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TRPA1 Mediates the Inflammatory Actions of Environmental Irritants and Proalgesic Agents

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SUMMARY

TRPA1 is an excitatory ion channel targeted by pungent irritants from mustard and garlic. TRPA1 has been proposed to function in diverse sensory processes, including thermal (cold) nociception, hearing, and inflammatory pain. Using TRPA1-deficient mice, we now show that this channel is the sole target through which mustard oil and garlic activate primary afferent nociceptors to produce inflammatory pain. TRPA1 is also targeted by environmental irritants, such as acrolein, that account for toxic and inflammatory actions of tear gas, vehicle exhaust, and metabolic byproducts of chemotherapeutic agents. TRPA1-deficient mice display normal cold sensitivity and unimpaired auditory function, suggesting that this channel is not required for the initial detection of noxious cold or sound. However, TRPA1-deficient mice exhibit pronounced deficits in bradykinin-evoked nociceptor excitation and pain hypersensitivity. Thus, TRPA1 is an important component of the transduction machinery through which environmental irritants and endogenous proalgesic agents depolarize nociceptors to elicit inflammatory pain.

INTRODUCTION

Somatosensory neurons enable us to detect a wide range of endogenous and environmental stimuli, including pressure, temperature, irritants, and products of inflammation. A subset of these neurons, collectively referred to as nociceptors, can be activated by stimuli capable of eliciting tissue damage or pain (Julius and Basbaum, 2001). A fundamental goal toward understanding the mechanisms of somatosensation, nociception, and pain is to identify molecules that contribute to stimulus detection and promote sensitization or excitation of primary sensory nerve fibers. In this regard, significant attention has focused on members of the TRP family of cation channels that serve as receptors for a variety of natural plant products that robustly excite nociceptors to elicit pain and inflammation (Clapham, 2003; Wang and Woolf, 2005). These include TRPV1, a heat-activated channel that also functions as the receptor for capsaicin (the pungent ingredient in chili peppers) and TRPM8, a cold-activated channel that is targeted by menthol and other cooling agents (Jordt et al., 2003).

TRPA1 (formerly ANKTM1) is yet another member of the TRP channel family that has attracted attention for its potential role in nociception. Heterologously expressed TRPA1 channels can be activated by isothiocyanate or thiosulfinate compounds, which constitute the pungent ingredients of mustard oil and garlic, respectively (Bandell et al., 2004; Bautista et al., 2005; Jordt et al., 2004; Macpherson et al., 2005). Topical application of these agents has long been known to excite sensory nerve fibers, thereby producing acute pain and neurogenic inflammation through peripheral release of neuropeptides (substance P and CGRP), purines, and other transmitters from activated nerve endings. This, in turn, produces robust hypersensitivity to thermal and mechanical stimuli. TRPA1 is selectively expressed by a subset of unmyelinated, peptidergic nociceptors that also express TRPV1, consistent with its involvement in this signaling pathway (Bautista et al., 2005; Story et al., 2003). Indeed, neuronal sensitivity to mustard oil correlates with TRPA1 expression in vivo (Jordt et al., 2004; Nagata et al., 2005), but whether this channel represents the exclusive target of mustard-oil action has not been directly determined. A related and very important issue concerns the extent to

which TRPA1 serves as a receptor for other environmental or metabolic irritants that act on sensory nerve endings within the lung, bladder, or other visceral and vascular organs to produce inflammation, vasodilation, and edema.

A topic of particularly vigorous debate concerns endogenous physiological roles for TRPA1 in sensory transduction. Story et al. have reported that heterologously expressed TRPA1 channels are activated when ambient temperatures drop below 17°C, leading them to propose that TRPA1 functions as a detector of noxious cold in vivo (Story et al., 2003). However, cold sensitivity of recombinant TRPA1 channels has not been universally observed. Moreover, the percentage of neurons in rat trigeminal ganglia that express TRPA1 or respond to mustard oil is significantly greater than that exhibiting cold sensitivity, and thus, a role for TRPA1 in cold transduction remains controversial (Jordt et al., 2004; Nagata et al., 2005; Reid, 2005). In addition to its proposed role as a cold sensor, histological and RNAi studies have led to the suggestion that TRPA1 constitutes a component of mechanically gated transduction channels in auditory hair cells (Corey et al., 2004). However, heterologously expressed TRPA1 channels have not been shown to be mechanosensitive, nor have hair cells been shown to respond to mustard oil or other TRPA1 agonists. Thus, direct physiological, pharmacological, or genetic evidence to support a role for TRPA1 in mechanosensation is still lacking.

In addition to being directly gated by physical or chemical stimuli, many TRP channels are activated or modulated downstream of neurotransmitter or growth-factor receptors that stimulate phospholipase C (PLC) (Montell et al., 2002). In fact, in vitro studies have shown that TRPA1 can be activated in this manner, raising the possibility that it functions as a "receptor-operated" channel that depolarizes nociceptors in response to proalgesic or proinflammatory agents that activate PLC (Bandell et al., 2004; Jordt et al., 2004). One such agent is bradykinin, a nonapeptide that is produced in response to tissue injury, inflammation, or ischemia and which binds to PLC-coupled (BK_2) receptors on sensory neurons (McMahon et al., 2006). Bradykinin elicits acute pain through immediate excitation of nociceptors, followed by a longer lasting sensitization to thermal and mechanical stimuli (Dray and Perkins, 1993). Genetic and electrophysiological studies suggest that bradykinin-evoked thermal hypersensitivity is produced through PLC-mediated potentiation of TRPV1 (Cesare et al., 1999; Chuang et al., 2001; Premkumar and Ahern, 2000). In contrast, the molecular mechanism (or mechanisms) underlying acute depolarization are less clear but likely involve activation of other cationic channels in addition to TRPV1 (Dunn and Rang, 1990; Kollarik and Undem, 2004). Whether TRPA1 contributes to acute excitation or sensitization of nociceptors by bradykinin, or other proalgesic agents, has yet to be determined.

Here we address a number of these questions by characterizing cellular and behavioral deficits in mice lacking functional TRPA1 channels. First, we prove that TRPA1 constitutes the obligate site of mustard oil or garlic action on nociceptors since mice lacking this channel are completely unresponsive to isothiocyanate or thiosulfinate compounds. We also show that TRPA1 is targeted by other environmental irritants, such as unsaturated aldehydes present in smoke or produced by drug metabolism, which elicit pain syndromes associated with inflammation and edema. With regard to thermal sensitivity, we found that lack of TRPA1 has no effect on the prevalence of cold-sensitive primary afferent neurons or the magnitude of their responses, suggesting that TRPA1 plays no significant role in acute cold detection. Similarly, TRPA1-deficient mice exhibit no overt vestibular defects and display normal auditory responses, suggesting that TRPA1 is not required for hearing. In contrast, TRPA1-deficient mice showed substantially decreased responses to bradykinin at the cellular and behavioral level. These observations indicate that TRPA1 is an important component of the transduction machinery that depolarizes nociceptors in response to endogenous and environmental irritants or proalgesic agents that elicit inflammatory pain.

RESULTS

TRPA1 Is the Sole Site of Mustard-Oil and Garlic Action

We generated TRPA1-deficient mice through targeted deletion of a genomic region encoding the presumptive pore-loop domain of the ion channel (Figure 1A; see also Figure S1 in the Supplemental Data available with this article online). The resulting *TRPA1*$^{-/-}$ mice were normal in overall appearance and viability, and matings between heterozygous animals produced wild-type, heterozygous, and homozygous mutant male and female offspring with expected Mendelian ratios. RT-PCR using primers that span the deleted coding region showed that normal TRPA1 transcripts were absent from trigeminal ganglia of *TRPA1*$^{-/-}$ animals (Figure 1B). In contrast, TRPM8-derived PCR products were readily observed in ganglia from *TRPA1*$^{+/+}$ or *TRPA1*$^{-/-}$ mice.

Most, if not all, TRPA1-positive cells are peptidergic (Bautista et al., 2005; Story et al., 2003), and we therefore asked whether the prevalence of substance P-expressing neurons is affected by the absence of TRPA1. Wild-type and mutant mice exhibited the same prevalence (25%) of substance P immunoreactive cells within trigeminal or dorsal root ganglia (Figure 1C). Similarly, capsaicin receptor expression was comparable in wild-type and mutant mice as determined by immunohistochemical staining with a TRPV1-specific antiserum (Figure 1C).

To determine whether TRPA1 is solely responsible for sensitivity to mustard oil (MO) or garlic, we used live-cell calcium imaging to quantify responses of cultured trigeminal neurons to the relevant pungent compounds, allyl isothiocyanate or allicin, respectively. Twenty-five percent of neurons from newborn (P0) wild-type mice showed rapid and robust responses to bath-applied MO (50 μM; Figures 2A and 2B) or allicin (200 μM; Figure 2B). As observed previously in rat neurons (Bautista et al., 2005), all

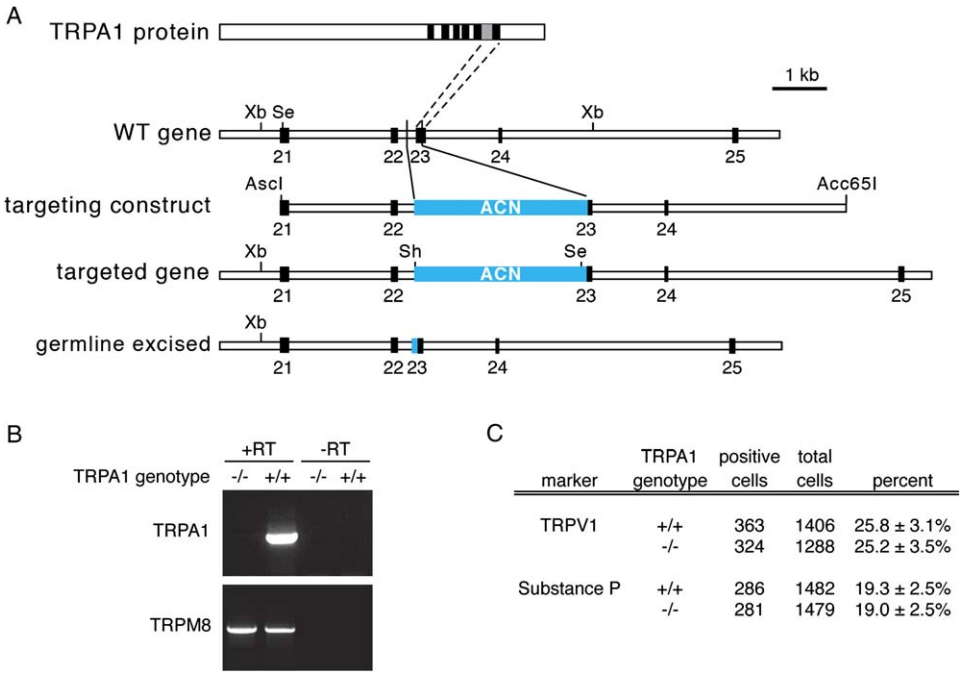

Figure 1. Generation of TRPA1-Deficient Mice

(A) Targeting strategy for disruption of the *TRPA1* gene. Transmembrane and pore-loop protein domains are indicated by black and gray bars, respectively. Exons are numbered and shown as black bars on genomic map. Xb, XbaI; Se, SpeI; Sh, SphI. Blue bar depicts self-excising ACN neomycin expression cassette.

(B) TRPA1 primers designed to amplify a 683 bp fragment within the coding region (beginning at exon 23; see Figure S1) do not generate a product using first-strand template cDNA from *TRPA1*$^{-/-}$ trigeminal ganglia. As a positive control, an 859 bp portion of TRPM8 was amplified from normal and TRPA1-deficient ganglia.

(C) Immunostaining of trigeminal ganglion shows normal prevalence of TRPV1- and substance P-expressing neurons in *TRPA1*$^{-/-}$ mice.

MO-sensitive trigeminal neurons from wild-type mice were dually responsive to capsaicin (1 μM), accounting for approximately half of all TRPV1-expressing cells. In contrast, neurons from TRPA1-deficient mice were completely insensitive to MO or allicin (Figures 2A and 2B), demonstrating that TRPA1 is the obligate site of action for both isothiocyanate and thiosulfinate irritants. Importantly, the prevalence of capsaicin-responsive cells was identical in wild-type versus mutant neurons, consistent with the anatomical results described above. Similar results were obtained with neurons from dorsal root ganglia (wild-type: 24% MO-sensitive, 65% capsaicin-sensitive; TRPA1-deficient: 0% MO-sensitive, 63% capsaicin-sensitive).

Interestingly, cultures prepared from *TRPA1*$^{+/-}$ mice showed only one-third as many MO-sensitive neurons compared to wild-type controls (10% versus 25%, respectively), and responses were generally smaller or developed more slowly in neurons from heterozygous animals (Figure 2B and data not shown). This haploinsufficiency phenotype suggests that the number of functional TRPA1 channels is limiting such that changes in their expression or sensitivity can alter neuronal excitation in a linear and dynamic fashion. It should be mentioned, however, that RT-PCR analysis using primers located upstream of the deleted region amplify a fragment from trigeminal ganglia of *TRPA1*$^{-/-}$ mice (Figure S1), indicating that a truncated transcript is produced from the disrupted allele. Thus, it is formally possible that this truncated transcript produces a protein fragment (lacking the pore loop and sixth transmembrane domain) having dominant-negative activity, which could contribute to the apparent haploinsufficiency phenotype. Unfortunately, direct detection of such a protein fragment is not possible with existing antibodies, which are directed to the carboxy-terminal tail of the channel. Finally, while the putative TRPA1 fragment could have other effects through interaction with unspecified protein partners, sensory ganglia from TRPA1-deficient mice show no obvious deficits in capsaicin sensitivity, menthol sensitivity, or calcium handling (Figure 2 and data not shown), and thus we have no direct evidence to support a dominant-negative effect. In any event, our results clearly show that functional ablation of the *TRPA1* gene leads to selective and complete loss of isothiocyanate and thiosulfinate sensitivity without generally affecting cell type specification or sensitivity to other chemical stimuli.

Topical application of mustard oil to the hindpaw produces an acute noxious (nocifensive) response, followed by neurogenic inflammation and robust hypersensitivity

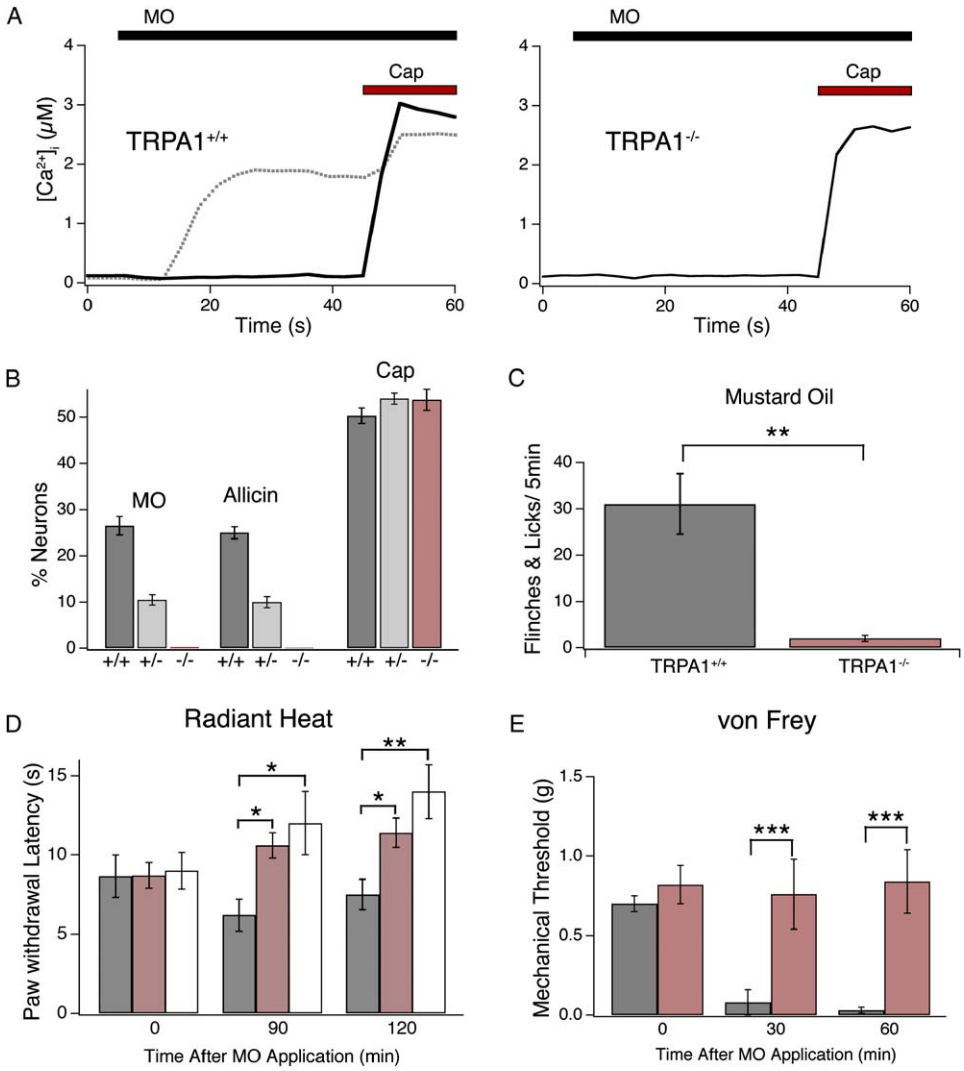

Figure 2. TRPA1-Deficient Mice Are Insensitive to Mustard Oil

(A) Trigeminal neurons from *TRPA1*$^{+/+}$ (left) and *TRPA1*$^{-/-}$ (right) mice were exposed to mustard oil (MO, 100 μM) followed by capsaicin (Cap, 1 μM), and responses were assessed by calcium imaging. Among Cap-sensitive neurons from wild-type animals, both MO-sensitive (dotted trace) and MO-insensitive (solid trace) subpopulations were observed. No MO-sensitive cells were detected from TRPA1-deficient mice.

(B) Prevalence of neurons responding to MO (100 μM), allicin (100 μM), or Cap (1 μM) was determined for *TRPA1*$^{+/+}$ (black), *TRPA1*$^{+/-}$ (gray), and *TRPA1*$^{-/-}$ (red) mice.

(C) Behavioral responses (paw licks or flinches) to MO were measured for *TRPA1*$^{+/+}$ and *TRPA1*$^{-/-}$ mice (n = 6 mice/genotype; 5 min duration).

(D) Changes in radiant heat threshold were measured after application of MO to the hindpaw of *TRPA1*$^{+/+}$ (black) or *TRPA1*$^{-/-}$ (red) mice. Control measurements were made using the untreated contralateral hindpaw (white). Baseline responses were not significantly different between genotypes. Significant differences were observed when comparing *TRPA1*$^{+/+}$ ipsilateral paw latencies to the *TRPA1*$^{+/+}$ contralateral paw or *TRPA1*$^{-/-}$ ipsilateral paw ($p < 0.001$, $F = 10$; two-way ANOVA). Differences across genotypes at 90 and 120 min post-MO were further analyzed using one-way ANOVA with Tukey's HSD post hoc analyses.

(E) Changes in von Frey hair threshold were measured after MO application to the hindpaw. Baseline responses were not significantly different. Significant differences were observed between and *TRPA1*$^{+/+}$ and *TRPA1*$^{-/-}$ at 30 and 60 min post-MO ($p < 0.001$, $F = 16$; two-way ANOVA) and were further analyzed using one-way ANOVA with Tukey's HSD post hoc analyses. Comparisons between wild-type and knockout animals were made using littermates, and experiments were performed blind to genotype. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n = 9 per genotype.) All error bars represent standard error of the mean (SEM).

to thermal and mechanical stimuli. We found that acute behavioral responses (licking and flinching) were abolished in *TRPA*$^{-/-}$ mice, whereas wild-type controls showed characteristically robust behavior (Figure 2C). Moreover, TRPA1-deficient animals showed substantially reduced mustard-oil-evoked swelling of the treated hindpaw compared to wild-type mice (7% versus 22% increase in thickness). We also measured sensitivity to

thermal and mechanical stimuli before and after mustard-oil treatment. For wild-type mice, paw-withdrawal latencies from a radiant heat source were characteristically reduced after mustard-oil treatment. These animals also showed a dramatic decrease in the mechanical threshold for eliciting paw withdrawal (Figures 2D and 2E). In contrast, TRPA1-deficient mice showed no hypersensitivity to either stimulus following mustard-oil treatment. The slight decrease in thermal sensitivity observed in mutant animals reflects behavioral desensitization as evidenced by analysis of the contralateral (control untreated) hindpaw of wild-type mice, where the same degree of desensitization was observed. These experiments demonstrate that TRPA1 is essential for mediating sensitivity to mustard oil at both the cellular and behavioral level. Importantly, wild-type and mutant mice did not differ in baseline thermal or mechanical thresholds, suggesting that TRPA1 is not required for normal acute sensitivity to heat or pressure.

TRPA1 Mediates Responses to Acrolein and Related Volatile Irritants

A variety of volatile compounds activate sensory nerve endings in airways, inducing neurogenic inflammation and exacerbating chronic cough or asthma (Handzel, 2000; Leikauf, 2002). One such irritant is acrolein (2-propenal), a highly toxic and reactive α,β-unsaturated aldehyde present in tear gas, vehicle exhaust, and smoke from burning vegetation, including tobacco products (Hales et al., 1988, 1992). Acrolein can induce apnea, shortness of breath, cough, airway obstruction, and mucous secretion (Ghilarducci and Tjeerdema, 1995). Acrolein is also a toxic metabolite of cyclophosphamide and ifosfamide, chemotherapeutic agents widely used in the treatment of cancer, severe arthritis, multiple sclerosis, and lupus (Fleming, 1997; Nicol, 2002). Responses to acrolein are mediated by capsaicin-sensitive, peptidergic primary afferent fibers (Ahluwalia et al., 1994; Maggi et al., 1992; Morris et al., 1999; Springall et al., 1990; Turner et al., 1993). Additionally, the structurally related irritant 2-pentenal activates capsaicin-sensitive trigeminal neurons in culture (Inoue and Bryant, 2005). However, heterologously expressed TRPV1 does not respond to acrolein (Dinis et al., 2004), and TRPV1-deficient mice react normally to this irritant (Symanowicz et al., 2004), ruling out a role for the capsaicin receptor as a direct target of acrolein action.

TRPA1 is expressed by capsaicin-sensitive neurons and is therefore an attractive candidate for mediating the inflammatory actions of acrolein and structurally related irritants (Figure 3). Because α,β-unsaturated double bonds are crucial for MO and allicin activity, we hypothesized that TRPA1 may also be sensitive to α,β-unsaturated aldehydes such as acrolein. Indeed, we found that acrolein or 2-pentenal (10 μM) produced robust calcium increases in TRPA1-transfected cells but not in those expressing TRPV1, TRPV2, or TRPM8 (Figure 4A and data not shown). Moreover, congeners that have little or no activity

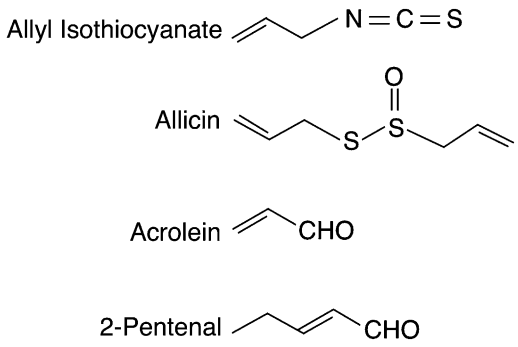

Figure 3. TRPA1 Is Activated by Structurally Related Isothiocyanate, Thiosulfinate (Allicin), and Aldehyde (Acrolein and 2-Pentenal) Irritants

at sensory neurons, such as propionaldehyde, acrylic acid, and 1-propanol, were inactive at the cloned receptor (data not shown). We observed an identical pharmacological profile with TRPA1-expressing oocytes, where acrolein elicited large sustained membrane currents that were attenuated by ruthenium red, which blocks a number of TRP channels, including TRPA1. Dose-response analysis revealed a half maximal effective concentration (EC_{50}) of 5 ± 1 μM (Figure 4B). In trigeminal cultures from wild-type mice, acrolein and pentenal evoked large increases in calcium specifically within the subset of capsaicin- and MO-sensitive neurons. Acrolein-evoked responses were completely absent in cultures from TRPA1-deficient mice (Figure 4C), demonstrating that TRPA1 is, indeed, an essential site for acrolein action.

Sensitivity to Cold Is Preserved in the Absence of TRPA1

To address the controversial role of TRPA1 in the detection of cold, we compared the sensitivity of wild-type and TRPA1-deficient trigeminal neurons to cold thermal stimuli. Sixteen percent of sensory neurons from wild-type animals showed robust increases in calcium when the bath temperature was reduced from ~23°C to 6°C. As previously observed with rat sensory neurons (McKemy et al., 2002; Reid and Flonta, 2002), the majority of these cold-sensitive cells (78%) responded to a saturating dose of menthol (250 μM), suggesting that TRPM8 accounts for their cold sensitivity (Figure 5A). The remaining cold-sensitive cells (22%) were insensitive to menthol or MO, consistent with the involvement of a TRPM8/TRPA1-independent mechanism for cold sensitivity in this subpopulation of trigeminal neurons. Furthermore, the prevalence and magnitude of cold- or menthol-evoked responses were identical in trigeminal neurons from *TRPA1*$^{-/-}$ and *TRPA1*$^{+/+}$ ganglia (Figure 5A). Similar results were obtained with cold-sensitive neurons from normal and mutant dorsal root ganglia (wild-type: 64% menthol-sensitive, 36% menthol-insensitive; TRPA1-deficient: 60% menthol-sensitive, 40% menthol-insensitive).

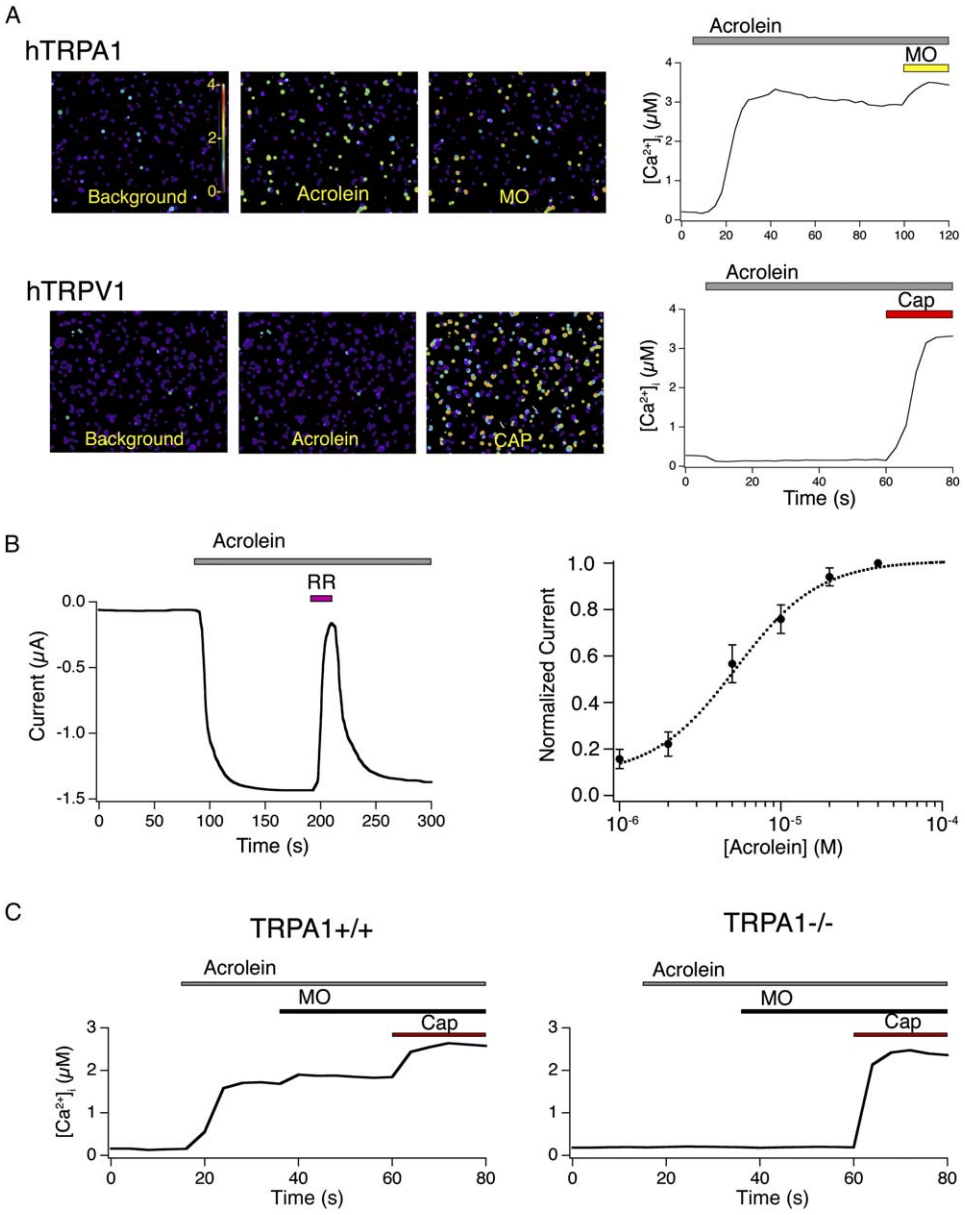

Figure 4. TRPA1 Is Required for Acrolein Sensitivity

(A) Acrolein (20 μM) evokes calcium influx into hTRPA1-expressing HEK293 cells (top). Cells transfected with hTRPV1 (bottom) are insensitive to acrolein but respond to capsaicin (1 μM). Pseudocolor images of Fura-2-loaded cells; scale bar shows intracellular calcium concentration (μM, left). Graphs display calcium responses as a function of time (right).

(B) Representative current trace (left) and dose-response curve (right) for activation of hTRPA1 by acrolein in voltage-clamped ($V_h = -60$ mV) *Xenopus* oocytes. Currents were normalized to maximal response obtained with saturating (>100 μM) acrolein; $EC_{50} = 5 \pm 1$ μM (n = 5 oocytes/data point).

(C) Responses of trigeminal neurons from *TRPA1*$^{+/+}$ (left) and *TRPA1*$^{-/-}$ (right) mice to acrolein (100 μM), MO (100 μM), and Cap (1 μM) were measured by calcium imaging. Left trace displays average of 100 MO-sensitive neurons; right trace shows average of 100 Cap-sensitive neurons. All error bars represent standard error of the mean (SEM).

We also assessed behavioral cold sensitivity by measuring acute nocifensive responses to acetone-evoked evaporative cooling or contact with a cold surface. There were no significant differences in the number of acetone-evoked paw flinches by wild-type or TRPA1-deficient mice (Figure 5B). Moreover, latencies for paw withdrawal or shivering in the cold-plate assay did not differ with genotype (Figure 5C). Finally, when allowed to choose between adjacent surfaces adjusted to room temperature versus 15°C or 10°C, all animals spent >98% of the allotted time (5 min) on the room-temperature side, irrespective of genotype (data not shown). These findings

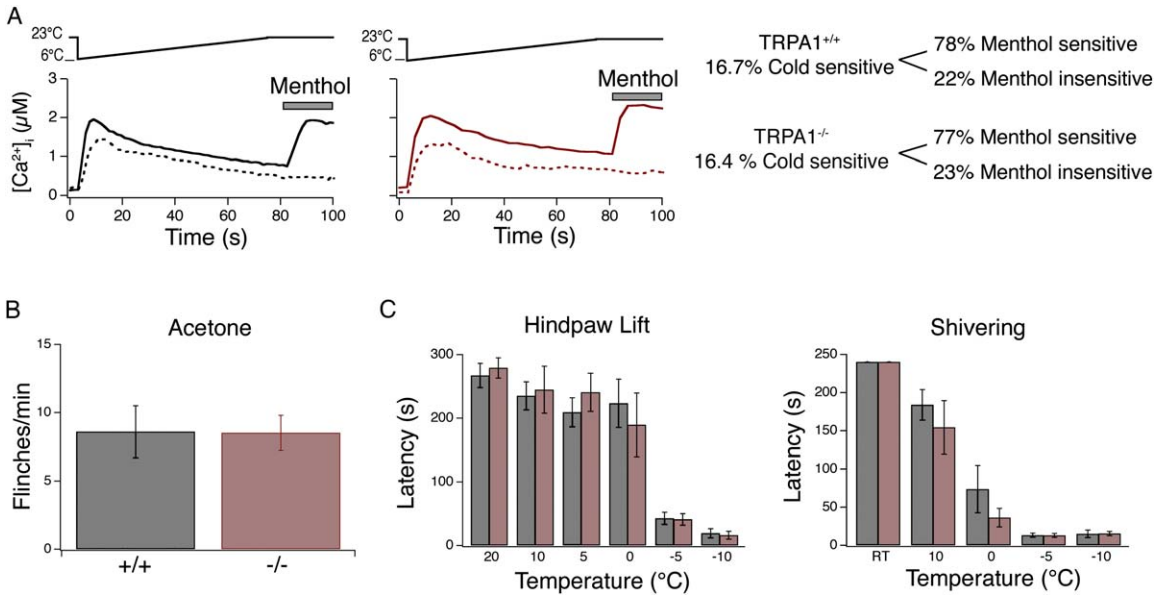

Figure 5. TRPA1 Is Not Required for Normal Cold Sensitivity

(A) Trigeminal neurons from *TRPA1*$^{+/+}$ (left, black) or *TRPA1*$^{-/-}$ (middle, red) mice were challenged with cold (6°C) perfusate followed by menthol (500 μM), and responses were measured by calcium imaging. Among cold-sensitive neurons, both menthol-sensitive (solid) and -insensitive (dashed) populations were observed independent of genotype.

(B) Licking and flinching in response to evaporative cooling was measured following application of acetone (20 μl) to the hindpaw (n = 13 mice/genotype). No significant differences were observed (one-way ANOVA with Tukey's HSD post hoc analyses).

(C) Paw-withdrawal latency (left) or time to first shiver (right) was measured following placement of mice on an aluminum surface cooled to the indicated temperature. Responses were not significantly different (lift latency: $p < 0.93$, $F = 0.009$; shiver latency: $p < 0.25$, $F = 2$; two-way ANOVA). Comparisons were made using littermates while blind to genotype (n = 13 mice/genotype). All error bars represent standard error of the mean (SEM).

are consistent with a lack of involvement of TRPA1 in the acute detection of cold.

TRPA1-Deficient Mice Have Normal Auditory Responses

Sensory hair cells in the inner ear use mechanosensitive channels to transduce auditory and vestibular stimuli into electrical signals. TRPA1 has been proposed as a candidate mechanosensitive transduction channel in the vertebrate inner ear (Corey et al., 2004). If TRPA1 is an essential component of this transduction machinery, then mice lacking the channel should display aberrant vestibular and/or auditory phenotypes. However, we did not observe overt vestibular phenotypes in TRPA1-deficient mice, such as circling, altered gait or balance, or poor righting behavior. Additionally, motor responses to auditory stimuli (Preyer's test) were normal in these mice. To evaluate the status of inner hair-cell function, we analyzed auditory brainstem responses to a variety of sound-pressure levels. TRPA1-deficient mice were indistinguishable from wild-type littermates in that they exhibited similar characteristic responses to both broadband clicks and pure tones of 8, 16, and 32 kHz stimuli (Figure 6A). Moreover, *TRPA1*$^{+/+}$ and *TRPA1*$^{-/-}$ mice yielded similar distortion product (Figure 6B), indicative of normal outer hair-cell function.

TRPA1 Is Required for Bradykinin-Evoked Excitation of Sensory Neurons

Based on heterologous-expression studies, we and others proposed that TRPA1 functions as a receptor-operated channel, depolarizing nociceptors in response to proalgesic or proinflammatory agents—such as bradykinin, ATP, monoamines, and neurotrophins—that activate PLC signaling pathways (Bandell et al., 2004; Jordt et al., 2004). To test this possibility, we used calcium imaging to examine responses of trigeminal neurons to bradykinin (10 nM) (Figure 7A). In cultures from wild-type ganglia, 19% of neurons showed a moderate rise in calcium due to release from intracellular stores. Another 30% of neurons exhibited responses of greater magnitude, virtually all of which (97%) were MO sensitive. A significant component of this larger response was due to calcium influx, as it was attenuated by chelation of extracellular calcium (EGTA) or addition of ruthenium red (Figures 7A and 7B). Importantly, the magnitude of bradykinin-evoked calcium responses (peak or integral) correlated with the size of the MO-evoked response but not with capsaicin (Figure 7C) or high KCl (75 mM; data not shown), consistent with the notion that bradykinin elicits robust calcium influx by activating TRPA1 in a subset of sensory neurons. This was confirmed by the fact that trigeminal neurons from *TRPA1*$^{-/-}$ mice showed a significant attenuation of responses to bradykinin (Figures 7A–7C).

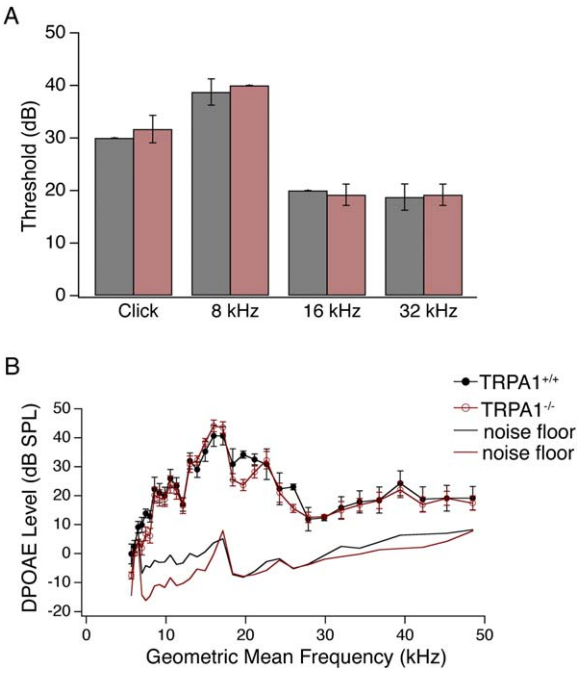

Figure 6. TRPA1-Deficient Mice Have Normal Hearing

(A) ABR thresholds were measured from the right ears of *TRPA1*$^{+/+}$ (black, n = 5) and *TRPA1*$^{-/-}$ (red, n = 6) mice in response to broadband clicks and 3 ms pure tones of 8, 16, and 32 kHz (mean response ± SD). No significant differences were observed between genotypes.

(B) Mean DP-grams for *TRPA1*$^{+/+}$ (n = 5) and *TRPA1*$^{-/-}$ (n = 6) mice (5–6 weeks old) were tested by measuring levels of the $2f_1$-f_2 DPOAE over a geometric-mean frequency range from 5.6 to 48.5 kHz, using an f_2/f_1 of 1.25 and primary tone stimuli at $L_1 = L_2 = 75$ SPL. *TRPA1*$^{+/+}$ and *TRPA1*$^{-/-}$ mice produced similar distortion product. Noise floors for both genotypes are displayed (solid lines). All error bars represent standard error of the mean (SEM).

Bradykinin elicits thermal hyperalgesia by sensitizing TRPV1 to heat (Cesare and McNaughton, 1996; Chuang et al., 2001). Although TRPV1 does not behave as a receptor-operated channel per se, bradykinin-evoked PLC activation decreases its normal activation threshold (~43°C) such that small but appreciable currents can be observed even at room temperature (Chuang et al., 2001). Because TRPA1 is always coexpressed with TRPV1, this raises the possibility that TRPV1 acts in concert with TRPA1 to activate nociceptors in response to bradykinin. Indeed, bradykinin-evoked responses were significantly attenuated in *TRPV1*$^{-/-}$ trigeminal neurons, resembling the deficit observed with *TRPA1*$^{-/-}$ cells (Figures 7A–7C). Moreover, while the magnitude of MO-evoked responses was normal in TRPV1-deficient neurons, the correlation between MO and bradykinin sensitivity was lost (Figure 7C), providing evidence for a functional coupling between TRPA1 and TRPV1 in the bradykinin response pathway.

Given the requirement for TRPA1 in the cellular actions of bradykinin, we asked whether TRPA1-deficient mice also exhibit behavioral deficits in bradykinin-evoked hyperalgesia. We injected bradykinin into the hindpaw of mice and measured paw withdrawal latencies from a radiant heat source. Wild-type mice showed a characteristic decrease in latency within minutes of injection, whereas *TRPA1*$^{-/-}$ mice showed no evidence of hypersensitivity (Figure 7D). The slight decrease in thermal sensitivity seen in mutant animals reflects behavioral desensitization as evidenced by analysis of the contralateral (control untreated) hindpaw of wild-type mice, where the same decrease in sensitivity was observed. Injection with complete Freund's adjuvant (CFA), which produces wholesale inflammation via both neurogenic and nonneurogenic mechanisms, induced robust sensitization in wild-type or mutant animals (Figure 7E). This important control shows that TRPA1-deficient mice are not generally incapable of mounting an inflammatory response and, unlike TRPV1-deficient mice, are not generally defective in their ability to develop thermal hyperalgesia irrespective of the inflammatory mechanism. Our findings suggest that TRPA1 is a downstream target for specific components of the inflammatory milieu, such as bradykinin, that elicit nociceptor excitation and pain hypersensitivity.

DISCUSSION

TRPA1 has been proposed to contribute to a variety of sensory processes, including thermal (cold) nociception, mechanosensation, and inflammatory hyperalgesia. While our results do not support a requirement for this channel in the initial detection of cold or sound, they clearly demonstrate a role for TRPA1 in mechanisms of neurogenic inflammation and pain hypersensitivity. This is dramatically illustrated for a variety of exogenous irritants—including isothiocyanates, thiosulfinates, and α,β-unsaturated aldehydes—whose ability to excite nociceptors is entirely dependent on TRPA1 expression. We also show that TRPA1 is an important downstream target of inflammatory agents, such as bradykinin, that produce nociceptor excitation and/or hypersensitivity via their actions on PLC signaling pathways.

TRPA1 and Environmental Irritants

Different plant genera (e.g., *Capsicum* versus *Brassica* and *Allium*) have evolved chemically distinct defensive strategies that target different molecular sites (TRPV1 versus TRPA1) on primary afferent neurons of the pain pathway. Despite such segregation at the molecular level, these agents elicit similar physiological responses (irritation and inflammation) by exciting an overlapping subpopulation of peptidergic nociceptors. Mustard-oil treatment has long been used as an important experimental paradigm for generating and studying mechanisms of inflammatory pain. Our findings now prove that TRPA1 is the obligate site through which MO and chemically related irritants mediate their actions on the primary afferent nervous system.

Based on in vitro studies, it has been suggested that the pungency of garlic is mediated through activation of both TRPA1 and TRPV1 (Macpherson et al., 2005). However,

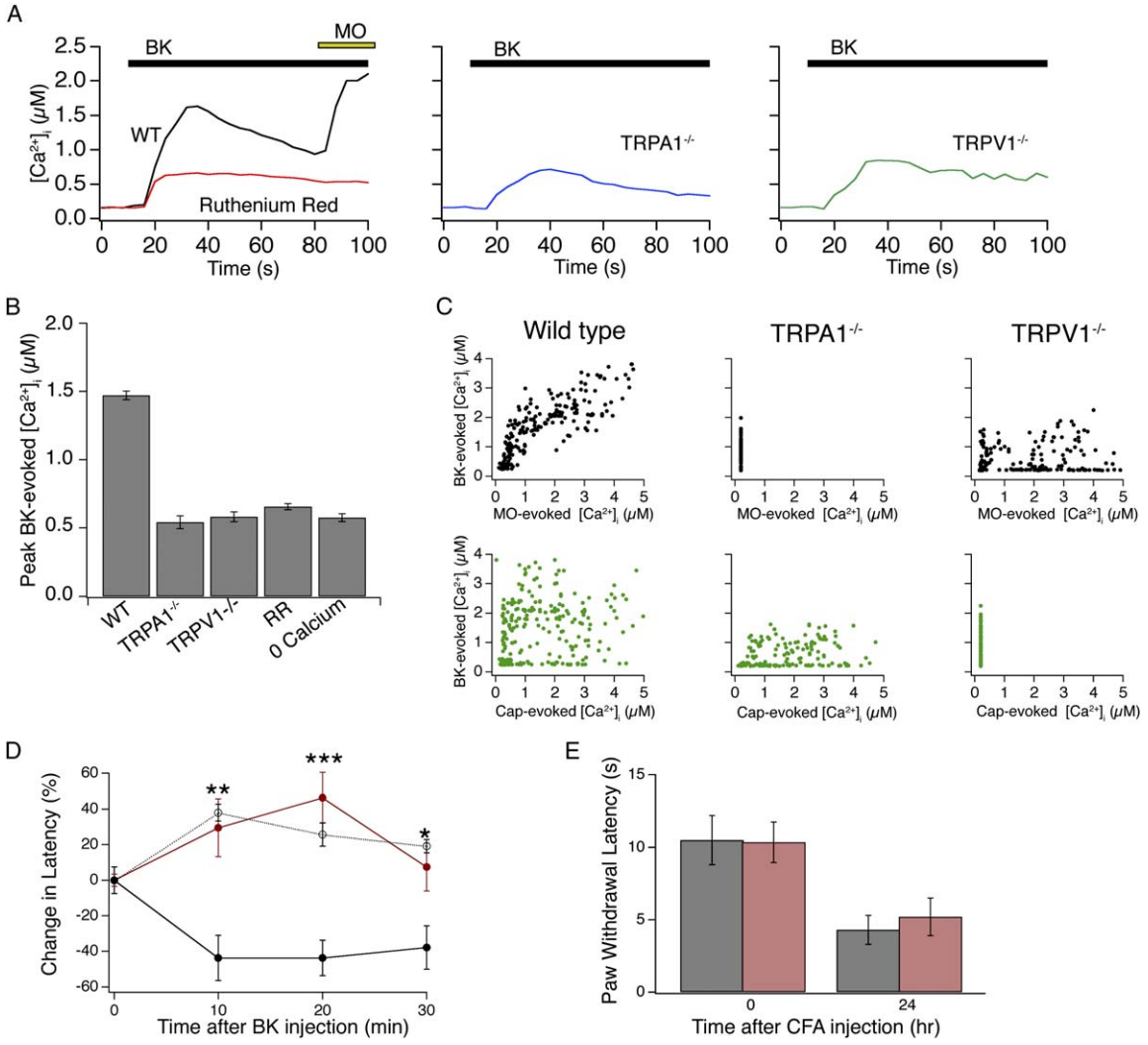

Figure 7. Both TRPA1 and TRPV1 Are Required for Bradykinin-Evoked Thermal Hyperalgesia

(A) Application of bradykinin (BK, 10 nM) to cultured trigeminal neurons from wild-type mice elicits robust, ruthenium red (RR) sensitive calcium influx in MO-responsive cells (left). TRPA1-deficient neurons displayed small BK-evoked responses resembling those observed with wild-type cells in the presence of RR (middle). Similar results were obtained with neurons from TRPV1-deficient animals (right). RR caused no reduction of calcium responses in TRPA1- or TRPV1-deficient neurons (data not shown).

(B) Bar graph compares average peak intracellular calcium responses for all genotypes or for wild-type neurons in the presence of RR or absence of extracellular calcium (n = 300–1000 cells/category). Similar results were obtained when comparing integrals of responses (data not shown).

(C) Sensitivity to BK and MO are correlated in sensory neurons. Scatter plots display relationship between BK- and MO- or Cap-evoked calcium responses in neurons from wild-type, *TRPA1*$^{-/-}$, or *TRPV1*$^{-/-}$ mice. Each point denotes peak calcium response from a single cell.

(D) Paw-withdrawal latencies from a radiant heat source were measured at various times after BK injection. Baseline responses were not significantly different. Significant differences in latencies were observed between *TRPA1*$^{+/+}$ ipsilateral paw (black) compared to the control *TRPA1*$^{+/+}$ contralateral paw (dashed) or the *TRPA1*$^{-/-}$ ipsilateral paw (red) ($p < 0.000015$, $F = 25$; two-way ANOVA). Differences at 10, 20, and 30 min were further analyzed using one-way ANOVA with Tukey's HSD post hoc analyses ($p < 0.009$, $p < 0.0007$, and $p < 0.05$, respectively). No significant differences were observed between *TRPA1*$^{+/+}$ contralateral and *TRPA1*$^{-/-}$ ipsilateral latencies.

(E) Paw-withdrawal latencies from a radiant heat source were measured before and after CFA injection. No significant difference was observed between wild-type (black) and mutant (red) animals ($p < 0.4$, $F = 0.9$; two-way ANOVA). Differences before and after CFA treatment were significant in both *TRPA1*$^{+/+}$ and *TRPA1*$^{-/-}$ mice (one-way ANOVA with Tukey's post hoc; $p < 0.000009$ and $p < 0.000005$, respectively). All error bars represent standard error of the mean (SEM).

we have shown that cultured neurons from TRPV1-deficient and wild-type mice do not differ in their sensitivity to allicin or diallyl disulfide, suggesting that TRPV1 does not contribute significantly to the pungency of these agents in vivo (Bautista et al., 2005). The complete loss of allicin-evoked responses among trigeminal neurons from *TRPA1*$^{-/-}$ mice supports this conclusion and demonstrates that TRPA1 is the sole site through which the

pungent ingredients of mustard and garlic mediate their excitatory actions.

Original estimates of TRPA1-expressing neurons within mouse dorsal root ganglia were significantly lower than the prevalence of MO-sensitive neurons observed in trigeminal cultures from rat. Moreover, neuronal responses to the TRPA1 agonist cinnamaldehyde were less prevalent than those evoked by isothiocyanates (Bandell et al., 2004). Based on these observations, it has been suggested that cinnamaldehyde is a more selective TRPA1 agonist than MO and that the latter may act at multiple receptor sites. Our results with TRPA1-deficient mice now show that cinnamaldehyde underestimates the prevalence of TRPA1-positive neurons and that sensitivity to MO is specific to cells expressing TRPA1. Indeed, this is consistent with more recent studies examining the expression of TRPA1 in trigeminal or dorsal root ganglia using histological methods (Bautista et al., 2005; Kobayashi et al., 2005; Nagata et al., 2005).

Another important finding of our study is that TRPA1 is essential for the action of acrolein. Acrolein initiates neuropeptide release from capsaicin-sensitive nerve endings in the lung or bladder wall, and thus the involvement of primary afferent fibers in the initial steps of acrolein toxicity was well established (Ahluwalia et al., 1994; Morris et al., 1999; Springall et al., 1990; Turner et al., 1993). However, the molecular target through which acrolein and other α,β-unsaturated aldehydes mediate their effects remained unknown. These reactive and volatile irritants pose numerous health risks associated with their ability to promote neurogenic inflammation through activation of sensory nerve endings in the airways (Handzel, 2000; Leikauf, 2002; Undem and Carr, 2001). Indeed, acrolein is a major causative agent of smoke poisoning, producing lung edema and other forms of respiratory irritation (Ghilarducci and Tjeerdema, 1995; Leikauf et al., 1989). Identification of TRPA1 as a nociceptor-specific site of acrolein toxicity provides a pharmacological target for treating pulmonary edema and respiratory irritation caused by environmental irritants of this chemical class—a problem that is especially relevant to individuals with chronic cough or asthma.

Acrolein toxicity is also an important factor in the side-effect profile of chemotherapeutic regimens that use cyclophosphamide or ifosfamide to suppress tumor growth or autoimmunity. Metabolism of these drugs leads to the production and accumulation of acrolein in urine (Cox, 1979; Takamoto et al., 2004), resulting in complications such as hemorrhagic cystitis, in which permeabilization of the bladder vasculature causes severe bleeding, pain, and termination of therapy (Nicol, 2002). As in the lung, this pathophysiological response is mediated by excitation of sensory nerve endings in the bladder wall, followed by neuropeptide release, vasodilation, vascular permeabilization, and other neuroinflammatory effects (Maggi et al., 1992). TRPA1 antagonists may therefore increase the efficacy of cyclophosphamide-based chemotherapy by blocking the neuroinflammatory actions of acrolein and related metabolites.

TRPA1 and the Detection of Cold

TRPA1 was initially proposed to function as a detector of noxious cold, a hypothesis that was based on two main observations: (1) Cells heterologously expressing TRPA1 could be activated by temperatures below 17°C, and (2) only 4% of mouse dorsal root ganglion neurons were found to express TRPA1 transcripts, consistent with the idea that this channel mediates cold sensitivity among a small subpopulation of menthol-insensitive (TRPM8-negative) primary afferent neurons (Bandell et al., 2004; Story et al., 2003). However, cold-evoked responses have not been uniformly observed in cells expressing cloned rat or human TRPA1, even with extended exposure to temperatures as low as 5°C (Jordt et al., 2004; Nagata et al., 2005). Moreover, the prevalence of MO-responsive neurons exceeds the total number of cold-sensitive cells that we observed in rat or mouse trigeminal cultures, including the menthol-sensitive (TRPM8-positive) and insensitive (TRPM8-negative) populations. This discrepancy could have been explained by the existence of multiple MO receptor subtypes, but our results now show that TRPA1 is the only site at which isothiocyanate compounds mediate their effects.

Perhaps most importantly, our findings demonstrate that TRPA1 is not required for acute cold sensitivity at the cellular or behavioral level. Analysis of cultured trigeminal neurons shows no significant difference between normal and TRPA1-deficient mice in the prevalence of cold-sensitive neurons or the magnitude of their response to temperatures as low as 6°C. Thus, TRPA1 is unlikely to account for cold sensitivity exhibited by a subpopulation of menthol-insensitive neurons that we, and others, have observed in cultures from mouse or rat sensory ganglia. These responses are characterized by a thermal activation threshold of ~15°C (versus ~26°C for TRPM8), but little else is known about their biophysical or pharmacological properties, such as whether they result from the opening of cationic channels or the closing of background potassium channels (Reid, 2005). Furthermore, our behavioral studies revealed no significant differences between *TRPA1*$^{+/+}$ and *TRPA*$^{-/-}$ mice in their sensitivity to noxious cold based on their responses to a cooled surface or to acetone-evoked evaporative cooling, suggesting that TRPA1 does not function as an initial detector of noxious cold.

TRPA1 and Inflammatory Hypersensitivity

Bradykinin has long been known to have dual effects on nociceptors, including acute depolarization and a longer-lasting hypersensitivity to thermal or mechanical stimuli (Dray and Perkins, 1993; McMahon et al., 2006). However, the cellular signaling mechanisms underlying these actions have remained elusive, especially in regard to the identity of channels that mediate acute excitation. A common feature of many TRP channels is their ability to be activated or modulated by PLC-coupled signaling pathways (Montell et al., 2002), making them likely candidates for mediating either of these responses. Indeed,

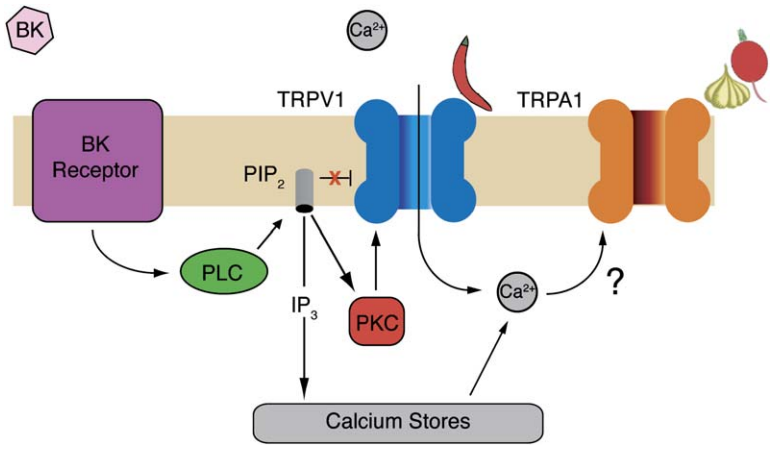

Figure 8. Model Depicting Functional Interactions between Bradykinin Receptors, TRPA1, and TRPV1

BK binds to G protein-coupled receptors on the surface of primary afferent neurons to activate phospholipase C (PLC), leading to the hydrolysis of membrane phosphatidylinositol bisphosphate (PIP_2), activation of protein kinase C (PKC), and release of calcium from intracellular stores. Consequently, the capsaicin receptor (TRPV1) is sensitized, leading to channel opening and calcium influx. We hypothesize that the mustard-oil and garlic receptor (TRPA1) opens in response to both calcium influx through TRPV1 and calcium release from intracellular stores.

TRPA1 resembles canonical receptor-operated TRP channels in that activation of the PLC pathway produces rapidly developing inward currents, even in the absence of other known stimuli. TRPA1 has therefore been proposed as a likely candidate to mediate the acute excitatory actions of bradykinin or other such proalgesic agents (Bandell et al., 2004; Jordt et al., 2004). Our findings are consistent with such a role for this channel in vivo.

PLC activation does not robustly activate TRPV1, and thus we were at first surprised to find that acute excitatory responses to bradykinin were diminished in *TRPV1*$^{-/-}$ neurons. However, PLC activation does elicit modest TRPV1 gating and calcium influx, even at room temperature (Chuang et al., 2001). Moreover, TRPA1 is always coexpressed with TRPV1, and our previous studies suggest that TRPA1 can be activated by increases in intracellular calcium (Jordt et al., 2004). If so, then bradykinin could elicit modest calcium influx through TRPV1, which would then enhance the activation of TRPA1 in these cells. In this scenario, TRPA1 contributes the bulk of the excitatory response. This is further supported by our observation that the magnitudes of MO- and bradykinin-evoked responses are no longer correlated in TRPV1-deficient neurons, as expected if TRPV1 acts upstream of TRPA1. Moreover, the haploinsufficiency phenotype that we observed (i.e., *TRPA1*$^{+/-}$ neurons show reduced MO sensitivity compared to wild-type) suggests that TRPA1 levels are limiting and that changes in its expression or physiological regulatory mechanisms (such as intracellular calcium levels) could have substantial effects on sensory neuron excitation. Our observations are consistent with a model in which TRPA1 is activated by bradykinin in two ways: through PLC-mediated increases in intracellular calcium (or other metabolites) and via calcium influx through TRPV1 (Figure 8). It is also possible that loss of TRPV1 somehow affects TRPA1 expression or vice versa. However, this seems less likely since we observed no significant changes in capsaicin or MO sensitivity in TRPA1- or TRPV1-deficient neurons, respectively. Additionally, loss of TRPA1 or TRPV1 could conceivably have an effect on bradykinin receptor expression, signaling, or calcium handling. However, our measurements of bradykinin-evoked calcium transients suggest that this is not the case for the following reasons: (1) the number of bradykinin-responsive cells is not altered in TRPV1 or TRPA1 mutants, (2) the magnitude of the calcium-release transient does not change with genotype—only the influx component is decreased in mutants, and (3) normal depolarization-evoked calcium responses are observed in neurons of all genotypes. Clearly, further studies will be required to elucidate the exact nature of the functional interaction between these excitatory TRP channels.

Consistent with the cellular deficits in bradykinin signaling, TRPA1-deficient mice were also refractory to bradykinin-evoked thermal hyperalgesia. However, in contrast to phenotypes observed with *TRPV1*$^{-/-}$ mice (Caterina et al., 2000; Davis et al., 2000), *TRPA1*$^{-/-}$ animals exhibited robust thermal hypersensitivity when challenged with complete Freund's adjuvant, which activates multiple inflammatory pathways. Because TRPV1 is itself a thermosensor, it stands to reason that animals lacking this channel exhibit deficits in thermal hyperalgesia irrespective of the mechanism of tissue injury or inflammation. In contrast, TRPA1 appears to act upstream of thermal and mechanical detectors, where it is more specifically responsive to a subset of inflammatory mediators that activate PLC pathways. Other proalgesic agents (e.g., protons and bioactive lipids) may still excite nociceptors through parallel, TRPA1-independent pathways to produce pain hypersensitivity. As such, TRPA1 antagonists may be useful for selective attenuation of specific aspects of inflammatory responses contributing to pain.

EXPERIMENTAL PROCEDURES

TRPA1 Gene Disruption

The targeting construct deleted most of exon 23, encoding the putative pore and part of the sixth transmembrane domain of mouse TRPA1 (residues 901–951). Genomic DNA flanking this exon (2.5 and 8.8 kb) was PCR amplified (KOD polymerase, Novagen/Toboyo) from clone 191A9 of the CITB BAC library (Invitrogen), subcloned, and sequenced. Fragments were transferred to pACN, a vector with a neomycin cassette that is removed through self-excision in the male germline

(Bunting et al., 1999). Linearized targeting construct was transfected into E14Tg2A.4 mouse ES cells (UCSF Core Facility). G418-resistant clones were screened for homologous recombination by PCR and verified by Southern blotting using 5′ and 3′ flanking probes as well as a neomycin cassette probe. Targeted ES cell clones were injected into C57Bl/6 blastocysts and chimeric mice mated to C57Bl/6 females. Heterozygotes were mated to produce paired littermates for all studies. Germline transmission of the mutated allele and excision of the selection cassette were verified by PCR analysis and Southern blotting. Gender of newborn pups was determined by PCR amplification of Y chromosome-specific (Sry) sequences (Koopman et al., 1991).

Histology and PCR

Cryostat sections (10 μm thick) were prepared from paraformaldehyde-fixed ganglia as described (Caterina et al., 1999) and incubated overnight at 4°C with affinity-purified guinea pig anti-mouse TRPV1 (1:2000; Julius lab) or guinea pig anti-substance P antiserum (1:20,000; gift of J. Maggio). Primary antisera were visualized with AlexaFluor 594-coupled goat anti-guinea pig secondary antibody (Invitrogen). For RT-PCR, total RNA was isolated from trigeminal ganglia of *TRPA1*$^{-/-}$ or *TRPA1*$^{+/+}$ mice, and 1 μg was used to synthesize first-strand cDNA (SuperScript II, Invitrogen) according to manufacturer's protocol. PCR was carried out using primers corresponding to nucleotides 2695–2727 and 3352–3378 for mTRPA1 and 2072–2093 and 2907–2931 for mTRPM8.

Calcium Imaging and Oocyte Electrophysiology

Dissociation and culturing of mouse sensory neurons, heterologous expression of TRPA1 in HEK293 cells or oocytes, calcium imaging, and two-electrode voltage clamp were carried out as described (Bautista et al., 2005; Jordt et al., 2004). Neuronal imaging was performed using ganglia from littermates while blind to genotype.

Behavior

Mice (20–35 g) were housed with 12 hr light/dark cycle at 21°C, and experiments were performed under the policies of the International Association for the Study of Pain and the UCSF Animal Care and Use Committee. 50% mechanical threshold was determined with von Frey hair filaments using the "up and down paradigm" (Chaplan et al., 1994), starting with 0.1 g and ending with 2.0 g filament as cutoff value. Noxious heat thresholds were determined using an adaptation of the radiant heat test (Hargreaves et al., 1988) with a cutoff value of 20 s to prevent injury. Nocifensive responses and thermal and mechanical hyperalgesia following MO application or bradykinin injection (25 g male mice) were measured as described (Caterina et al., 2000; Chuang et al., 2001). Hindpaw thickness was determined with spring-loaded caliper before and 1 and 2 hr after agonist application/injection. For acetone-evoked evaporative cooling, animals were habituated in a plastic enclosure on a mesh floor, and 0.05 ml acetone applied from a distance of 2 mm from the hindpaw. Flinching and licking was measured as described (Martinez-Caro and Laird, 2000) and repeated five times every 5 min. For cold-plate tests, the temperature of an aluminum block was controlled by fluid circulation (+20°C to −10°C). Time to first response of forelimb shivering or hindpaw lifting was recorded with a 300 s cutoff to prevent injury. Complete Freund's adjuvant (10 mg in saline, Sigma, CFA) was injected into one hindpaw (20 μl), and thermal latencies and paw thickness were measured after 24 hr. Data were analyzed using two- or one-way ANOVA with Tukey's HSD post hoc analysis. Behavioral tests were performed by one individual blind to genotype.

Auditory Brainstem Responses

Ear twitch responses were determined for wild-type and TRPA1-deficient littermates (n = 5 per genotype) with a handclap (Preyer's reflex) to grossly assess the hearing status. Mice (5–6 weeks old) were anesthetized with avertin, and auditory brainstem response (ABR) measurements were recorded as described (Dou et al., 2004). Briefly, a ground needle electrode and recording needle were placed subcutaneously in the scalp, and a calibrated electrostatic speaker coupled to a hollow ear bar was placed inside the pinna. Broadband clicks and pure tones (8, 16, and 32 kHz) were presented in the ear in 10 dB increments, from 0 to 100 dB SPL. The ABR sweeps were computer averaged (time locked with onset of 128–1024 stimuli, at 20/s) out of the continuous electroencephalographic activity. Hearing threshold was determined as the lowest intensity of sound required to elicit a characteristic waveform.

Distortion Product Otoacoustic Emissions (DPOAE)

Mice were anesthetized with ketamine (95 mg/kg) and xylazine (4 mg/kg). The f_1 and f_2 primary tones were generated by a two-channel frequency synthesizer (HP 3326A) presented over two tweeters (Realistic) and delivered through a soft rubber probe. Ear-canal sound pressure was measured with a commercial acoustic probe (Etymotic Research 10B$^+$). Ear-canal sound pressure was sampled and synchronously averaged (n = 8) by a digital signal processor for frequencies <20.1 kHz and a dynamic signal analyzer (HP 3561A) for frequencies >20.1 kHz. DP-grams were collected over a range of geometric mean frequencies between 5.6 and 48.5 kHz (f_2 = 6.3–54.2 kHz), in 0.5 octave intervals at stimulus levels of $L_1 = L_2$ = 75 dB SPL, with f_2/f_1 = 1.25.

Supplemental Data

Supplemental Data include one figure and can be found with this article online at http://www.cell.com/cgi/content/full/124/6/1269/DC1/.

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