 0.33 ± 0.01 in hypotonic solution; n = 55).

We observed tachyphylaxis upon consecutive hypotonic stimulation (data not shown). Therefore, to avoid

confounding desensitization and the blocking effect of the drugs tested, we challenged the cells with a 30% hypotonic stimulus; when the hypotonic-induced [Ca^{2+}]$_i$ increase reached a plateau, the perfusion was switched to a hypotonic solution containing either La^{3+} (1 μM) or RR (10 μM).

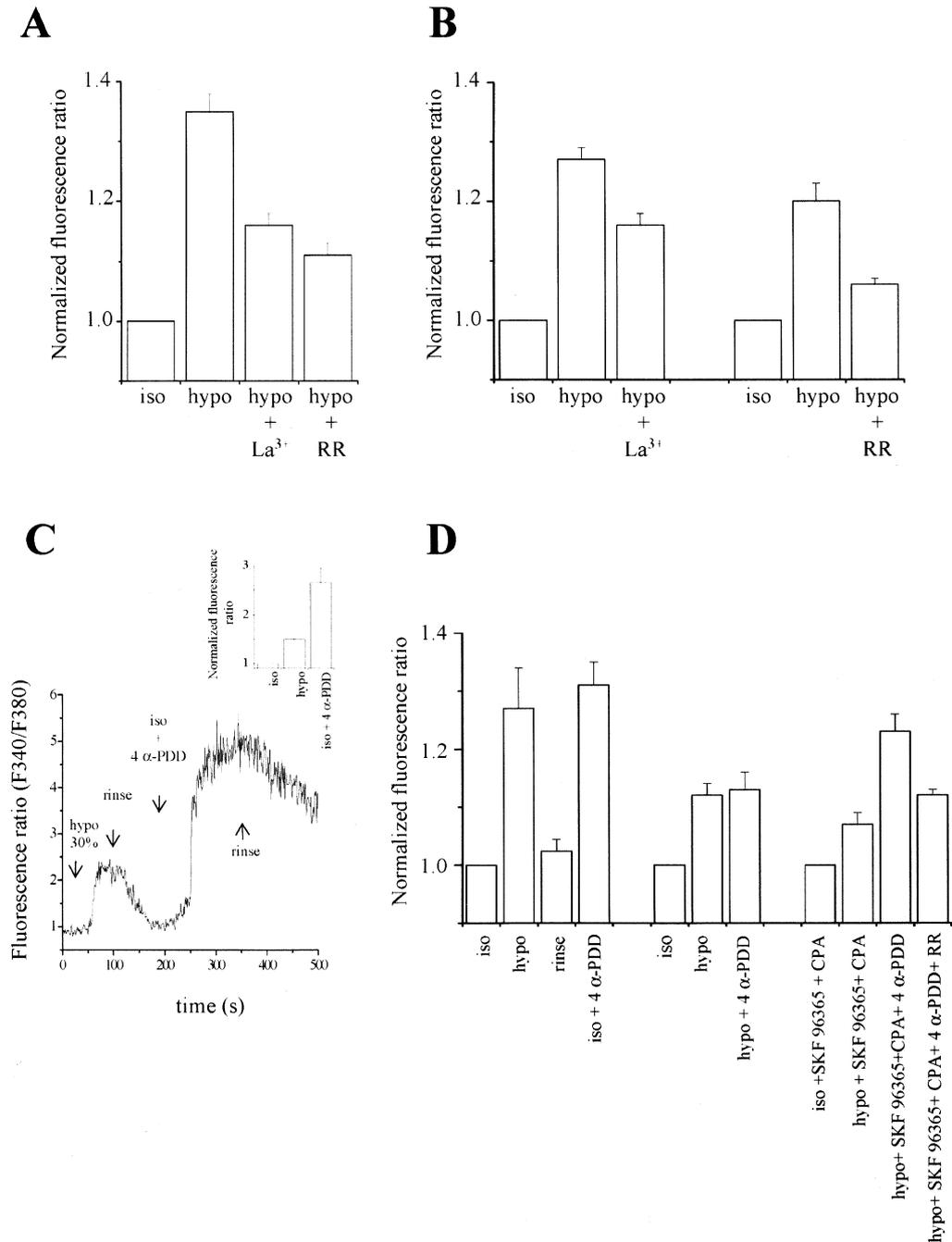Perfusion of La^{3+} (1 μM) on CHO-TRPV4 cells induced

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Figure 4. Effect of TRPV4 Blockers and 4α-PDD on the Hypotonic-Induced [Ca^{2+}]$_i$ Increase

(A) Effect of the perfusion of La^{3+} (1 μM) and RR (10 μM) on hypotonic-induced [Ca^{2+}]$_i$ increase in CHO-TRPV4 cells (n = 10).

(B) La^{3+} blocked 45% (n = 5) and RR blocked 70% (n = 4) of the hypotonic-induced increase in [Ca^{2+}]$_i$ in nociceptors.

(C) Example of a CHO-TRPV4 response to an isotonic solution + 4α-PDD (10 μM). Insert: mean ± SEM of the increase of the fluorescence ratio when CHO-TRPV4 cells are challenged with a 30% hypotonic solution or an isotonic solution + 4α-PDD (10 μM), n = 10.

(D) Mean ± SEM of the normalized fluorescence ratio for different extracellular solutions. For the left part of the graph n = 9, for the middle part n = 24, and for the right part n = 6. For all graphs, results are expressed as the mean ± SEM of the normalized fluorescence ratio.

a 45% reduction of the hypotonic-induced increase in [Ca^{2+}]$_i$ (Figure 4A, p < 0.05, paired Student's t test; n = 10). RR (10 μM) blocked 60% of the hypotonic-induced increase of [Ca^{2+}]$_i$ in CHO-TRPV4 cells (Figure 4A, p < 0.05, paired Student's t test, n = 9). As seen in Figure 4B, the hypotonic stimulus-induced increase in [Ca^{2+}]$_i$

in nociceptors was inhibited by 45% during the perfusion of La^{3+} (Figure 4B, p < 0.05, paired Student's t test, n = 5) and by 70% during the perfusion of RR (Figure 4B, p < 0.05, paired Student's t test, n = 4). These results support the involvement of TRPV4 in nociceptor osmosensitivity.

4α-PDD Causes an Increase in [Ca²⁺]ᵢ in Nociceptors

The phorbol ester 4α-PDD (4α-phorbol 12,13-didecanoate), an analog of phorbol 12-myristate 13-acetate (PMA) that does not activate protein kinase C, is reported to be an *agonist* of TRPV4 (Watanabe et al., 2002a). As shown previously, perfusion of 4α-PDD (10 μM) on CHO-TRPV4 cells was more efficacious than a 30% hypotonic stimulus (Watanabe et al., 2002a). As shown in Figure 4C, the mean fluorescence ratio during hypotonic stimulation was 1.35 ± 0.03 versus 2.7 ± 0.3 during the perfusion of isotonic solution containing 4α-PDD (10 μM, $p < 0.05$, paired Student's t test, $n = 10$). DRG neurons (diameters <40 μm) were challenged with a 30% hypotonic solution for 3 min, rinsed for 3 min, and then challenged with an isotonic solution containing 4α-PDD (10 μM) for 3 min. Of 77 neurons tested, 33 (42%) were responsive to hypotonic stimulation, with 51% of those neurons (17/33) sensitive to both the 30% hypotonic and 4α-PDD stimuli. We suggest that neurons responsive to both stimuli are likely to express TRPV4. We focused further on the nociceptors (capsaicin-responsive neurons with a diameter <40 μm) that responded to both stimuli. As shown in Figure 4D, unlike in transfected cells, the magnitude of the 4α-PDD-induced increase in [Ca²⁺]ᵢ in nociceptors was not significantly different from that induced by the hypotonic stimulus ($p > 0.05$, paired Student's t test, $n = 9$).

Watanabe et al. (2002a) showed in TRPV4-transfected cells that 4α-PDD potentiated the hypotonic-induced increase in [Ca²⁺]ᵢ. To investigate this potential effect on nociceptors, neurons were first perfused with a 30% hypotonic solution and when the [Ca²⁺]ᵢ increase reached a plateau, the perfusion was switched to hypotonic + 4α-PDD (10 μM). As shown in Figure 4D, addition of 4α-PDD did not significantly modify the hypotonic-induced increase in the [Ca²⁺]ᵢ ($p > 0.05$, paired Student's t test, $n = 24$).

In TRPV4-transfected cells, increasing the level of intracellular calcium was reported to inhibit TRPV4 activation by 4α-PDD (Watanabe et al., 2002a). To investigate if it was also the case in nociceptors, blockers of intracellular stores were used. Nociceptors were preincubated for 20 min with CPA (20 μM) + SKF 96365 (20 μM), challenged with a 30% hypotonic + CPA + SKF 96365, and when the [Ca²⁺]ᵢ increase reached a plateau, 4α-PDD (10 μM) was added. As shown in Figure 4D, in the presence of intracellular blockers, addition of 4α-PDD to a hypotonic stimulus induced a significant increase in the fluorescence ratio (normalized fluorescence ratio was 1.07 ± 0.02 before 4α-PDD and 1.23 ± 0.05 after 4α-PDD, $p < 0.05$, paired Student's t test, $n = 6$). The 4α-PDD-induced potentiation was blocked by subsequent application of RR (10 μM, Figure 4D). This result suggests that release of calcium from intracellular stores increases [Ca²⁺]ᵢ to a level that inhibits a subsequent activation of TRPV4 by another stimulus. Thus, activation of TRPV4 in nociceptors appears to be tightly regulated by [Ca²⁺]ᵢ.

TRPV4 Protein in Peripheral Nerve

To detect TRPV4 protein expression, we generated an affinity-purified polyclonal anti-TRPV4 antibody using a
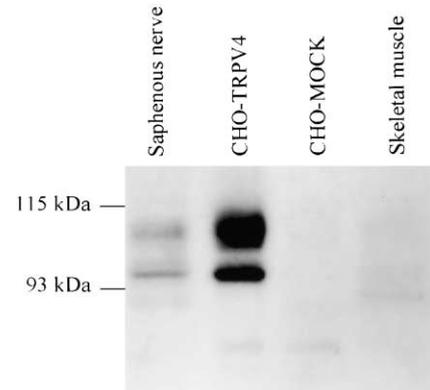

Figure 5. TRPV4 Protein Is Detected in Saphenous Nerves

Western blot analysis shows two protein bands at the predicted molecular weight (98 kDa to 107 kDa) for TRPV4 protein in the saphenous nerve and CHO-TRPV4 cells. CHO-MOCK cells and skeletal muscle did not present any band. To obtain similar levels of the TRPV4 protein expression on the gel, only 4 μg/lane of total protein were loaded for CHO-TRPV4 and CHO-MOCK, whereas 16 μg/lane were loaded for the saphenous nerve and skeletal muscle.

synthetic peptide corresponding to the C terminus of rat TRPV4, the same epitope used by others (Delany et al., 2001; Guler et al., 2002). The specificity of the antibody was confirmed by testing it in non-TRPV4-expressing tissues and cells, including skeletal muscle and CHO-MOCK cells, using Western blot, immunochemistry, and flow cytometry (data not shown). Using this antibody, TRPV4 protein could not be detected in DRG (Guler et al., 2002). Therefore, we performed further experiments in which the saphenous nerve was ligated to dam transport of proteins from the cell body toward the periphery (Parada et al., 2003). A 5 mm section of saphenous nerve, proximal to the ligature, was removed 3 days after ligation, and Western blot analysis was performed on the tissue to visualize TRPV4 protein. As shown in Figure 5, TRPV4 protein was detected in the saphenous nerve segment. As reported previously in TRPV4-transfected cells (Xu et al., 2003), a doublet between 98 and 107 kDa was detected, suggesting varying degrees of glycosylation of the TRPV4 protein. These findings demonstrate that TRPV4 is present in sensory nerve fibers, presumably being transported toward the peripheral nerve endings.

Primary Afferent Nerve Fibers Respond to Hypotonic Stimulus and This Response Is Enhanced by PGE₂

Because TRPV4 protein appears to be transported to peripheral nerve endings, we tested if we could elicit TRPV4-mediated osmodetection in saphenous nerve C-fibers. Thirteen single cutaneous C-fibers from the saphenous nerve were recorded in vivo. None of the fibers showed spontaneous activity. Intradermal injection of hypotonic solution (5 μl deionized water) elicited activity in 7 of the 13 fibers (Figure 6A, upper trace). The effect of hypotonic stimulus was brief, and after approximately 25 s the fibers stopped firing. When PGE₂ (2.5 μl, 100 ng) was intradermally injected prior to a second hypotonic challenge, the number of action po-
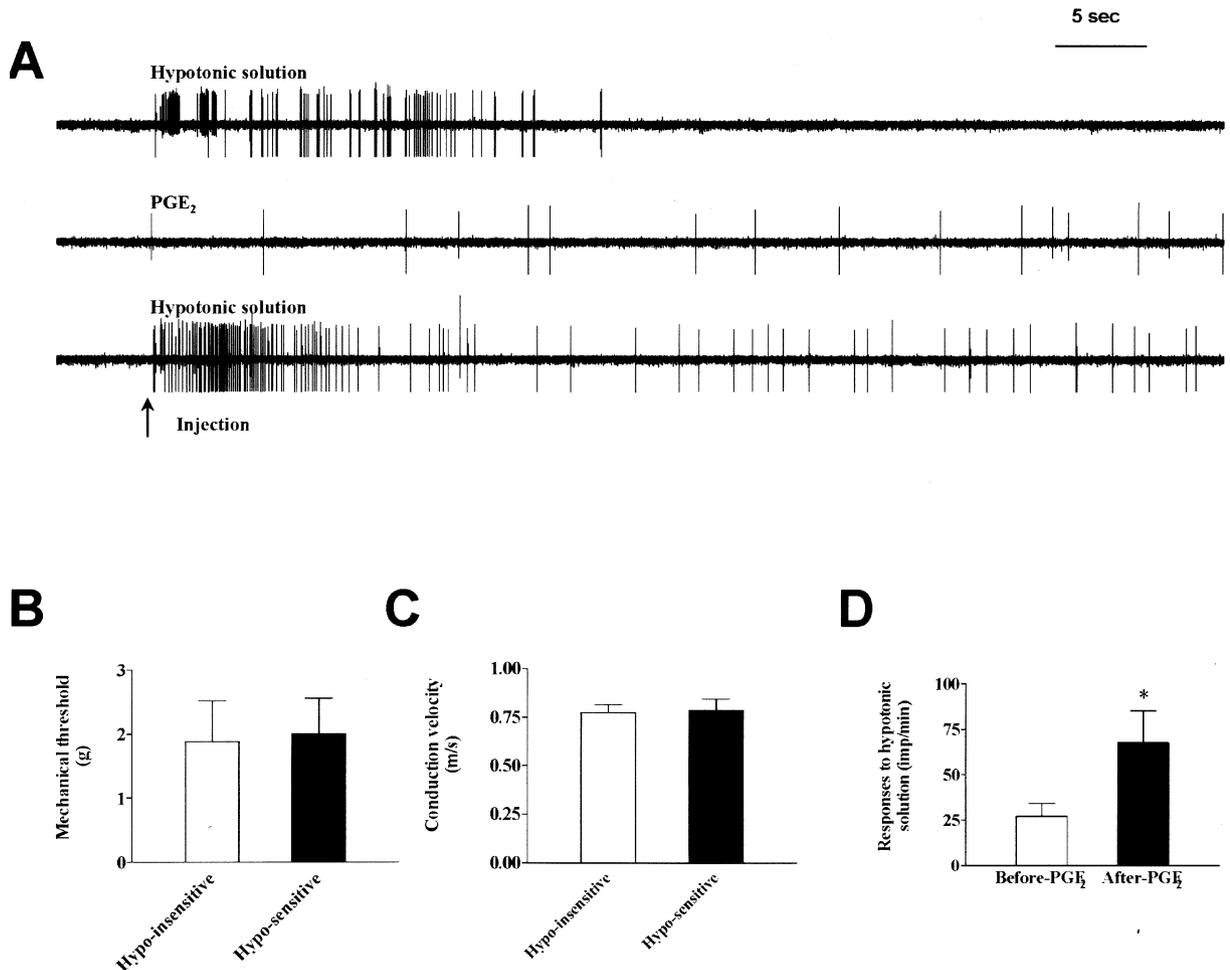
Figure 6. Primary Afferent Response to Hypotonic Stimulation Is Enhanced by PGE$_2$

(A) Recording of cutaneous C-fibers from the saphenous nerve. The fiber did not have spontaneous activity. When challenged with an intradermal injection of hypotonic solution (5 μl deionized water), the fiber started firing a burst of action potentials (upper trace). Subsequent intradermal injection of PGE$_2$ (2.5 μl, 100 ng) produced a low level of spontaneous activity (middle trace). A second hypotonic challenge following the injection of PGE$_2$ elicited a train of action potentials (bottom trace).

(B) Mean value ± SEM of mechanical thresholds from hypotonic responsive (n = 7) and nonresponsive C-fibers (n = 6).

(C) Mean value ± SEM of conduction velocities of the two groups of C-fibers.

(D) Mean value ± SEM of the response of hypotonic sensitive C-fibers before and after intradermal injection of PGE$_2$ (2.5 μl, 100 ng), paired Student's t test, p < 0.05.

tentials in C-fibers evoked by hypotonic solution was significantly increased (Figure 6A, bottom trace). Neither the mechanical threshold nor the conduction velocity of the hypotonicity-responsive C-fibers was significantly different from those of the nonresponsive C-fibers (Figures 6B and 6C). As shown in Figures 6A and 6D, intradermal injection of PGE$_2$ resulted in a significant increase in the mean number of action potentials evoked by the hypotonic stimulus (27 ± 7 before PGE$_2$ versus 68 ± 17 after PGE$_2$, p < 0.05, paired Student's t test, n = 7). Furthermore, 2 of the hypotonicity-insensitive C-fibers became sensitive after PGE$_2$. Of note, only 3 of the 7 hypotonicity-sensitive C-fibers showed a decrease in their mechanical thresholds after application of PGE$_2$. Therefore, PGE$_2$-induced sensitization of the response to hypotonic stimuli can occur independent of changes in mechanical threshold.

Hypotonic Stimulation of Sensitized Nociceptors Is Noxious and Is Mediated by TRPV4

We next investigated whether hypotonic stimulus could induce nociceptive behavior in rat via the activation of TRPV4 channels. Injection of 10 μl hypotonic (deionized water) or isotonic solution (0.9% NaCl solution) in the hindpaw did not induce nociceptive behavior (Figure 7A). When PGE$_2$ was administered prior to the injection of 10 μl hypotonic solution, significant flinching behavior was observed during the 5 min period following the hypotonicity challenge (Figure 7A, mean ± SEM, 8.8 ± 1.2 after PGE$_2$ versus 0.2 ± 0.2 before PGE$_2$, p < 0.05, paired Student's t test, n = 6). However, when PGE$_2$ was administered 30 min prior to the injection of 10 μl of isotonic solution, again no flinching behavior was elicited (Figure 7A, mean ± SEM, 1.2 ± 0.7 after PGE$_2$ versus 0.0 ± 0 before PGE$_2$, p > 0.05, paired Student's
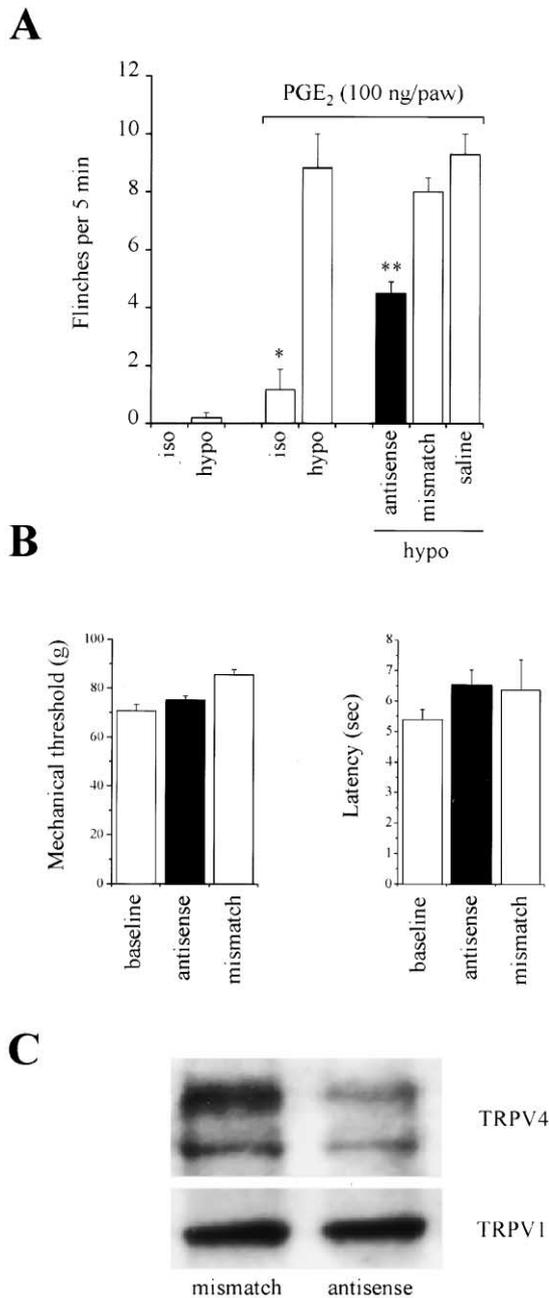
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Figure 7. Hypotonicity Is Noxious after Exposure to PGE$_2$

(A) Mean ± SEM of flinches induced by injection of an isotonic or hypotonic solution in the rat hindpaw. Injection of 10 μl of isotonic or hypotonic solution did not induce pain-related behavior. After PGE$_2$ sensitization of the hindpaws, injection of 10 μl hypotonic solution elicits a significant number of flinches, whereas isotonic solution did not. Rats treated with TRPV4 antisense showed a 50% decrease in the number of hypotonic-induced flinches during the first 5 min following the hypotonic injection (mean ± SEM, 8.8 ± 1.2 in control versus 4.5 ± 0.4 for the antisense-treated, p < 0.05 unpaired Student's t test, n = 10). Rats treated with either saline or mismatch sequence did not show a significant difference in the number of flinches compared to control (p > 0.05, unpaired Student's t test and Tukey's multiple comparison test). Of note, there were no significant differences in the PGE$_2$-induced mechanical hyperalgesia among the three groups of rats (data not shown, p > 0.05, Tukey's multiple comparison test).

(B) There was no significant difference in the mechanical thresholds

t test, n = 6). Hindpaws in groups injected with isotonic or hypotonic solution were similarly sensitized to mechanical stimulation by intradermal injection of PGE$_2$ (data not shown, p > 0.05, unpaired Student's t test, n = 6).

To assess whether TRPV4 could be responsible for the nociceptive behavior (flinches) elicited after sensitization of the C-fibers with PGE$_2$, three groups of rats were prepared. Rats were treated by spinal intrathecal administration of either TRPV4 antisense, mismatch sequence, or saline (0.9% NaCl) solution for 3 days. There were no statistically significant differences in the PGE$_2$-induced mechanical hyperalgesia among the three groups of rats (data not shown, p > 0.05, Tukey's multiple comparison test). As shown in Figure 7A, rats treated with TRPV4 antisense showed a 50% decrease in the number of flinches during the first 5 min following the hypotonic injection (mean ± SEM, 8.8 ± 1.2 in control versus 4.5 ± 0.4 for the antisense-treated, p < 0.05, unpaired Student's t test, n = 10). Rats treated with either saline or mismatch sequence did not show a significant difference in the number of flinches induced by hypotonicity when compared to control (mean ± SEM, 9.3 ± 0.7, n = 8 for saline-treated; 8 ± 0.5, n = 13 for mismatch-treated versus 8.8 ± 1.2, n = 6 for control, p > 0.05, Tukey's multiple comparison test).

Recently, intact hot and touch sensation was observed in TRPV4 knockout mice (Suzuki et al., 2003). Thus, we tested change in mechanical threshold and latency of the hindpaw to withdraw from a hot stimulus. As shown in Figure 7B, there was no significant difference in the mechanical or thermal thresholds between the antisense- and mismatch-treated rats. The thresholds in both groups of treated animals did not significantly differ from the control baseline measured (p > 0.05, Tukey's multiple comparison test, n = 6 for each condition).

Western blot analysis showed a specific 41% ± 17% diminution of TRPV4 protein expression level (p = 0.017, unpaired Student's t test, n = 8 for antisense-treated and n = 9 for mismatch-treated) in the saphenous nerve in antisense-treated rats (Figure 7B). VR1 and tubulin antibodies were used as a test of antisense specificity and as a loading control, respectively. The level of expression of TRPV1 protein remained unchanged under the TRPV4 antisense and mismatch treatment (p > 0.05, unpaired Student's t test, n = 6 for both groups). These data suggest that TRPV4 is involved in the transduction of the hypotonic-induced nociceptive behavior elicited after sensitization with PGE$_2$.

(left panel, p > 0.05, Tukey's multiple comparison test, n = 6) or in the latencies to withdraw the hindpaw from a heat source (right panel, p > 0.05, Tukey's multiple comparison test, n = 6) between antisense- or mismatch-treated rats.

(C) Selective knockdown of TRPV4 protein expression in saphenous nerve. Top: the antisense-treated rats showed a 41% ± 17% decrease in the level of TRPV4 protein compared to the mismatch-treated rats (p = 0.017, unpaired Student's t test, n = 8 for antisense and n = 9 for mismatch-treated rats). The amount of protein in both lanes was confirmed to be comparable (16.8 μg/lane) by reprobing with an α-tubulin antibody. Bottom: TRPV1 protein level remained unchanged in the TRPV4 antisense-treated rats (p > 0.05, unpaired Student's t test, n = 6).

A

B

C

Figure 8. N-Terminal TRPV4 Splice Variant

(A) Nucleotide sequence alignment of the region corresponding to the amplified RT-PCR product with the primer pair TRPV4-1. The missing nucleotide sequence from the 201 bp product is highlighted. Bold lines indicate the localization of the TRPV4-F2 primer.

(B) Schematic representation of the localization of the two pairs of TRPV4 primers on the rat TRPV4 gene. From a single cell responsive to both a hypotonic stimulus and 4α-PDD, a first round of PCR was performed using the forward primer of the primer pair TRPV4-1 (TRPV4-F1) and the reverse primer of pair TRPV4-2 (TRPV4-R2). The second round of PCR was done using either the TRPV4-1 or TRPV4-2 primer pairs. The TRPV4-1 primer pair yields the 201 bp and the 428 bp products. Enzymatic restriction was performed to verify the 428 bp PCR product; the enzymes Afl II (yielding 173 + 255 bp products) and Sac I (yielding 334 + 94 bp products) were used. To verify the 298 bp PCR product amplified with the TRPV4-2 primer pair, the enzymes Hinc II (yielding 136 bp + 162 bp products) and Hinf I (yielding 187 bp + 111 bp products) were used. This neuron expresses both the potential splice variant and TRPV4 mRNAs.

(C) Autoradiograph of a rat multiple tissue premade membrane probed with a RNA probe corresponding to the 428 bp PCR product. Heart, kidney, and liver show two bands of 2.8 and 3.2 Kb. Lung only shows the 2.8 Kb band, whereas spleen only shows the 3.2 Kb band. Skeletal muscle and testis did not show any band.

Detection of a N-Terminal Splice Variant of TRPV4

As shown in Figure 1C, in neurons responsive to the 30% hypotonic stimulus, we amplified by single-cell RT-PCR a 201 bp band with the TRPV4-1 primer pair. Enzymatic restriction performed with two different enzymes (Sac I and Sma I) ruled out the possibility of a nonspecific PCR product (data not shown). The two PCR products (the expected 428 bp and the 201 bp) were subcloned into a pCR-II TOPO vector (Invitrogen Life Technologies) and DNA sequencing was performed. Sequence analysis showed that the 201 bp product lacked a 227 bp portion of DNA (Figure 8A) corresponding to exon 4 and the first 61 nucleotides of exon 5 (Figure 8B). A new pair of primers, identified as TRPV4-2, targeted to the splice variant sequence by localization of the forward primer (denoted as TRPV4-F2) on the splice locus, was designed (Figure 8B). DRG neurons were challenged with a 30% hypotonic solution and subsequently with an isotonic solution containing 4α-PDD (10 μM). Cytoplasm from neurons responsive to both stimuli in calcium imaging was harvested and single-cell RT-PCR was performed. Enzymatic restriction analysis was performed for all the cells expressing TRPV4 mRNA. The enzymes Afl II, yielding 173 bp and 255 bp products, and Sac I, yielding 334 bp and 94 bp products, were used to verify that the 428 bp band amplified with the TRPV4-1 primer pair was specific. The enzymes Hinc II, yielding 136 bp and 162 bp products, and Hinf I, yielding 187 bp and 111 bp products, were used to verify that the 298 bp band amplified with the TRPV4-2 primer pair was also specific (Figure 8B). For three different responsive neurons, the PCR product amplified with TRPV4-2 primers was subcloned into a pCR-II TOPO vector. Sequence analysis confirmed 100% homology with the expected "spliced" sequence. Our preliminary results in single-cell RT-PCR did not show a differential distribution of the two TRPV4 mRNAs 15 of 16 neurons that were responsive to hypotonic stimulation expressed both mRNAs.

To confirm our findings, Northern blot experiments were performed using the 428 bp PCR product sequence as a probe. In RNA from DRG, we observed a 3.2 Kb band and another faint band at approximately 2.8 Kb (data not shown). Therefore, in order to confirm the existence of a second band, we performed Northern blot experiments with our probe on tissue known to express higher levels of TRPV4. In heart, kidney, and liver, we detected two transcripts of 2.8 and 3.2 Kb, as previously shown (Strotmann et al., 2000); lung only shows the 2.8 Kb band, whereas spleen only shows the 3.2 Kb band (Figure 8C). The Northern blot analysis and single-cell RT-PCR experiments support the existence of a TRPV4 splice variant.

Discussion

Exposure to hypotonic extracellular solution induces cell swelling due to the osmotic entry of water into the cell. Because swelling induces membrane stretch, previous studies testing the effect of hypotonic stimuli on sensory cells (Harada et al., 1994) focused on the potential implication of stretch-activated channels in the response. TRPV4 is not activated by stretch but more

likely by tyrosine kinase phosphorylation (Strotmann et al., 2000; Xu et al., 2003). It was shown recently that a subpopulation of capsaicin-sensitive trigeminal neurons responded with variable magnitude to hypotonicity and a nonselective cation channel was proposed to be involved (Viana et al., 2001). TRPV4 mRNA is present in trigeminal neurons (Liedtke et al., 2000) and is also detected in dorsal root ganglia (Guler et al., 2002). Recent reports characterize TRPV4 as a polymodal receptor activated by hypotonicity, heat, acid, and possibly by an unidentified intracellular ligand (Nilius et al., 2003; Suzuki et al., 2003). Based on these findings, we investigated the potential role of TRPV4 in hypo-osmotic detection in nociceptive DRG neurons and the involvement of osmodetection in peripheral pain mechanisms.

Mechanism for the Hypotonicity-Induced Calcium Increase in Nociceptors

We show the existence of a mechanism for transduction of hypotonic stimulation in a subpopulation of small- to medium-diameter nociceptors. The mechanisms involved in the hypotonicity-induced increase in $[Ca^{2+}]_i$ can be grouped into two categories (Lang et al., 1998): activation of calcium influx and/or release of calcium from intracellular stores. Our data with Ca^{2+}-free solutions show that entry of extracellular calcium is essential for the hypotonic-induced increase in $[Ca^{2+}]_i$ in DRG nociceptors. However, the hypotonicity-induced $[Ca^{2+}]_i$ increase is smaller in the presence of calcium store blockers. Most of the blocking effects seemed due to SKF 96365, which was shown to block members of the TRPC subfamily of TRP channels (Okada et al., 1998; Zhu et al., 1998; Inoue et al., 2001). Thus, we cannot exclude the possibility of a direct interaction of that drug with TRPV4 channels; we suggest that the TRPV4-mediated extracellular influx of Ca^{2+} may trigger the release of Ca^{2+} from intracellular stores as it was shown for TRPV1 and capsaicin (Eun et al., 2001).

Supporting that idea, we also show that, in nociceptors, 4α-PDD is unable to potentiate the hypotonicity-induced $[Ca^{2+}]_i$ increase unless the intracellular stores were blocked and depleted. TRPV4 appears to be tightly regulated by intracellular and extracellular calcium level in nociceptors, which is in agreement with other reports (Strotmann et al., 2003; Watanabe et al., 2003). This finding is significant because in some neuropathies, such as diabetic peripheral neuropathy, alteration in neuronal mechanisms are linked to changes in intracellular signaling systems that can influence $[Ca^{2+}]_i$ levels such as mitochondrial dysfunction (Srinivasan et al., 2000). It has also been shown that impaired mitochondrial function leads to opening of TRP channels (Agam et al., 2000).

A TRPV4 N-Terminal Splice Variant?

Our results in single-cell RT-PCR and Northern blot analysis are preliminary but support the existence of a TRPV4 N-terminal splice variant in rat. Although the entire sequence of the detected splice variant has not yet been determined, we can infer that the splice occurs in the region of amino acids 184–260. The splice variant likely lacks one ankyrin repeat, which would potentially influence its interaction with the cytoskeleton or the interaction between its potential constitutive subunits.

Other investigators have shown that the amino-terminal domain of TRPV4 is key for its response to tonicity and heat (Liedtke et al., 2000; Watanabe et al., 2002b) and is the site of tonicity-dependent tyrosine phosphorylation (Xu et al., 2003). Further experiments are needed to determine the full sequence of this potential splice variant and to distinguish its function.

Hypotonicity Excites Nociceptive Neurons

Our finding brings a new perspective to the physiological role of TRPV4. Our electrophysiological experiments in vitro and in vivo are consistent, extracellular hypotonicity excites a subset of nociceptors, and we provide evidence implicating TRPV4 channels in this pathway.

Prostanoids such as PGE_2 are generated by most cells in response to mechanical, thermal, or chemical injury or during inflammation. PGE_2 acts on EP prostanoid receptors, present on sensory neurons, and stimulates adenylyl cyclase and/or PLC activity. The most prominent cellular signaling pathway in which TRP channels play a role are mediated via phospholipase C (PLC) (Minke and Cook, 2002). These findings led us to the hypothesis that PGE_2 may enhance the effect of a hypotonic stimulus on nociceptors. Thus, hypotonicity became noxious to the animal when nociceptive fibers were sensitized by PGE_2.

Our results with the TRPV4 antisense-treated rats are in agreement with the findings that in TRPV4 knockout mice, mechanical and thermal thresholds are unchanged (Suzuki et al., 2003). However, they state that hypotonicity is the less sensitive stimulus for TRPV4. Suzuki et al. drew their conclusions after testing hypotonicity on transfected cells that do not always express TRPV4 at the membrane but rather in intracellular localization (Guler et al., 2002), and they did not test any pain-related behavior after a hypotonic challenge. We show here that hypotonicity excites TRPV4 in vitro and in vivo and that in sensitized states (such as inflammation, for example), hypotonicity becomes even a more efficient stimulus and is able to be noxious in the animal. That finding is of primary interest because it is the first demonstration that TRPV4, or any osmosensitive channel, is involved in pain transduction in sensory neurons.

Elucidation of a relationship between osmolarity and neuronal excitability is of considerable clinical impact. Disturbances of osmolarity or cell volume is common to different types of disease such as diabetes (Puliyel and Bhambhani, 2003), alcoholism (Vamvakas et al., 1998), schizophrenia (Canuso and Goldman, 1999), and asthma (Fujimura et al., 1998). Studies on the gastrointestinal tract show that hypotonic solutions increase the activity of sensory vagal neurons (Mei and Garnier, 1986; Kobashi and Adachi, 1996). In the periphery, water is known to be an irritant and there are many examples of diseases or painful conditions such as "tropical immersion foot" in which patient's feet are painful following a prolonged immersion in water (Tsai and Maibach, 1999). Nociception-related neuropeptides including substance P are suspected to mediate the intense itching in the aquagenic pruritus, which develops after contact with water in the absence of cutaneous signs (Lotti et al., 1994); and in aquadynia, a water-related pain syndrome, VIP release may be involved (Misery et al., 2003). It has

also been shown that hyperosmolarity elicits substance P release from rat C-fibers (Garland et al., 1995). An extended literature is available on hyperosmolarity and neuropathies probably because of the importance of diabetes usually characterized by hyperglycemia. However, the existence of hypoglycemic neuropathies (Mohseni, 2001) and the fact that in diabetic ketoacidosis some patients have hypo-osmolar plasma suggest the possible importance of hypo-osmolarity in neuropathies. In addition, the fact that DRG lack a blood-nerve barrier supports the idea of their possible role as sensor of our body's internal osmotic milieu.

Little information is yet available concerning the consequences of fluctuations of extracellular osmolarity in the periphery during inflammatory states or in neuropathies. Our results suggest that TRPV4 is a transducer of osmotic stimulation and, more specifically, a transducer involved in inflammatory pain. These findings represent critical developments in the delineation of TRPV4 function, but tantalizing questions remain. Is TRPV4 involved in inflammatory states following injuries? Does dysfunction of TRPV4 play a role in neuropathies? What is the relevance of the splice variant? Answers to these questions may indicate that TRPV4 is a potential pharmacological target for the development of novel analgesics in inflammatory pain.

Experimental Procedures

DRG Cell Culture

L2-L6 dorsal root ganglion (DRG) were harvested from adult rat and dissociated as described previously (Reichling and Levine, 1997). Cells were maintained in Dulbecco's modified Eagle's (MEM) medium supplemented with 50–100 ng/ml NGF, 100 units/ml penicillin/streptomycin, MEM vitamins, and 10% heat-inactivated Fetal Calf Serum. The components of the culture media were purchased from Invitrogen Life Technologies. Dissociated cells were plated on cover slips treated with poly-DL-ornithine (0.1 mg/ml; Sigma) and laminin (5 μg/ml; Invitrogen Life Technologies) and incubated at 37°C in 96.5% air, 3.5% CO_2.

Recording Solutions for Electrophysiology or Calcium Imaging

Extracellular solutions (Table 1) were made fresh, and pH (7.38) and osmolarity were measured before each experiment.

All drugs or blockers tested were diluted daily from stock solutions in dimethylsulfoxide (DMSO). Capsaicin, SKF 96365, thapsigargin, cyclopiazonic acid (CPA), lanthanum, and 4α-PDD were purchased from Sigma and ruthenium red from Research Biochemicals International (RBI).

In Vitro Electrophysiological Recordings

DRG neurons were recorded in whole-cell current-clamp mode between 24 and 72 hr after dissociation. Experiments were carried out at 20°C–23°C with an Axoclamp 200 A patch amplifier (Axon Instruments). Patch clamp electrodes had a resistance of 3–4 MΩ and were filled with the following intracellular solution: 140 mM KCl, 2 mM NaCl, 3 mM $MgCl_2$, 10 mM HEPES, and 5 mM EGTA (pH 7.3) with KOH (290 mOsm). The volume of the chamber was 200 μl and the extracellular solutions were perfused with a ValveBank 8 perfusion system (Automate Scientific) at a flow rate of 1–2 ml/min.

Data were sampled (10 kHz) and filtered (2 kHz) with pClamp 8 (Axon Instruments) and analyzed with Origin software (OriginLab).

Single-Cell RT-PCR

Single-cell RT-PCR experiments were conducted as described previously (Alessandri-Haber et al., 2002). Briefly, at the end of recordings, cytoplasm was harvested and reverse transcription was immediately performed using Superscript II (Invitrogen). Two suc-

Table 1. Composition of Extracellular Solutions

	Osmolarity (mOsm)	NaCl (mM)	KCl (mM)	KH$_2$PO$_4$ (mM)	MgSO$_4$ (mM)	MgCl$_2$ (mM)	CaCl$_2$ (mM)	NaHCO$_3$ (mM)	D-mannitol (mM)	HEPES (mM)
Isotonic	312	124	5	1.2	0	1.3	2.4	26	0	0
Isotonic Ca0	314	76	5	1.2	1.3	2.4	0	26	100	0
Hypotonic 30%	219	74	5	1.2	0	1.3	2.4	26	0	0
Hypotonic 30% Ca0	212	76	5	1.2	1.3	2.4	0	26	0	0
Isotonic[a]	312	88	5	0	0	1	2.4	0	110	10
Hypotonic 15%[a]	260	88	5	0	0	1	2.4	0	50	10
Hypotonic 30%[a]	219	88	5	0	0	1	2.4	0	10	10
Hypotonic 30% Ca0[a]	219	88	5	0	0	3.4	0	0	10	10

Data is of solutions used for electrophysiology experiments
[a] Solutions used for calcium imaging experiments

cessive PCR amplifications (initial Taq activation step at 94°C for 15 min, then 94°C for 30 s, 60°C for 1 min, 72°C for 1 min, and 72°C for 7 min) were performed for 28 and 38 cycles using Hotstar™ Taq (Qiagen). Two different primers pairs were used for TRPV4 (accession number AF263521): TRPV4-1 (nucleotides 523–950) and TRPV4-2 (nucleotides 631–928). The housekeeping gene GAPDH (accession number AF 106860, nucleotides 320–556) was used as a positive control. Gels were analyzed with AlphaEaseFC™ software (Alpha Innotech Corporation).

In Vivo Single-Fiber Electrophysiology
Rats were anesthetized with sodium pentobarbital (initially 50 mg/kg, i.p.), with additional doses given throughout the experiment to maintain areflexia. Single cutaneous C-fibers from the saphenous nerve were recorded as described previously (Chen and Levine, 1999). Fibers with conduction velocities less than 2 m/s were classified as C-fibers (Willis, 1985). The action potential corresponding to the C-fiber whose receptive field had been identified was determined by the latency delay technique (Handwerker et al., 1991). Mechanical threshold was determined with calibrated von Frey hairs (Ainsworth) and defined as the lowest force that elicited two or more spikes within 1 s, in at least 6 of 10 trials. Hypotonic solution (5.0 μl, deionized water) and PGE$_2$ (2.5 μl, 100 ng, Sigma) were intradermally injected 1 mm from the center of a fiber's mechanical receptive field.

Measurement of Intracellular Ca^{2+} Concentration
DRG neurons were recorded between 24 and 72 hr after dissociation. Neurons were loaded with 5 μM fura-2 acetoxymethyl ester (fura-2 AM; Molecular Probes) for 20 min in isotonic solution (312 mOsm). The volume of the chamber was 300 μl and the perfusion was carried out at 20°C–23°C 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 hydrolysis of the fura-2 AM.

The measurement of [Ca^{2+}]$_i$ was performed by digital ratiometric imaging of fura-2 AM with an intensified charge-coupled device camera ICCD (Stanford Photonics, Inc). 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 and acquired with METAFLUOR® imaging system software (Universal Imaging Corporation). The vehicle for the fura-2 AM, DMSO, was tested separately on DRG neurons to verify that it did not induce any response.

In absence of specific blockers of TRPV4 and given the ionic complexity of DRG neurons, experiments were performed in conditions that were less likely to activate other ions channels: low NaCl concentration, HEPES buffer, room temperature, and where only the amount of mannitol was modified to change the solutions osmolarity but in which the activation of TRPV4 may not be maximal.

CHO-K1 Transfection and Cell Culture
Negative control cell line (CHO-MOCK) was generated by transfecting CHO-K1 cells (ATCC, Manassas) with 10 μg pcDNA3.1 plasmid DNA (Invitrogen Life Technologies) per 10 cm dish using a standard calcium phosphate/HEPES protocol (Sambrook et al., 1989). Cells

were split 48 hr after transfection and selected with 800 μg/ml geneticin (Invitrogen); discrete colonies were isolated with cloning rings.

CHO-K1 cells stably transfected with TRPV4 (CHO-TRPV4) were kindly provided by Drs. J. Friedman and W. Liedtke (Rockefeller University, NY). CHO-MOCK and CHO-TRPV4 cell lines were cultured in F12 Ham's medium supplemented with 10% Fetal Bovine Serum, 200 mM glutamine, 100 mcg/ml streptomycin/100 units/ml penicillin G, and 250 μg/ml geneticin (Invitrogen). Cells were plated on cover slips 48 hr before calcium imaging experiments.

Northern Blot
Northern blot experiments were performed using a rat Multiple Tissue Northern blot premade membrane (rat MTN blot, Clontech). The 428 bp PCR product was used as the probe after ligation to the T7 polymerase promoter (Lig'nScribe, Ambion) and transcription with the RNA StripEZ kit (Ambion). The antisense RNA probe was then purified with the RNaid kit (Bio 101, Inc). In parallel, 5 μg of rat poly(A)$^+$ RNA was extracted from DRG, separated by electrophoresis on a 1% denaturant agarose gel, and transferred to a Bright Star membrane. Hybridization was carried out using the Northern Max kit (Ambion).

Antibody Generation and Purification
A synthetic peptide (CDGHQQGYAPKWRAEDAPL) corresponding to the C terminus of the rat TRPV4 was synthesized and conjugated to keyhole limpet hemocyanin (KLH) via its N terminus by Invitrogen Life Technologies. Rabbits were immunized with the KLH-conjugated peptide (240 μg peptide for the first immunization and 120 μg thereafter) according to standard procedures at Lampire Biologicals. The resulting immune serum was purified using affinity chromatography made from the synthetic peptide and Sulfolink coupling gel (Pierce Biotechnology). Purification was performed by incubating 1 ml of antiserum/ml of affinity resin for 2 hr at room temperature with constant rotation followed by washing with 20 bed volumes of binding buffer (100 mM sodium borate and 0.02% sodium azide [pH 8.0]). The purified antibody was eluted with 10 bed volumes of ImmunoPure Gentle Ag/Ab elution buffer (Pierce Biotechnology).

Western Blot
Saphenous nerves from anesthetized rats (178–198 g male Sprague-Dawley rats) were ligated with silk surgical suture (4-0) 1 cm above the knee-level bifurcation of the nerves. A 5 mm section of saphenous nerve proximal to the ligation was removed 3 days after the surgery. Protein membrane preparation and Western blot analysis were performed as previously described (Parada et al., 2003). The membrane was probed with affinity-purified anti-TRPV4 antibody (1:750) followed by incubation with horseradish peroxidase-conjugated (HRP) goat anti-rabbit IgG (1:10,000 Santa Cruz biotechnology). Membranes were incubated with enhanced chemiluminescence reagents (Pierce Biotechnology) and exposed to films. To determine the specificity of the TRPV4 antisense probe and to normalize the loaded samples, affinity-purified goat polyclonal TRPV1 antibody (1:500, Santa Cruz biotechnology) and α-tubulin (1:2000, Sigma) were used to reprobe the membrane. Respectively, a HRP-conjugated rabbit anti-goat IgG (1:4000, Santa Cruz biotechnology)

or HRP-conjugated goat anti-mouse IgG (1:10,000, Sigma) were used as the secondary antibodies.

Oligodeoxynucleotides
Oligodeoxynucleotides (ODNs) were synthesized by Invitrogen Life Technologies. The antisense ODNs sequence 5′-CATCACCAGGAT CTGCCATACTG-3′ was directed against a unique region of the rat TRPV4 channel. The mismatch ODN sequence was designed by mismatching 7 bases (denoted by bold face) of the TRPV4 antisense sequence, 5′-**CAACAGGAGGTT**CAGGC**AAA**CTG-3′. ODNs were reconstituted in nuclease-free 0.9% NaCl (10 μg/μl, LabChem) and were intrathecally administered once daily at a dose of 40 μg for 3 days.

Nociceptive Behavior
Behavioral experiments were performed on 220–250 g male Sprague-Dawley rats (Charles River). Experimental protocols were approved by the UCSF Committee on Animal Research and conformed to NIH guidelines for use of animals in research.
Intrathecal ODNs Treatment
Intrathecal ODNs treatment was as described in Papir-Kricheli et al. (1987). Rats were anesthetized with 2.5% isoflurane inhalation anesthetic (97.5% O_2), a 30-gauge needle was inserted into the subarachnoid space on the midline between the L4 and L5 vertebrae, and 20 μl of ODNs was injected at 1 μl/s, using a 3/10 cc BD micro-syringe.
Flinching Test
Flinching tests were as described in Taylor et al. (1995). Flinch tests were run on the 4th day following initiation of the ODNs treatment (10 hr postinjection). Animals were placed in an open acrylic observation chamber for 30 min to accommodate them to their surroundings. Rats were removed and restrained while 10 μl hypotonic (deionized water) or isotonic solution (0.9% NaCl solution) was intradermally administered into the dorsum of the hindpaw using a 30-gauge needle connected with a 100 μl Hamilton syringe by polyethylene tubing. Rats were observed immediately after the injection for a 5 min period.
Mechanical Thresholds
Mechanical thresholds in rat were evaluated by the Randall-Sellito paw-withdrawal flexion-reflex test (Aley et al., 2001) with an Ugo-Basile analgesymeter (Stoelting). Baseline mechanical thresholds were determined before and after PGE_2 treatment as the mean of two measurements performed 5 min apart. PGE_2 was administered intradermally 30 min prior to the hypotonic or isotonic test in the same site of injection. PGE_2-induced hypersensitivity was quantified as the change in mechanical nociceptive threshold.
Thermal Thresholds
Thermal thresholds in rat were determined by the Hargreave test (Hargreaves et al., 1988). Light from a halogen bulb lamp was delivered to the plantar surface of the rat hindpaws through the base of the glass panel. Time taken for the rat to lift its hindpaw was noted. The intensity of the radiant heat was selected in order to reach a basal latency of 5–7 s. A cut-off time of 15 s was used to avoid tissue damage. Each latency value is the mean of three applications of the radiant heat stimulus, separated by 5 min intervals.

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