A Transient Receptor Potential Vanilloid 4-Dependent Mechanism of Hyperalgesia Is Engaged by Concerted Action of Inflammatory Mediators

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The transient receptor potential vanilloid 4 (TRPV4) is a primary afferent transducer that plays a crucial role in neuropathic hyperalgesia for osmotic and mechanical stimuli, as well as in inflammatory mediator-induced hyperalgesia for osmotic stimuli. In view of the clinical importance of mechanical hyperalgesia in inflammatory states, the present study investigated the role of TRPV4 in mechanical hyperalgesia induced by inflammatory mediators and the second-messenger pathways involved. Intradermal injection of either the inflammatory carrageenan or a soup of inflammatory mediators enhanced the nocifensive paw-withdrawal reflex elicited by hypotonic or mechanical stimuli in rat. Spinal administration of TRPV4 antisense oligodeoxynucleotide blocked the enhancement without altering baseline nociceptive threshold. Similarly, in *TRPV4*^{−/−} knock-out mice, inflammatory soup failed to induce any significant mechanical or osmotic hyperalgesia. *In vitro* investigation showed that inflammatory mediators engage the TRPV4-mediated mechanism of sensitization by direct action on dissociated primary afferent neurons. Additional behavioral observations suggested that multiple mediators are necessary to achieve sufficient activation of the cAMP pathway to engage the TRPV4-dependent mechanism of hyperalgesia. In addition, direct activation of protein kinase A or protein kinase C ϵ , two pathways that mediate inflammation-induced mechanical hyperalgesia, also induced hyperalgesia for both hypotonic and mechanical stimuli that was decreased by TRPV4 antisense and absent in *TRPV4*^{−/−} mice. We conclude that TRPV4 plays a crucial role in the mechanical hyperalgesia that is generated by the concerted action of inflammatory mediators present in inflamed tissues.

Key words: pain; mechanotransduction; silent nociceptor; dorsal root ganglion; sensory nerve; inflammatory mediators

Introduction

Transient receptor potential vanilloid 4 (TRPV4), a member of the transient receptor potential family of ligand-gated ion channels (Liedtke et al., 2000; Strotmann et al., 2000), functions as a sensory transducer for osmotic-induced nociception (Alessandri-Haber et al., 2003). In addition, TRPV4 participates in sensation of intense noxious mechanical stimuli in mice (Liedtke and Friedman, 2003; Suzuki et al., 2003a) and in mechanical hyperalgesia in a model of small-fiber painful peripheral neuropathy in rat (Alessandri-Haber et al., 2004). Although TRPV4 plays an important role in the transduction of noxious mechanical stimuli, it apparently does not contribute to detection of threshold-level pain sensation (Liedtke and Friedman, 2003; Suzuki et al., 2003a).

In view of its role in sensation of intense and neuropathic pain, but not in threshold-level pain, we hypothesized that TRPV4-dependent hyperalgesia might be a mechanism specific to patho-

logical pain states, perhaps including inflammatory pain. Inflammatory pain is of particular interest because it is the predominant cause of clinical pain conditions. Confirmation of our hypothesis could have important implications for the treatment of pain because it would identify TRPV4 as a target for the development of a new class of analgesic drugs to treat common pain conditions.

To test our hypothesis, we investigated whether TRPV4 contributes to inflammatory hyperalgesia induced by either the inflammatory carrageenan or by a soup of inflammatory mediators [bradykinin (BK), substance P (SP), prostaglandin E₂ (PGE₂), serotonin (5-HT), and histamine] (Steen et al., 1996; Kress et al., 1997; Vyklicky et al., 1998; Levine and Reichling, 1999; Linhart et al., 2003). Nociceptive behavioral tests were performed in both rats that received repeated intrathecal injections of antisense oligodeoxynucleotide (ODN) for TRPV4 to transiently downregulate the level of expression of TRPV4 protein and in C57BL/6 mice lacking functional TRPV4 gene. We also performed *in vitro* experiments to test whether TRPV4 function is altered by direct action of the inflammatory mediators on primary afferent neurons. To elucidate the cellular mechanisms that cause TRPV4-dependent mechanisms to be activated in primary afferents in inflamed tissue, we examined interactions between TRPV4 and two key intracellular second-messenger pathways that mediate inflammatory hyperalgesia, protein kinase A (PKA) and protein kinase C ϵ (PKC ϵ) (Levine and Reichling, 1999).

Received Aug. 11, 2005; revised Feb. 6, 2006; accepted Feb. 28, 2006.

This work was supported by National Institutes of Health Grant NS053880. We thank Drs. Wolfgang Liedtke and Jeffrey Friedman for kindly providing the TRPV4 knock-out mice.

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DOI:10.1523/JNEUROSCI.5385-05.2006

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Materials and Methods

Animals. Experiments were performed on 180–200 g adult male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) and on C57BL/6 mice lacking functional TRPV4 gene (*TRPV4*^{−/−} mice) (Liedtke and Friedman, 2003) and TRPV4 wild-type littermates (*TRPV4*^{+/+}). Experimental protocols were approved by the University of California, San Francisco Committee on Animal Research and conformed to National Institutes of Health guidelines for the use of animals in research.

Drugs. PGE₂, histamine, 5-HT, SP, 8-bromoadenosine 3', 5'-cAMP (8-Br-cAMP), Rp-adenosine 3', 5'-cyclic monophosphothioate (Rp-cAMPS), H89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline-sulfonamide dihydrochloride), and carrageenan were purchased from Sigma (St. Louis, MO). BK was purchased from ICN Biomedicals (Aurora, OH), cAMP-dependent protein kinase A catalytic subunit (PKACS) was purchased from New England Biolabs (Beverly, MA), PKCε translocation inhibitor (PKCεI) was purchased from Calbiochem (La Jolla, CA), and HDAPIGYD-protein kinase C activator (pseudoreceptor for activated RACK, *ψ*ε-RACK) was synthesized by SynPep (Dublin, CA).

For behavioral experiments, stock solutions of SP, BK, histamine, 5-HT, 8-Br-cAMP, PKACS, *ψ*ε-RACK, and carrageenan were made in 0.9% NaCl. Stock solution of PGE₂ was made in 10% ethanol. For all drugs used in behavioral experiments, final experimental concentrations were made in 0.9% NaCl on the day of the experiment (final concentration of ethanol <1%).

For calcium imaging experiments, stock solutions of H89, PKCεI, BK, and PGE₂ were made in 10% dimethylsulfoxide (DMSO), and Rp-cAMPS, histamine, SP, and 5-HT were made in distilled H₂O. Final experimental concentrations were made in the isotonic or hypotonic solution (Hypo) on the day of the experiment. Of note, the final concentration of DMSO is 100 μM, and the osmolality of the solution is not measurably different from the hypotonic solution alone.

Mechanical threshold in rat. Mechanical nociceptive thresholds were evaluated by the Randall–Sellito paw-withdrawal test with an Ugo Basile algometer (Stoelting, Chicago, IL) as described previously (Aley and Levine, 2001; Aley et al., 2001). Baseline mechanical thresholds were recorded as the mean of three measurements (at 10 min intervals) before the injection of hypotonic solution or pharmacological reagents. To induce hyperalgesia, a solution of either carrageenan (1% w/v, 5 μl), inflammatory soup, PGE₂ (100 ng/2.5 μl), PGE₂ and 5-HT (1 μg each/2.5 μl), 8-Br-cAMP (1 μg/2.5 μl), PKA catalytic subunit (10 U/2.5 μl), or *ψ*ε-RACK (1 μg/2.5 μl) were injected intradermally into the dorsum of the rat hindpaw, 30 min before behavioral testing. For statistical analysis, each paw was considered to be an independent observation.

For the 8-Br-cAMP dose–response experiment, rats were treated with either TRPV4 antisense or mismatch ODN for 3 d, and, on day 4 after initiation of ODN treatment, mechanical nociceptive thresholds were evaluated before and 15 min after intradermal injection of increasing doses of 8-Br-cAMP (0.1, 0.3, 1, 3, 10, 100, 300, and 1000 ng). The two ODN-treated and control groups were tested in parallel on the same day.

Flinch test in rat. As described previously (Zheng and Chen, 2001; Zhang et al., 2003; Alessandri-Haber et al., 2004; Houck et al., 2004), rats were acclimated in a transparent observation chamber for 30 min. Rats were then restrained while 10 μl of hypotonic (deionized water, 17 mOsm) or isotonic (0.9% NaCl, 283 mOsm) solution was administered intradermally into the dorsum of the hindpaw via a 30 gauge needle connected to a 100 μl syringe by polyethylene tubing. Immediately after the injection, rats were observed in the chamber for a 5 min period. For experiments involving TRPV4 antisense ODN treatment, the flinch tests were performed on day 4 after initiation of ODN treatment (12 h after the last ODN injection). PGE₂ (100 ng/2.5 μl), carrageenan (1%, w/v, 5 μl), the inflammatory soup, 8-Br-cAMP, PKACS, or *ψ*ε-RACK were injected intradermally 45 min before administration of the hypotonic stimulus, at the same site of injection.

Mechanical nociception in mice. Mice were acclimated 15–20 min in a transparent box with a metal mesh floor. A calibrated von Frey hair monofilament (Stoelting) was applied through the mesh floor to the plantar skin of the hindpaw. Mechanical threshold was measured as the

total number of paw withdrawals in response to a series of five applications (at 3 min intervals) of a 0.17 mN von Frey hair. The response to hyperalgesia-inducing agents was measured 30 min after intraplantar injection of agent.

Dorsal root ganglion cell culture. L2–L6 dorsal root ganglia (DRGs) were harvested from *TRPV4*^{+/+} and *TRPV4*^{−/−} mice and dissociated and cultured as described previously (Alessandri-Haber et al., 2003). Briefly, dissociated cells were plated on coverslips treated with poly-DL-ornithine (0.1 mg/ml; Sigma) and laminin (5 μg/ml; Invitrogen, Carlsbad, CA), incubated at 37°C in 96.5% air, 3.5% CO₂. Neurons were maintained in culture for 3 d in DMEM supplemented with 50–100 ng/ml nerve growth factor, 100 U/ml penicillin/streptomycin, MEM vitamins, and 10% heat-inactivated fetal calf serum (all from Invitrogen).

Calcium imaging. Calcium imaging was performed using the fluorescent calcium indicator fura-2 AM between 24 and 72 h after dissociation, as described previously (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 performed 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 nonhydrolyzed fura ester.

Measurement of the concentration of free calcium ions ([Ca²⁺]_i) was performed by ratiometric imaging with an intensified charge-coupled device (CCD) camera. Fluorescence was excited at 340 and 380 nm, and emitted light was long-pass filtered at 510 nm. The fluorescence ratio, F_{340}/F_{380} , was calculated with Metafluor software (Universal Imaging Corporation, Downingtown, PA). Calcium calibration was performed with a fura-2 calcium imaging calibration kit (Invitrogen), and apparent free [Ca²⁺]_i was calculated from the equation $[Ca^{2+}]_i = K_d \times ((R - R_{min}) / (R_{max} - R)) \times (F_{380max} / F_{380min})$, where R_{min} is the ratio at zero free Ca²⁺, R_{max} is the ratio at saturating Ca²⁺ (e.g., 39 μM), F_{380max} is the fluorescence intensity exciting at 380 nm, for zero Ca²⁺, and F_{380min} is the fluorescence intensity at saturating free Ca²⁺.

Given the absence of specific pharmacological blockers of TRPV4, we minimized conductance via other ion channels by using a combination of room temperature HEPES buffer and variation of osmolality only by modifying D-mannitol concentration. Thus, the standard isotonic solution (312 mOsm) contained the following (in mM): 88 NaCl, 5 KCl, 1 MgCl₂, 2.4 CaCl₂, 110 D-mannitol, and 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 to 10 mM. Osmolality and pH were measured before each experiment. The vehicle for the fura-2 AM, DMSO, at its final working dilution did not induce any response in DRG neurons.

To measure the response of DRG neurons to hypotonicity, we averaged the value of the fluorescence ratio when the stimulus-induced increase in [Ca²⁺]_i reached a maximum plateau. This fluorescence ratio was then normalized with respect to the baseline value: fluorescence ratio during hypotonic stimulation divided by resting fluorescence ratio in isotonic solution. The fluorescence ratio was then converted to apparent free [Ca²⁺]_i. At the end of each experiment, a short exposure to solution containing 20 mM KCl was performed to confirm that all cells studied exhibited electrical excitability that is typical of healthy neurons.

For experiments involving sensitization with the inflammatory soup (a combination of 10 μM each BK, SP, histamine, 5-HT, and PGE₂), neurons were challenged with a 30% hypotonic solution for 3 min, washed for 6 min with isotonic solution, perfused with an isotonic solution containing the soup for 5 min, and then challenged with 30% hypotonic solution containing the soup for 3 min. Intracellular calcium was calculated from the normalized fluorescence ratio during a 30% hypotonic solution during exposure to the soup divided by the normalized fluorescence ratio in 30% hypotonic solution before exposure to soup.

For experiments testing the effect of inhibitors of PKA or PKCε on the hypotonic increase in fluorescence ratio, neurons were first challenged with hypotonic solution for 3 min, rinsed with isotonic solution for 6 min, perfused with isotonic solution containing the inhibitor for 15 min, and challenged with a 30% hypotonic solution containing the inhibitor for 3 min. The effect of the blockers was calculated from the normalized fluorescence ratio during exposure to 30% hypotonic solution in the

presence of inhibitor divided by the normalized fluorescence ratio in 30% hypotonic solution before inhibitor.

cAMP ELISA. Dorsal root ganglia were harvested from rats and dissociated as described above and maintained in culture for 2 d (two ganglia per dish). Cells were washed with culture medium and then incubated for 20 min at 37°C with 100 μ M PGE₂, 100 μ M 5-HT, or PGE₂ plus 5-HT (each at 10 μ M). The medium was aspirated, and 0.1N HCl was added for 10 min to lyse the cells. Lysates were processed immediately with a cAMP ELISA kit (Assay Designs, Ann Arbor, MI). Four independent experiments were performed.

ODN preparation and administration. The TRPV4 antisense ODN sequence, 5'-CATCACCAGGATCTGCCATACTG-3' (Invitrogen), was directed against a unique region of the rat TRPV4 channel (GenBank accession number AF263521). The mismatch ODN sequence was designed by mismatching seven bases (denoted by bold face) of the TRPV4 antisense sequence: 5'-CAACAGGAGGTT**C**AGGCAA**A**CTG-3'.

ODN was reconstituted in nuclease-free 0.9% NaCl (10 μ g/ μ l) and was administered into the spinal intrathecal space at a dose of 40 μ g, once a day for 3 d. As described previously (Alessandri-Haber et al., 2003), for this procedure, rats were anesthetized with 2.5% isoflurane (97.5% O₂), a 30 gauge needle was inserted into the subarachnoid space on the midline between L4 and L5 vertebrae, and 20 μ l ODN was injected at 1 μ l/s by microsyringe.

We have demonstrated previously that TRPV4 is present in sensory nerve fibers, presumably being transported from the cell body toward the peripheral nerve endings, and that our antisense ODN treatment procedure induces a significant and specific decrease in TRPV4 protein expression level in ligated saphenous nerve (Alessandri-Haber et al., 2003, 2004).

Data analysis. Calcium imaging and behavioral data are presented as mean $\pm$ SEM, and comparisons between groups were performed by Student's *t* test or ANOVA followed by Tukey's multiple comparison *post hoc* test. Significance was defined as *p* < 0.05.

Results

TRPV4 contributes to inflammatory hyperalgesia

To investigate whether TRPV4 contributes to inflammatory hyperalgesia, we tested whether the inflammogen carrageenan or a soup of inflammatory mediators (bradykinin, substance P, PGE₂, 5-HT, and histamine) would enhance the TRPV4-dependent nociceptive response to an injection of hypotonic solution (10 μ l of deionized water) in rat hindpaw. As shown in Figure 1A, carrageenan and inflammatory soup induced, respectively, a 4.6- and a 3.6-fold increase in the number of flinches induced by hypotonicity (3.9 ± 0.3 , *n* = 24 for hypotonicity alone; 18 ± 2 , *n* = 6 after carrageenan; and 14 ± 2 , *n* = 10 after inflammatory soup). To assess the contribution of TRPV4 in this nociceptive test, rats were pretreated with a spinal intrathecal administration of antisense or mismatch ODN for TRPV4, daily for 3 d. Antisense ODN (compared with mismatch) reduced the number of hypotonicity-induced flinches, in the presence of carrageenan or inflammatory soup, by 72 and 43%, respectively (Fig. 1A).

Intradermal injection of carrageenan or inflammatory soup 20 min before mechanical stimulation of the rat hindpaw also induced hyperalgesia to mechanical stimuli. As shown in Figure 1B, pretreatment with TRPV4 antisense prevented the mechanical hyperalgesia induced by both carrageenan and the inflammatory soup. In contrast, pretreatment with TRPV4 antisense did not affect the baseline nociceptive mechanical threshold in control rats (Fig. 1B, inset).

As an independent test of the role of TRPV4 in mechanical hyperalgesia induced by inflammatory soup, we tested C57BL/6 mice lacking the TRPV4 gene (*TRPV4*^{-/-}) (Liedtke and Friedman, 2003). As shown in Figure 2A, in the absence of inflammatory soup, the baseline withdrawal response to von Frey hair stimulation was not significantly different between *TRPV4*^{-/-}

Figure 1. TRPV4 mediates inflammatory hyperalgesia. **A**, Inflammatory mediators enhance nociceptive flinching induced by the injection of hypotonic solution. Intradermal injection of a hypotonic solution in rat hindpaw induced nociceptive flinches. Injection of carrageenan (Carr) or inflammatory soup (Soup) before the hypotonic solution induced a 4.6- and a 3.6-fold increase, respectively, in the number of flinches. Treatment with TRPV4 antisense ODN reduced the number of hypotonicity-induced flinches in the presence of carrageenan by 72% (5 ± 1 , *n* = 6 for antisense-treated vs 18 ± 2 , *n* = 6 for mismatch-treated rats; *p* < 0.05, unpaired Student's *t* test) and the ones in the presence of the inflammatory soup by 43% (9 ± 2 , *n* = 6 for antisense-treated vs 16 ± 2 , *n* = 6 for mismatch-treated rats; *p* < 0.02, unpaired Student's *t* test). **B**, Intradermal injection of carrageenan or inflammatory soup induced a decrease in mechanical nociceptive thresholds in the rat. Spinal intrathecal injection of TRPV4 antisense ODN prevented the mechanical hyperalgesia induced by carrageenan ($3.5 \pm 3\%$, *n* = 6 for antisense-treated vs $26 \pm 0.8\%$, *n* = 6 for mismatch-treated rats; *p* < 0.001, unpaired Student's *t* test) or by inflammatory soup ($-1 \pm 4\%$, *n* = 6 for antisense-treated vs $30 \pm 2\%$, *n* = 6 for mismatch-treated rats; *p* < 0.0001, unpaired Student's *t* test). Inset, Treatment with TRPV4 antisense had no effect on mechanical threshold in naive rats (*n* = 58 for mechanical threshold baseline and *n* = 20 for each ODN-treated rats; *p* > 0.05, ANOVA), whereas it prevented the decrease in threshold caused by inflammatory soup or carrageenan.

Figure 2. Inflammatory soup does not induce mechanical hyperalgesia in *TRPV4*^{-/-} mice. **A**, The baseline withdrawal response frequency to von Frey hair stimulation was not significantly different between *TRPV4*^{+/+} and *TRPV4*^{-/-} mice ($n = 10$ for *TRPV4*^{+/+} and $n = 12$ for *TRPV4*^{-/-} mice; $*p > 0.05$, unpaired Student's *t* test). However, in the presence of inflammatory soup, the withdrawal response frequency was increased by 2.8-fold in *TRPV4*^{+/+} mice ($30 \pm 5\%$ before and $84 \pm 3\%$ after inflammatory soup; $n = 10$; $p < 0.0001$, paired Student's *t* test), whereas the response remained unchanged in *TRPV4*^{-/-} mice ($n = 12$; $p > 0.05$, paired Student's *t* test). **B**, Small-diameter DRG neurons from *TRPV4*^{+/+} and *TRPV4*^{-/-} mice were first challenged with a 30% hypotonic solution for 3 min and then challenged with a 30% hypotonic solution containing the inflammatory soup (bradykinin, histamine, serotonin, substance P, and PGE₂, 10 μ M each). **C**, In *TRPV4*^{+/+} mice, the increase in $[Ca^{2+}]_i$ induced by a 30% hypotonic challenge was significantly enhanced in the presence of inflammatory soup (1.7 ± 0.1 μ M before and 2.3 ± 0.2 μ M after the soup; $n = 23$; $p < 0.05$, paired Student's *t* test). In contrast, in *TRPV4*^{-/-} mice, the response of DRG neurons to hypotonicity was unaffected ($n = 21$; $p > 0.05$, paired Student's *t* test). Iso, Isotonic solution; caps, capsaicin.

mice and wild-type littermates (*TRPV4*^{+/+}). After intraplantar injection of inflammatory soup, the frequency of paw withdrawal was increased by 2.8-fold in *TRPV4*^{+/+} mice ($30 \pm 5\%$ before and $84 \pm 3\%$ after inflammatory soup; $n = 10$; $p < 0.0001$, paired Student's *t* test), whereas it remained unaffected in *TRPV4*^{-/-} mice ($n = 12$; $p > 0.05$, paired Student's *t* test).

TRPV4 mediates the direct sensitizing action of inflammatory mediators on sensory neurons

To test whether the inflammatory soup-induced enhancement of TRPV4-dependent nociception can be explained by a direct action of the soup on primary afferent nerve endings, we measured calcium ion influx in sensory neurons isolated *in vitro*. Small-diameter DRG neurons (≤ 25 μ m) were isolated from *TRPV4*^{+/+} and *TRPV4*^{-/-} mice. As shown in Figure 2B, neurons were first challenged with a 30% hypotonic solution (212 mOsm) for 3 min and then challenged with a 30% hypotonic solution containing the inflammatory soup (212 mOsm). At the end of each experiment, neurons were identified as putative nociceptors if application of capsaicin (1 μ M, 10 s) induced a significant increase in the

concentration of free $[Ca^{2+}]_i$; all neurons included in the analysis were capsaicin responsive. In agreement with our previous results (Alessandri-Haber et al., 2005), hypotonicity induced a significantly smaller increase in $[Ca^{2+}]_i$ ($\sim 16\%$) in neurons from *TRPV4*^{-/-} mice compared with those from *TRPV4*^{+/+} mice (1.46 ± 0.05 μ M, $n = 21$ for *TRPV4*^{-/-} mice vs 1.7 ± 0.1 μ M, $n = 23$ for *TRPV4*^{+/+} mice; $p < 0.05$, unpaired Student's *t* test). The difference in the response to hypotonic stimuli between the TRPV4 genotypes is not large but is in agreement with our hypothesis; although TRPV4 participates in osmosensation in DRG neurons, it is not, however, a major osmosensor under physiological conditions. Our current and previous results suggest that TRPV4 plays a central role in the transduction of hypotonic- or hypertonic-induced nociceptive behavior only during inflammatory or neuropathic states (Alessandri-Haber et al., 2003, 2004, 2005).

Indeed, as shown in Figure 2C, inflammatory soup caused a 35% enhancement of the hypotonicity-induced increase in free $[Ca^{2+}]_i$ in *TRPV4*^{+/+} mice ($n = 23$; $p < 0.05$, paired Student's *t* test), whereas the inflammatory soup-induced enhancement was absent in *TRPV4*^{-/-} mice ($n = 21$; $p > 0.05$, paired Student's *t* test). Moreover, in the presence of inflammatory soup, the $[Ca^{2+}]_i$ in neurons from *TRPV4*^{+/+} mice was 1.6-fold the $[Ca^{2+}]_i$ level in neurons from *TRPV4*^{-/-} mice (2.3 ± 0.2 μ M, $n = 21$ for *TRPV4*^{+/+} mice vs 1.41 ± 0.07 , $n = 21$ for *TRPV4*^{-/-} mice) (Fig. 2C).

The increase in the $[Ca^{2+}]_i$ response to hypotonicity in neurons from *TRPV4*^{+/+} mice after a challenge with inflammatory soup was not large, but the response was probably underestimated because of the following: (1) although we record from a subpopulation of DRG neurons (i.e., diameter of ≤ 25 μ m and capsaicin responsive), most of these neurons do not express TRPV4, and (2) in our experimental conditions designed to minimize activation of other ion channels (i.e., HEPES buffer, room temperature, and low sodium concentration), the response of TRPV4 to hypotonicity was probably not maximized. For example, it has been shown that increasing temperature to physiological levels potentiates the response of TRPV4 to hypotonicity or other stimuli (Liedtke et al., 2000; Guler et al., 2002; Gao et al., 2003). Importantly, DRG neurons from *TRPV4*^{-/-} mice do not show an increase in $[Ca^{2+}]_i$ in the presence of inflammatory soup. Therefore, we consider this experiment to be more a qualitative measure (inflammatory soup increases the $[Ca^{2+}]_i$ or does not) rather than a quantitative measurement of an increase in $[Ca^{2+}]_i$. These data suggest that the inflammatory mediator-enhanced TRPV4-dependent nociceptive behavior in response to osmotic stimuli is the consequence of the direct action of the mediators on primary afferent neurons.

The combined action of multiple inflammatory mediators is required to engage TRPV4-dependent mechanisms of hyperalgesia

We attempted to identify which mediators are responsible for engaging TRPV4, hypothesizing that some components of the inflammatory soup, when administered alone, would elicit TRPV4-dependent hyperalgesia. As expected, intradermal injection of PGE₂ or 5-HT alone induced hyperalgesia to mechanical stimuli (Fig. 3A). However, treatment with TRPV4 antisense had no effect on the PGE₂-induced mechanical hyperalgesia (100 ng of PGE₂, $n = 26$ for antisense and mismatch treated) (Fig. 3A). Conceivably, this failure to engage TRPV4-dependent mechanisms of hyperalgesia occurred because the dose of PGE₂ was insufficient. Therefore, we investigated the effect of 1 μ g of PGE₂,

Figure 3. TRPV4 participation in mechanical hyperalgesia requires a threshold level of cAMP reached only by combined mediators. **A**, Injection of 1 μg of PGE₂ in rat hindpaw induced a slightly greater mechanical hyperalgesia compared with the injection of 100 ng of PGE₂ (mean ± SEM; 32 ± 0.7%, $n = 48$ for 100 ng of PGE₂ vs 37 ± 1%, $n = 12$ for 1 μg of PGE₂; $p < 0.001$, unpaired Student's t test). However, treatment with antisense ODN for TRPV4 did not reduce the 100 ng nor the 1 μg PGE₂-induced mechanical hyperalgesia ($n = 26$ for antisense- and mismatch-treated for 100 ng of PGE₂ and $n = 6$ for 1 μg PGE₂ antisense- and mismatch-treated rats; $p > 0.05$, unpaired Student's t test). Injection of 100 ng or 1 μg of 5-HT in rat hindpaw induced a similar level of mechanical hyperalgesia ($n = 6$; $p > 0.05$, unpaired Student's t test). Again, treatment with antisense ODN for TRPV4 did not reduce the 5-HT-induced mechanical hyperalgesia ($n = 6$ for each ODN group; $p > 0.05$, unpaired Student's t test). In contrast, treatment with antisense ODN for TRPV4 prevented the decrease in paw-withdrawal threshold induced by the coinjection of PGE₂ and 5-HT (100 ng each; 0.6 ± 1.5%, $n = 8$ for antisense-treated and 33 ± 1%, $n = 8$ for mismatch-treated rats; * $p < 0.05$, unpaired Student's t test). **B**, Intraplantar injection of PGE₂ or 5-HT (100 ng and 1 μg each) similarly enhanced the withdrawal response frequency to mechanical stimuli in both TRPV4^{+/+} and TRPV4^{-/-} mice. In TRPV4^{+/+} mice, the coinjection of PGE₂ and 5-HT (100 ng each) induced a 3.9-fold increase in the withdrawal frequency response (23 ± 3%, $n = 18$ before and 90 ± 4%, $n = 6$ after coinjection of PGE₂ and 5-HT). In contrast, in TRPV4^{-/-} mice, the withdrawal response frequency after the coinjection of PGE₂ and 5-HT was not different from baseline ($n = 6$ after and $n = 23$ before treatment; * $p > 0.05$, unpaired Student's t test).

a dose that elicits the maximal hyperalgesic effect of this mediator (Khasar et al., 1994). Hyperalgesia induced by 1 μg of PGE₂ was somewhat greater compared with that induced by 100 ng of PGE₂ (32 ± 0.7%, $n = 48$ for 100 ng of PGE₂ vs 37 ± 1%, $n = 12$ for 1 μg of PGE₂; $p < 0.001$, unpaired Student's t test). However, treatment with TRPV4 antisense still had no significant effect on the mechanical hyperalgesia induced by the higher dose of PGE₂ (Fig. 3A). Similarly, mechanical hyperalgesia induced by 5-HT was also not affected by treatment with TRPV4 antisense (Fig. 3A). We then tested whether the combined administration of PGE₂ and 5-HT (each at a submaximal dose of 100 ng) would be sufficient to induce TRPV4-dependent mechanical hyperalgesia, and, as shown on Figure 3A, treatment with TRPV4 antisense prevented the mechanical hyperalgesia ($n = 8$ for each ODN group; $p < 0.05$, unpaired Student's t test).

As an independent confirmation, we investigated the withdrawal response frequency of TRPV4^{+/+} and TRPV4^{-/-} mice to mechanical stimuli after intraplantar injection of PGE₂ (100 ng and 1 μg), 5-HT (100 ng and 1 μg), or PGE₂ plus 5-HT (100 ng each). Pretreatment with PGE₂ or 5-HT (at both concentration) induced a similar 2.8-fold increase in the withdrawal response frequency in both TRPV4^{+/+} and TRPV4^{-/-} mice ($n = 6$ for 100 ng and 1 μg of PGE₂ and 1 μg of 5-HT; $n = 8$ for 100 ng of 5-HT; $p > 0.05$, ANOVA) (Fig. 3B). In contrast, whereas in TRPV4^{+/+} mice the coinjection of PGE₂ and 5-HT at 100 ng each induced a 3.9-fold increase in the withdrawal frequency response, in TRPV4^{-/-} mice the withdrawal response frequency was not different from baseline. Thus, although PGE₂ and 5-HT separately are unable to engage TRPV4, together they can.

In addition, intraplantar injection of bradykinin, substance P, or histamine (100 ng each) induced a similar 2.5-fold increase in the withdrawal frequency in TRPV4^{-/-} mice (73 ± 4%, $n = 6$ for bradykinin; 77 ± 8%, $n = 6$ for histamine; and 72 ± 5%, $n = 8$ for substance P). Therefore, we conclude that only the concerted action of more than one inflammatory mediator can engage TRPV4 in mechanical hyperalgesia.

TRPV4 activation appears to require a threshold level of cAMP, attainable only with combined mediators

Mechanical hyperalgesia induced by the soup of inflammatory mediators depends in part on the cAMP/PKA second-messenger pathway (Lotz et al., 1987; Taiwo and Levine, 1988; Taiwo et al., 1989, 1992; Malmberg et al., 1997; Aley and Levine, 1999; Cunha et al., 1999). PGE₂ and 5-HT, in particular, also induce hyperalgesia via the cAMP/PKA pathway when administered alone (Taiwo et al., 1989, 1992; Cardenas et al., 1997; Aley and Levine, 1999). Therefore, we hypothesized that the increase in cAMP induced by either PGE₂ or 5-HT alone is insufficient to activate TRPV4-dependent mechanisms of hyperalgesia; instead, simultaneous action of multiple inflammatory mediators would be required to achieve sufficient activation of the cAMP pathway to engage TRPV4. Our hypothesis suggests that a threshold level of cAMP must be achieved before TRPV4-dependent hyperalgesia can be produced. Therefore, we tested whether hyperalgesia produced by an exogenous membrane-permeable cAMP analog, 8-Br-cAMP, exhibits such features in its dose–response relationship. As shown in Figure 4A, intradermal injection of 8-Br-cAMP (0.1, 0.3, 1, 3, 10, 100, 300, and 1000 ng) dose dependently induced mechanical hyperalgesia starting at the dose of 3 ng (13 ± 2%; $n = 8$) and reaching a maximal effect at a dose of 100 ng (34 ± 3%; $n = 8$). TRPV4 antisense reduced the dose-dependent mechanical hyperalgesia induced by 8-Br-cAMP; the hyperalgesia induced by 100 ng of 8-Br-cAMP was reduced by 38% in

Figure 4. PGE₂-induced hyperalgesia switches to a TRPV4-dependent mechanism when a sufficient level of cAMP is reached. **A**, Rats were treated with either TRPV4 antisense or mismatch ODN for 3 d, and mechanical nociceptive thresholds were evaluated before and 15 min after injection of increasing doses of 8-Br-cAMP (0.1, 0.3, 1, 3, 10, 100, 300, and 1000 ng). The mechanical hyperalgesia induced by 10–1000 ng of 8-Br-cAMP was reduced in TRPV4 antisense-treated compared with mismatch-treated rats. **B**, Intradermal injection of 1 μ g of PGE₂ induced a significant decrease in mechanical threshold. The injection of 1 ng of 8-Br-cAMP had no effect on mechanical nociceptive threshold and injection of 1 ng of 8-Br-cAMP 30 min before the injection of 1 μ g of PGE₂ did not enhance PGE₂-induced mechanical hyperalgesia ($37 \pm 1\%$, $n = 12$ for PGE₂ alone and $34 \pm 2\%$, $n = 12$ for 8-Br-cAMP plus PGE₂; $p > 0.05$, unpaired Student's *t* test). However, in the presence of 8-Br-cAMP, treatment with TRPV4 antisense ODN prevented the PGE₂-induced mechanical hyperalgesia ($3 \pm 2\%$, $n = 12$ for antisense-treated vs $36 \pm 2\%$, $n = 8$ for mismatch-treated rats; $p < 0.0001$, unpaired Student's *t* test). **C**, Intraplantar injection of 1 ng of 8-Br-cAMP did not affect the withdrawal response frequency in TRPV4^{+/+} and TRPV4^{-/-} mice and injection of 100 ng of PGE₂ induced a threefold increase in the withdrawal response frequency in both TRPV4 genotypes. In contrast, after pretreatment with 1 ng of 8-Br-cAMP, the mechanical hyperalgesia induced by 100 ng of PGE₂ was absent in TRPV4^{-/-} mice ($n = 12$). Furthermore, in the presence of the inhibitor of activation by cAMP of cAMP-dependent protein kinase I and II, Rp-cAMPS, coinjection of PGE₂ and 5-HT (100 ng each) failed to increase the withdrawal response frequency in TRPV4^{+/+} mice ($27 \pm 6\%$, $n = 12$ for PGE₂ plus 5-HT plus Rp-cAMPS compared with $70 \pm 4\%$, $n = 6$ for PGE₂ plus 5-HT; $p < 0.05$, unpaired Student's *t* test).

antisense-treated compared with mismatch-treated rats ($30 \pm 3\%$, $n = 8$ for mismatch-treated rats; $19 \pm 2\%$, $n = 8$ for antisense-treated rats; $p < 0.05$, unpaired Student's *t* test) (Fig. 4A). Notably, sensitivity to TRPV4 antisense only became evident at the doses of 10–1000 ng. We conclude that a threshold level of cAMP is necessary to engage TRPV4-dependent mechanical hyperalgesia.

If our hypothesis is correct, then injection of a low dose of 8-Br-cAMP (1 ng) to boost the intracellular level of cAMP activity might enable 1 μ g of PGE₂ to engage TRPV4-dependent mechanisms of hyperalgesia. As shown in Figure 4, A and B, the 1 ng dose of 8-Br-cAMP alone had no effect on the mechanical nociceptive threshold. In addition, 1 ng of 8-Br-cAMP did not increase the magnitude of PGE₂-induced mechanical hyperalgesia (Fig. 4B). However, pretreatment with 1 ng of 8-Br-cAMP caused the hyperalgesia induced by PGE₂ to become sensitive to treatment with TRPV4 antisense ($3 \pm 2\%$, $n = 12$ for antisense-treated vs $36 \pm 2\%$, $n = 8$ for mismatch-treated rats; $p < 0.0001$, unpaired Student's *t* test) (Fig. 4B).

Furthermore, 1 ng of 8-Br-cAMP had no effect on the baseline withdrawal response frequency in either TRPV4^{+/+} or TRPV4^{-/-} mice. In contrast, after pretreatment with 8-Br-cAMP, the PGE₂-induced enhancement in the withdrawal response frequency was absent in TRPV4^{-/-} mice ($15 \pm 4\%$, $n = 12$ after pretreatment with 8-Br-cAMP and $65 \pm 3\%$, $n = 8$ before) (Fig. 4C). In addition, coinjection of the inhibitor of activation by cAMP of cAMP-dependent protein kinase I and II, Rp-cAMPS, with PGE₂ and 5-HT (100 ng each) prevented the enhancement in withdrawal response frequency in TRPV4^{+/+} mice ($70 \pm 4\%$, $n = 6$ for PGE₂ plus 5-HT and $27 \pm 6\%$, $n = 12$ for PGE₂ plus 5-HT plus Rp-cAMPS; $p < 0.05$, unpaired Student's *t* test) (Fig. 4C).

These results are consistent with the hypothesis that the switch to a TRPV4-dependent mechanism of hyperalgesia occurs because the increase in cAMP concentration induced by PGE₂ plus 5-HT (100 ng each) is higher than that induced by 1 μ g of PGE₂ or 5-HT alone. Levels of cAMP cannot be measured in primary afferent nerve endings *in vivo*, but we were able to use immunoassay methods *in vitro* to measure levels of cAMP accumulation in primary cultures of DRG neurons after exposure to inflammatory mediators.

We tested whether 100 μ M PGE₂ or 100 μ M 5-HT would induce a lesser increase in intracellular cAMP than that induced by PGE₂ plus 5-HT at 10 μ M each (concentrations chosen to be consistent with our experiments on the effect of inflammatory soup on cultured DRG neurons). Indeed, the increase in the intracellular concentration of cAMP induced by 100 μ M PGE₂ or 100 μ M 5-HT is significantly smaller than that induced by PGE₂ plus 5-HT at 10 μ M each ($16 \pm 3\%$, $n = 10$ after 100 μ M PGE₂; $12 \pm 6\%$, $n = 5$ after 100 μ M 5-HT; $25 \pm 3\%$, $n = 8$ after 10 μ M each PGE₂ plus 5-HT; $p < 0.05$). These results suggest that, when a sufficient level of cAMP is reached, PGE₂-induced hyperalgesia switches to a TRPV4-dependent mechanism, although the magnitude of hyperalgesia remains unchanged.

PKA appears to mediate cAMP activation of TRPV4-dependent hyperalgesia

An important intracellular target of cAMP is cAMP-dependent PKA, and we hypothesized that TRPV4 is modulated by the cAMP/PKA inflammatory mediator-activated second-messenger pathway. Therefore, we tested whether 8-Br-cAMP (1 μ g, 2.5 μ l) or PKACS (10 U/2.5 μ l) would enhance TRPV4-dependent nociceptive responses in mice.

Figure 5. TRPV4 participates in cAMP/PKA-mediated hyperalgesia. **A**, 8-Br-cAMP and PKACS induced, respectively, a threefold and a fourfold increase in the number of flinches induced by hypotonicity in rat (12 ± 1, *n* = 14 after 8-Br-cAMP, 15 ± 1, *n* = 10 after PKACS vs 3.9 ± 0.3, *n* = 24 for hypotonicity alone). Treatment with TRPV4 antisense ODN reduced the number of flinches induced by hypotonicity in the presence of 8-Br-cAMP or PKACS, respectively, by 41% (7.2 ± 0.8, *n* = 8 for antisense-treated vs 12.2 ± 0.9, *n* = 8 for mismatch-treated rats; **p* < 0.01, unpaired Student's *t* test) and 48% (8 ± 1, *n* = 8 for antisense-treated vs 16 ± 2, *n* = 7 for mismatch-treated rats; **p* = 0.0001, unpaired Student's *t* test). **B**, Intraplantar injection of PKACS induced a 2.5-fold increase in the withdrawal response frequency in TRPV4^{+/+} mice (25 ± 6% before and 63 ± 54% in the presence of PKACS; *n* = 8; **p* < 0.001, paired Student's *t* test). In contrast, PKACS did not affect the withdrawal response frequency in TRPV4^{-/-} mice (*n* = 18; *p* > 0.05, paired Student's *t* test). **C**, Injection of 8-Br-cAMP or PKACS in rat hindpaw induced a similar degree of mechanical hyperalgesia (35 ± 2%, *n* = 6 for PKACS vs 35 ± 1%, *n* = 17 for 8-Br-cAMP; **p* > 0.05, unpaired Student's *t* test). Treatment with TRPV4 antisense ODN attenuated both the 8-Br-cAMP- (4.9 ± 2.6%, *n* = 8 for antisense-treated vs 35.7 ± 1.3%, *n* = 8 for mismatch-treated rats; **p* < 0.0001, unpaired Student's *t* test) and the PKACS-induced mechanical hyperalgesia (−3.9 ± 3.8%, *n* = 8 for antisense-treated vs 32.7 ± 2.5%, *n* = 8 for mismatch-treated rats; **p* < 0.0001, unpaired Student's *t* test). In contrast, mismatch treatment had no effect on the decrease in mechanical threshold caused by PKACS or 8-Br-cAMP. **D**, Mean ± SEM of [Ca²⁺]_i for DRG neurons first challenged with a 30% hypotonic solution and then challenged with a 30% hypotonic solution containing either Rp-cAMPS (30 μM) or H89 (10 μM). In TRPV4^{+/+} mice, the hypotonicity-induced increase in [Ca²⁺]_i was significantly reduced in the presence of Rp-cAMPS or H89 (1.2 ± 0.1 μM, *n* = 4 for Hypo plus H89, 1.3 ± 0.08 μM, *n* = 8 for Hypo plus Rp-cAMPS vs 1.7 ± 0.1 μM, *n* = 12 for Hypo; **p* < 0.05, Tukey's multiple comparison test). In contrast, these inhibitors did not significantly affect the response of DRG neurons to hypotonicity in TRPV4^{-/-} mice (*p* > 0.05, ANOVA and Tukey's multiple comparison test).

As shown in Figure 5A, intradermal injection of 8-Br-cAMP and PKACS in the hindpaw induced threefold and fourfold increases, respectively, in the number of flinches induced by hypotonicity in rat. TRPV4 antisense reduced the number of flinches induced by hypotonicity in the presence of 8-Br-cAMP or PKACS, respectively, by 41 and 48% (Fig. 5A).

We then investigated the withdrawal response frequency of

TRPV4^{+/+} and TRPV4^{-/-} mice to mechanical stimuli before and after injection of PKACS. Pretreatment with PKACS induced a 2.5-fold increase in the withdrawal response frequency in TRPV4^{+/+} mice (25 ± 6% before and 63 ± 4% in the presence of PKACS; *n* = 8; *p* < 0.001, paired Student's *t* test) (Fig. 5B). In contrast, PKACS had no significant effect on the withdrawal response frequency in TRPV4^{-/-} mice (*n* = 18 before and after PKACS; *p* > 0.05, paired Student's *t* test).

In rat hindpaw, injection of PKACS induced a similar degree of mechanical hyperalgesia as did 8-Br-cAMP (35 ± 2% *n* = 6 for PKACS vs 35 ± 1%, *n* = 17 for 8-Br-cAMP; *p* > 0.05, unpaired Student's *t* test) (Fig. 5C), and treatment with TRPV4 antisense prevented the mechanical hyperalgesia induced by PKACS (−3.9 ± 3.8%, *n* = 8 for antisense-treated vs 32.7 ± 2.5%, *n* = 8 for mismatch-treated rats; *p* < 0.0001, unpaired Student's *t* test).

We further investigated whether TRPV4-dependent osmotransduction in dissociated DRG neurons *in vitro* depended on the cAMP/PKA pathway. DRG neurons (diameter of ≤25 μm) isolated from TRPV4^{+/+} and TRPV4^{-/-} mice were first challenged with a 30% hypotonic solution and then with a 30% hypotonic solution containing the PKA inhibitors Rp-cAMPS (30 μM) or H89 (10 μM). As shown in Figure 5D, the hypotonic-induced response in TRPV4^{+/+} neurons was significantly reduced in the presence of Rp-cAMPS or H89 (*n* = 12 for Hypo; *n* = 8 Hypo plus Rp-cAMPS; *n* = 4 for Hypo plus H89; *p* < 0.05, Tukey's multiple comparison test). In contrast, neither inhibitor had a significant effect on the hypotonic-induced response of TRPV4^{-/-} neurons (*n* = 16 for Hypo; *n* = 9 for Hypo plus Rp-cAMPS; *n* = 7 for Hypo plus H89; *p* > 0.05, Tukey's multiple comparison test). These findings suggest that TRPV4-mediated mechanisms of hyperalgesia are activated via the cAMP/PKA pathway.

TRPV4 also contributes to PKCε-dependent hyperalgesia

The PKCε second-messenger pathway is also required for full expression of inflammatory mediator-induced hyperalgesia (Khasar et al., 1994; Cesare et al., 1999). Therefore, we investigated whether PKCε-induced enhancement of hypotonic nociception also involves TRPV4-dependent mechanisms. The number of flinches induced by hypotonicity in rat hindpaw was increased by 3.3-fold by pretreatment with the PKCε activator *ψ*ε-RACK (13 ± 2, *n* = 6 after *ψ*ε-RACK vs 3.9 ± 0.3, *n* = 24 before; *p* < 0.0001, unpaired Student's *t* test) (Fig. 6A). As shown in Figure 6A, treatment with TRPV4 antisense reduced the number of flinches by 46% compared with mismatch.

We then investigated whether PKCε contributes to TRPV4-mediated osmotransduction in DRG neurons. Neurons isolated from TRPV4^{+/+} and TRPV4^{-/-} mice were first challenged with a 30% hypotonic solution and then with a 30% hypotonic solution containing the PKCε translocation inhibitor peptide PKCεI (2 μM). As shown in Figure 6B, the [Ca²⁺]_i was significantly reduced in the presence of PKCεI in TRPV4^{+/+} mice (1.3 ± 0.1 μM in the presence of PKCεI vs 1.7 ± 0.1 μM without; *n* = 13; *p* < 0.001, paired Student's *t* test). In contrast, PKCεI had no significant effect on the response of DRG neurons to hypotonicity in TRPV4^{-/-} mice (*n* = 12; *p* > 0.05; paired Student's *t* test).

We next investigated whether TRPV4 participates in PKCε-induced mechanical hyperalgesia (Fig. 6C). Treatment with antisense ODN for TRPV4 attenuated the mechanical hyperalgesia induced by the injection of *ψ*ε-RACK in the rat hindpaw (−0.03 ± 2.10%, *n* = 6 for antisense-treated vs 28.2 ± 1.7%, *n* = 6 for mismatch-treated rats; *p* < 0.0001, unpaired Student's *t* test).

Figure 6. TRPV4 participates in PKC ϵ -mediated hyperalgesia. **A**, The number of flinches induced by hypotonic stimulation was 3.3-fold higher in the presence of the PKC ϵ activator $\psi\epsilon$ -RACK (13 ± 2 , $n = 6$ after $\psi\epsilon$ -RACK vs 3.9 ± 0.3 , $n = 24$ before; $p < 0.0001$, unpaired Student's t test). Treatment with TRPV4 antisense reduced the number of flinches by 46% compared with mismatch-treated rats (5 ± 0.4 , $n = 6$ for antisense-treated vs 11 ± 2 , $n = 6$ for mismatch-treated rats; $*p < 0.02$, unpaired Student's t test). **B**, Mean $\pm$ SEM of $[Ca^{2+}]_i$. DRG neurons from TRPV4^{+/+} and TRPV4^{-/-} mice were first challenged with a 30% hypotonic solution and then challenged with a 30% hypotonic solution containing the PKC ϵ translocation inhibitor peptide (2 μ M). The hypotonicity-induced increase in $[Ca^{2+}]_i$ was significantly reduced in the presence of PKC ϵ translocation inhibitor peptide in TRPV4^{+/+} mice (1.3 ± 0.01 μ M in the presence of PKC ϵ inhibitor vs 1.7 ± 0.1 μ M without; $n = 13$; $*p < 0.001$, paired Student's t test). In contrast, the inhibitor had no effect on the response of DRG neurons to hypotonicity in TRPV4^{-/-} mice ($n = 12$; $p > 0.05$, paired Student's t test). **C**, Treatment with TRPV4 antisense attenuated the decrease in paw-withdrawal threshold induced by the injection of $\psi\epsilon$ -RACK (1 μ g, $-0.03 \pm 2.10\%$, $n = 6$ for antisense-treated vs $28.2 \pm 1.7\%$, $n = 6$ for mismatch-treated rats; $*p < 0.0001$, unpaired Student's t test). **D**, Intraplantar injection of $\psi\epsilon$ -RACK increased the withdrawal response frequency by 2.6-fold in TRPV4^{+/+} mice ($32 \pm 5\%$ before and $83 \pm 4\%$ in the presence of $\psi\epsilon$ -RACK; $n = 12$; $*p < 0.0001$, unpaired Student's t test). In contrast, it did not affect the response in TRPV4^{-/-} mice ($n = 14$; $p > 0.05$, unpaired Student's t test).

To confirm that PKC ϵ -induced hyperalgesia involves a TRPV4 mechanism, we tested whether $\psi\epsilon$ -RACK-induced hyperalgesia is diminished in TRPV4 knock-out mice. As shown in Figure 6D, $\psi\epsilon$ -RACK increased the withdrawal response frequency by 2.6-fold in TRPV4^{+/+} mice ($32 \pm 5\%$ without and $83 \pm 4\%$ in the presence of $\psi\epsilon$ -RACK; $n = 12$; $p < 0.0001$, paired Student's t test). In contrast, $\psi\epsilon$ -RACK had no significant effect on the withdrawal response frequency in TRPV4^{-/-} mice ($n = 14$; $p > 0.05$, paired Student's t test). These findings suggest that PKC ϵ -mediated component of hyperalgesia also involves a TRPV4 mechanism.

Discussion

After tissue injury, inflammatory mediators such as 5-HT, histamine, PGE₂, and bradykinin directly sensitize primary afferent neurons, resulting in hyperalgesia. Nociceptors then display a lowered threshold of activation, an increased spontaneous activity, and an increased response to a suprathreshold stimulus (Levine and Reichling, 1999; Millan, 1999). It has been demonstrated that inflammatory mediators increase nociceptor excitability by modulating ion channels, including transducers (for review, see Millan, 1999).

We demonstrate here for the first time, using two different models (i.e., transient downregulation of the level of expression of TRPV4 protein in the rat and TRPV4 knock-out mice) that, after exposure to inflammatory mediators, TRPV4 plays a crucial role in mechanical hyperalgesia. Because of the difference in size and behavior between rat and mice, different behavioral tests were performed to measure the mechanical hyperalgesia induced by inflammatory mediators in the hindpaws of the two species. In both species, we demonstrate that the cAMP/PKA and the PKC ϵ second-messenger pathways can engage TRPV4-dependent mechanical hyperalgesia. Our results suggest that the concerted action of inflammatory mediators is necessary to engage TRPV4 in mechanical hyperalgesia (i.e., each of the five inflammatory mediators tested alone does not engage TRPV4). Moreover, we show that at least two inflammatory mediators (i.e., PGE₂ and 5-HT) can act synergistically through cAMP to engage TRPV4-dependent mechanical hyperalgesia. However, the PKC ϵ pathway also participates in inflammatory mediator-induced hyperalgesia (Khasar et al., 1994; Cesare et al., 1999; Hucho et al., 2005), and the existence of crosstalk or interaction between the cAMP/PKA and PKC pathways has been demonstrated in sensory neurons (Sugita et al., 1997; Gu and Huang, 1998). In addition, the activation of PKC has been shown to potentiate the effect of PKA on sodium ion channels (Cantrell et al., 2002) and on the cystic fibrosis transmembrane conductance regulator channel (Chen et al., 2004). Therefore, we do not exclude a synergistic action of the inflammatory mediators through a cAMP- and PKC ϵ -dependent interaction, and additional experiments will be conducted to investigate this hypothesis.

Using DRG neurons isolated *in vitro*, we provide evidence that the inflammatory mediator-induced mechanism of TRPV4-dependent hyper-responsiveness may reside entirely within the sensory neuron. However, we cannot rule out that TRPV4 located in non-neuronal cells in the skin (i.e., keratinocytes) might make some additional contribution to the hyperalgesia.

Of note, it was demonstrated recently that thermal hyperalgesia induced by carrageenan was significantly reduced in TRPV4^{-/-} mice (Todaka et al., 2004). In addition, we demonstrated recently that TRPV4 also plays a major role in chemotherapy-induced mechanical hyperalgesia (Alessandri-Haber et al., 2004). Together with the present results, these observations suggest that TRPV4 has a central role in pathological pain induced by a variety of stimulus modalities rather than in normal discriminatory pain sensation.

Our data suggest that TRPV4 contributes specifically to pathological pain conditions because its activation requires relatively high levels of intracellular cAMP. It appears that such levels are not easily achieved by the action of a single inflammatory mediator, but that concerted action of multiple mediators (as would occur in inflamed tissues) is required. Consistent with this suggestion, it was shown that, although administration of cAMP analogs can lower mechanical thresholds in single cutaneous af-

ferent of rat skin *in vitro* (Kress et al., 1996), PGE₂ alone cannot (Lang et al., 1990).

TRPV4 is thought to be a transducer of noxious hypo-osmotic (Alessandri-Haber et al., 2003) and mechanical (Liedtke and Friedman, 2003; Suzuki et al., 2003a) stimuli. TRPV4 is expressed in small-diameter DRG neurons and in free nerve endings in the skin (Suzuki et al., 2003b). TRPV4 is a nonselective cation channel with an outwardly rectifying current–voltage relationship (Liedtke et al., 2000; Strotmann et al., 2000; Nilius et al., 2001; Voets et al., 2002) that lacks the hallmarks of a voltage-gated channel (Papazian et al., 1991; Mannikko et al., 2002; Lecar et al., 2003). Thus, the role of TRPV4 is most likely to contribute to hyperalgesia by enhancing transduction rather than by contributing directly to voltage-gated action potential currents.

TRPV4 is engaged by two key intracellular second-messenger pathways of inflammatory hyperalgesia

PKC has been shown to be involved in TRPV4 function *in vitro* (Gao et al., 2003; Xu et al., 2003), but this is the first report of a functional interaction between TRPV4 and a specific PKC isozyme. Interestingly, mice lacking either the PKC ϵ gene (Khasar et al., 1999) or the TRPV4 gene (Suzuki et al., 2003a) exhibited diminished nociception in the acetic acid writhing test. Importantly, stimulation of the cAMP pathway can lead to PKC ϵ translocation (Graness et al., 1997; Hucho et al., 2005). We have shown previously that, in sensitized nociceptors, the response of TRPV4 to mechanical and osmolar stimuli depend partly on Src tyrosine kinase phosphorylation (Alessandri-Haber et al., 2004, 2005). Of note, cAMP/PKA and PKC ϵ phosphorylation pathways can both directly interact with the Src tyrosine kinase family (Ping et al., 1999, 2002; Purcell and Carew, 2001; Stork and Schmitt, 2002).

Although a putative PKA phosphorylation site in the TRPV4 N-terminal region has been described previously (Nilius et al., 2004), this is the first study reporting a functional interaction between TRPV4 and the PKA pathway. We demonstrate that PGE₂-induced hyperalgesia to hypotonic stimuli is inhibited by 50% after treatment with antisense ODN for TRPV4 (Alessandri-Haber et al., 2003). Interestingly, we demonstrate that, to engage TRPV4 in inflammatory-induced mechanical hyperalgesia, a threshold level of cAMP is necessary. However, when this threshold is reached, mechanical hyperalgesia induced by an inflammatory mediator, such as PGE₂, clearly switches from a TRPV4-independent mechanism to a completely TRPV4-dependent mechanism. This suggests that, although high levels of cAMP engage TRPV4 in mechanical hyperalgesia, inactivation of TRPV4-independent mechanisms must also occur. At this time, it is difficult to formulate a detailed hypothesis for the underlying cellular mechanism. Many G-protein-coupled receptors for hormones can signal through different G-proteins. For example, although most of the functions of the β_2 -adrenergic receptor are mediated through G_s-protein and the cAMP/PKA pathway, this receptor can also couple to G_i-protein, which is cAMP-independent and initiates other signaling events (Xiao et al., 1995; Daaka et al., 1997). Similarly, in the heart, lower concentrations (10 nM) of epinephrine can activate G_s (10 nM epinephrine), whereas higher concentrations (1–100 μ M) activate G_i pathways (Heubach et al., 2004). In fact, we have demonstrated that the activation of a G_i/Ras/extracellular signal-regulated kinase signaling pathway produces inflammatory pain that is independent of PKA or PKC ϵ pathways (Aley et al., 2001). Future experiments will further address this question.

Is TRPV4 involved in the function of “silent” nociceptors?

Mechanical hyperalgesia depends in part on recruitment of previously “silent” nociceptors (Handwerker et al., 1991; Kress et al., 1992; Schmelz et al., 1994; Schmidt et al., 1995; Millan, 1999). Thus, in the setting of inflammation or peripheral neuropathy, mechano-insensitive (silent) nociceptors become responsive (Martin et al., 1987; Habler et al., 1988; Schaible and Schmidt, 1988a; McMahon and Koltzenburg, 1990; Schmidt et al., 1995, 2000). Interestingly, hyperalgesia to tonic mechanical stimulation is closely correlated with the discharge pattern of mechano-insensitive C-fibers (Adriaensen et al., 1984; Schmidt et al., 1995, 2000; Andrew and Greenspan, 1999). Additionally, an active contribution of silent C-mechano-insensitive fibers in chronic pain was demonstrated recently (Orstavik et al., 2003).

The cellular mechanisms responsible for the evanescent nature of silent nociceptors are unknown. Our observations suggest that, under normal conditions, TRPV4 may be inactive as a mechanical transducer but that it may be activated by the combined action of multiple inflammatory mediators or in neuropathy (Alessandri-Haber et al., 2004). Conceivably, primary afferent nerve fibers that express TRPV4 as their only mechanical transducer would exhibit properties of silent nociceptors, becoming mechanically responsive only in pathological conditions. Indeed, in parallel to our observations, inflammation induced by carrageenan or by inflammatory soup activates silent nociceptors (Schaible and Schmidt, 1985, 1988a; Xu et al., 2000), but PGE₂ alone is insufficient (Mizumura et al., 1987; Schaible and Schmidt, 1988b; Lang et al., 1990; Grubb et al., 1991; Handwerker et al., 1991; Kress et al., 1992).

Conclusion

This work confirms the hypothesis that TRPV4 plays a crucial role in the development of hyperalgesia in inflamed tissue. That a relatively high level of cAMP is required to activate TRPV4-dependent mechanisms of hyperalgesia may explain why this transducer makes little contribution to baseline nociception in normal tissue. Its predominant contribution to pathological pain raises the possibility that TRPV4 could present a promising target for the development of a novel class of analgesics that would selectively affect pathological pain.

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