Neuron, Vol. 41, 849–857, March 25, 2004, Copyright ©2004 by Cell Press

# Noxious Cold Ion Channel TRPA1 Is Activated by Pungent Compounds and Bradykinin

Report

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## Summary

**Six members of the mammalian transient receptor potential (TRP) ion channels respond to varied temperature thresholds. The natural compounds capsaicin and menthol activate noxious heat-sensitive TRPV1 and cold-sensitive TRPM8, respectively. The burning and cooling perception of capsaicin and menthol demonstrate that these ion channels mediate thermosensation. We show that, in addition to noxious cold, pungent natural compounds present in cinnamon oil, wintergreen oil, clove oil, mustard oil, and ginger all activate TRPA1 (ANKTM1). Bradykinin, an inflammatory peptide acting through its G protein-coupled receptor, also activates TRPA1. We further show that phospholipase C is an important signaling component for TRPA1 activation. Cinnamaldehyde, the most specific TRPA1 activator, excites a subset of sensory neurons highly enriched in cold-sensitive neurons and elicits nociceptive behavior in mice. Collectively, these data demonstrate that TRPA1 activation elicits a painful sensation and provide a potential molecular model for why noxious cold can paradoxically be perceived as burning pain.**

## Introduction

Among the five senses, the sense of touch is one of the most varied and least understood. Within this modality is the ability to sense mechanical forces, chemical stimuli, and temperature (Kandel et al., 2000). The distinct touch stimuli are sensed by dorsal root ganglia (DRG) and trigeminal neurons in the soma and head, respectively. These neurons convey information about the environment through specialized neurites that extend to the skin from cell bodies in the vertebral column. Recording directly from DRG nerve fibers has helped to classify some of these neurons as mechanosensitive or thermosensitive (hot, warm, or cool responsive). Still other neurons, called polymodal nociceptors, sense noxious thermal (cold and hot) and mechanical stimuli. The molecules that mediate the ability to sense thermal and mechanical forces have been a long-standing mystery.

Temperature has been recently shown to activate certain members of the transient receptor potential (TRP) family of cation channels (Patapoutian et al., 2003). TRPV1 is activated by noxious heat temperatures >42°C and by capsaicin, the active ingredient in chili peppers. Three other TRPV ion channels with distinct thresholds of heat activation have been described. In addition, single members of two distinct subfamilies of TRP channels have been implicated in cold sensation: TRPM8 is activated at 25°C, while TRPA1 (ANKTM1) is activated at 17°C. TRPA, the newest subfamily of TRP ion channels, is named after ANKTM1 (nomenclature proposed by D. Corey and C. Montell) (Corey, 2003). The TRPA1 ortholog in *Drosophila melanogaster* also acts as a temperature sensor (Viswanath et al., 2003). Together these temperature-activated channels represent a subset of TRP channels that we have dubbed thermoTRPs (Patapoutian et al., 2003). In agreement with a role in initiating temperature sensation, most of the thermoTRPs are expressed in subsets of DRG neurons that strikingly correlate with the physiological characteristics of thermosensitive DRG neurons: we have found neurons that express only TRPV1 (presumptive heat-responsive neurons), only TRPM8 (cool responsive), or both TRPV1 and TRPA1 (polymodal nociceptors) (Story et al., 2003).

In this study, we have screened natural sensory compounds for TRPA1 activation and show that constituents of cinnamon oil, mustard oil, and other natural oils that cause a burning sensation in humans activate TRPA1. A recent publication also described the activation of TRPA1 by mustard oil but failed to observe activation by cold (Jordt et al., 2004). Here, we confirm that cold is an important activator for TRPA1 (both in heterologous systems and, more importantly, in DRG cultures). Our results agree with existing physiological data demonstrating a separate noxious/burning component of cold (Arndt and Klement, 1991; Klement and Arndt, 1991). We further show that TRPA1 is coupled to bradykinin (BK) signaling and that phospholipase C (PLC) plays a crucial role in TRPA1 channel activity. Collectively, our results suggest that TRPA1 activation conveys a pain sensation independent of the quality of the stimulus and that this sensation is interpreted in conjunction with activity in other sensory neurons.

## Results

### TRPA1 Is Activated by Cinnamaldehyde and Other Sensory Compounds

Since TRPA1 marks neurons that can respond to both heat and cold stimuli, the sensory quality that TRPA1 activation conveys is crucial in understanding the coding of noxious temperature (Story et al., 2003). We searched for pharmacological activators of TRPA1. We focused on compounds mostly derived from food items used in oral care and confectionery products that have a sensory component distinct from taste and smell. Using a fluorometric imaging plate reader (FLIPR), we showed that mouse TRPA1-expressing CHO cells (mTRPA1) show a sharp increase in intracellular calcium

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upon application of eugenol, gingerol, methyl salicylate, allyl isothiocyanate, and cinnamaldehyde (Supplemental Table S1 at http://www.neuron.org/cgi/content/full/41/6/849/DC1 and data not shown). All these compounds are known to cause a pungent burning sensation in humans. We then tested these compounds against TRPM8 and TRPV1. Only allyl isothiocyanate and cinnamaldehyde were specific to mTRPA1, indicating that the burning sensation that these compounds cause is independent of TRPV1 (Supplemental Table S1; Figure 1). Methyl salicylate (MeS) (600 μM) was also specific to mTRPA1. However, 2 mM MeS activated TRPV1-expressing cells, corresponding to ~25% of the TRPV1 response observed from saturating amounts of capsaicin (data not shown). Compounds with cooling properties, such as spearmint, did not activate mTRPA1. Instead, these cooling compounds activated TRPM8, suggesting a similar mode of action to menthol.

We focused on the two mTRPA1-specific compounds. Using FLIPR, we determined the concentration for half-maximal activation to be 61 ± 9 μM for cinnamaldehyde and 22 ± 3 μM for allyl isothiocyanate (Figure 1A). We also performed ratiometric calcium imaging of CHO cells expressing mTRPA1 and recorded a robust increase in intracellular free calcium upon application of cinnamaldehyde and allyl isothiocyanate (Figure 1B). Ruthenium red, a known blocker of mTRPA1, blocked the cinnamaldehyde response (Figure 1C). Cinnamaldehyde and allyl isothiocyanate did not activate TRPV1-, TRPV4-, or TRPM8-expressing CHO cells (Figure 1D and data not shown).

We also characterized the cinnamaldehyde-induced current in mTRPA1-expressing CHO cells. In a whole-cell patch clamp setup, cinnamaldehyde elicited a robust desensitized current (data not shown). The current-voltage (IV) relationship in response to cinnamaldehyde and cold were identical, indicating that both activate the same ion channel (Figure 1E). Expression of either mouse or human TRPA1 (hTRPA1) in *Xenopus* oocytes rendered these cells responsive to cinnamaldehyde as well as to cold temperatures (Figure 1F). Oocytes expressing dTRPA1 (dANKTM1), the *Drosophila melanogaster* ortholog of mTRPA1, did not respond to cinnamaldehyde (data not shown). Repeated applications of cinnamaldehyde to hTRPA1-expressing oocytes showed strong sensitization in contrast to the desensitizing effect of cold (Supplemental Figures S1A and S1B at http://www.neuron.org/cgi/content/full/41/6/849/DC1). It has been shown for TRPM8 that subactivating menthol concentrations can cause a shift in the temperature activation threshold (McKemy et al., 2002; Peier et al., 2002). Calcium imaging experiments and oocyte electrophysiological recordings were undertaken to investigate whether cinnamaldehyde could also cause such a shift in temperature threshold. Perfusion with subthreshold concentrations of cinnamaldehyde did not affect the temperature threshold (data not shown). Moreover, we determined that the cinnamaldehyde concentration for half-maximal activation of TRPA1-expressing cells is 61 ± 9 μM at 23°C and 84 ± 9 μM at 35°C, suggesting that temperature did not significantly affect the response to cinnamaldehyde (Supplemental Figure S1C). In contrast, menthol EC50s at these temperatures are 7 ± 1 μM and 63 ± 4 μM, respectively (Supplemental Figure S1D). Similar to TRPA1, a strong cooperativity between subthreshold capsaicin and heat has not been observed for TRPV1 (Sprague et al., 2001).

### TRPA1 Is Activated by Bradykinin

Activation of TRPA1 by pungent natural products suggests a nociceptive role for TRPA1. BK is among the most potent algogenic substances released from tissue injury and inflammation, but little is known about the mechanism by which it causes acute excitation of sensory neurons (Levine et al., 1993; Couture et al., 2001). Bradykinin receptor (B2R), similar to TRPA1, is expressed in a subpopulation of capsaicin-responsive nociceptors (Kasai et al., 1998). We therefore examined whether TRPA1 is functionally coupled to B2R signaling. Whole-cell recording of mTRPA1-expressing CHO cells transiently transfected with B2R showed acute and immediate current responses to 1 μM BK (n = 5) (Figure 1G). No significant current was observed during BK application in control cells: CHO cells (n = 8), TRPA1 cells (n = 7), and B2R-only expressing cells (n = 6) (Figure 1H and data not shown). The currents evoked by BK, cold, and cinnamaldehyde have identical reversal potentials and rectification properties, arguing that BK-activated currents are due to TRPA1 activation (Figures 1E and 1I).

### Role of Phospholipase C in TRPA1 Activation

PLC and phospholipase A2 are activated by BK signaling. Since many TRP channels are modulated by PLC activity, we tested whether downstream affectors of PLC can modulate TRPA1 function (Minke, 2001). One of the major consequences of PLC activation is the release of calcium from intracellular stores. We therefore tested if passive release of calcium from the stores could activate TRPA1 using the smooth endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) pump blocker thapsigargin. Thapsigargin caused equivalent increases in intracellular calcium levels in naive CHO cells, CHO cells transiently transfected with human and mouse TRPA1, and stably transfected mTRPA1 (Figure 2A and data not shown). Another downstream effect of PLC activity is the generation of diacylglycerol (DAG). Therefore, we tested if 1-oleoyl-2-acetyl-sn-glycerol (OAG, a cell-permeable analog of DAG) could activate TRPA1. OAG application activated mTRPA1-expressing CHO cells, which could be blocked by ruthenium red (Figure 2B). OAG gives no significant response in naive CHO cells (data not shown). DAG can be converted to polyunsaturated fatty acid (PUFAs), such as arachidonic acid (AA), and both DAG and AA have been proposed to activate several ion channels, including TRPC3 and TRPV4, respectively (Clapham, 2003; Minke, 2001; Watanabe et al., 2003). AA also activated TRPA1-expressing CHO cells, and this activation was blocked by ruthenium red (Figures 2C and 2D). AA can be converted to numerous metabolites, including prostaglandins. We reasoned that if TRPA1 activation is due to downstream metabolites of AA, then a nonmetabolizeable AA analog would be unable to activate TRPA1. However, 5,8,11,14-eicosatetraynoic acid (ETYA) activated TRPA1-expressing CHO cells (Figure 2D). Therefore, AA metabolism is not required for the activation of TRPA1.

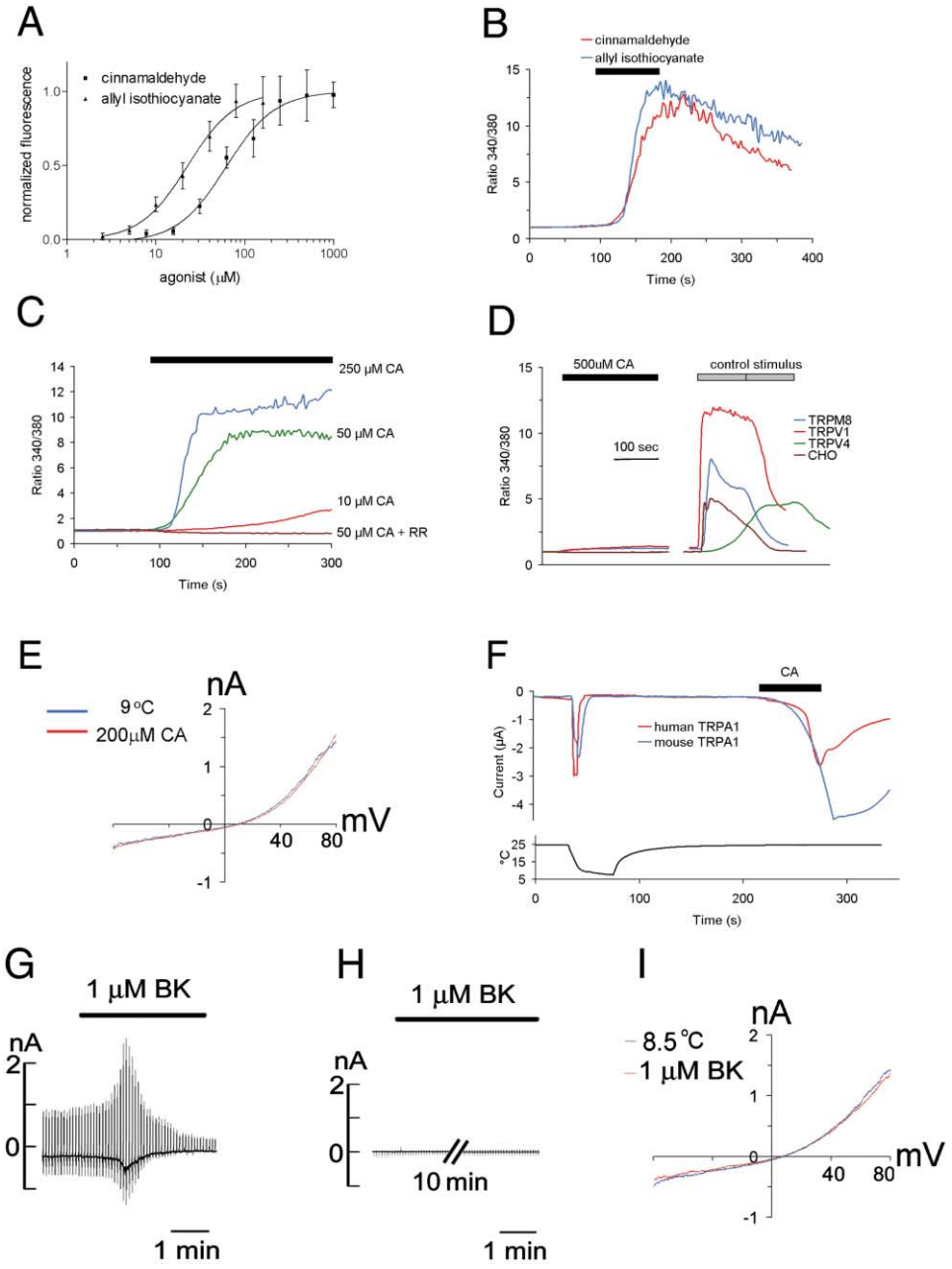



Figure 1. TRPA1 Is Activated by Noxious Compounds and Cold

(A) Dose-response curve of cinnamaldehyde and allyl isothiocyanate on mTRPA1-expressing CHO cells using FLIPR. Each datapoint represents an average of four to eight independent readings.

(B) Comparison of responses to 1.6× EC50 values of cinnamaldehyde (100 μM) and allyl isothiocyanate (33 μM) on mTRPA1 cells using ratiometric calcium imaging. Traces represent average fluorescent ratios of ~50 cells (B–D).

(C) Intracellular calcium increases in mTRPA1-expressing CHO cells upon application of cinnamaldehyde in the presence or absence of 10 μM ruthenium red.

(D) Calcium imaging of untransfected CHO cells and CHO cells expressing human TRPV1, mouse TRPM8, and rat TRPV4. Cells were perfused with 500 μM cinnamaldehyde (single black bar) followed by a control stimulus. Control stimuli are 100 s perfusion of 100 μM ATP, 1 μM capsaicin, and 50 μM menthol for naive CHO and TRPV1- and TRPM8-expressing cells, respectively, and 200 s application of hypoosmotic buffer (225 mOsm) for TRPV4.

(E) Current-voltage relationship of cinnamaldehyde-evoked current (200 μM) is identical to that evoked by cold temperatures from mTRPA1-expressing CHO cells.

(F) Inward current in human (red) and mouse (blue) TRPA1-expressing *Xenopus* oocytes evoked by cold temperature and by 50 μM (mTRPA1) and 100 μM (hTRPA1) cinnamaldehyde.

(G and H) Whole-cell recording of CHO cells expressing TRPA1/B2R (G) and B2R (H) upon application of 1 μM BK.

(I) Current-voltage relationship of BK-evoked current is identical to that evoked by cold temperatures.

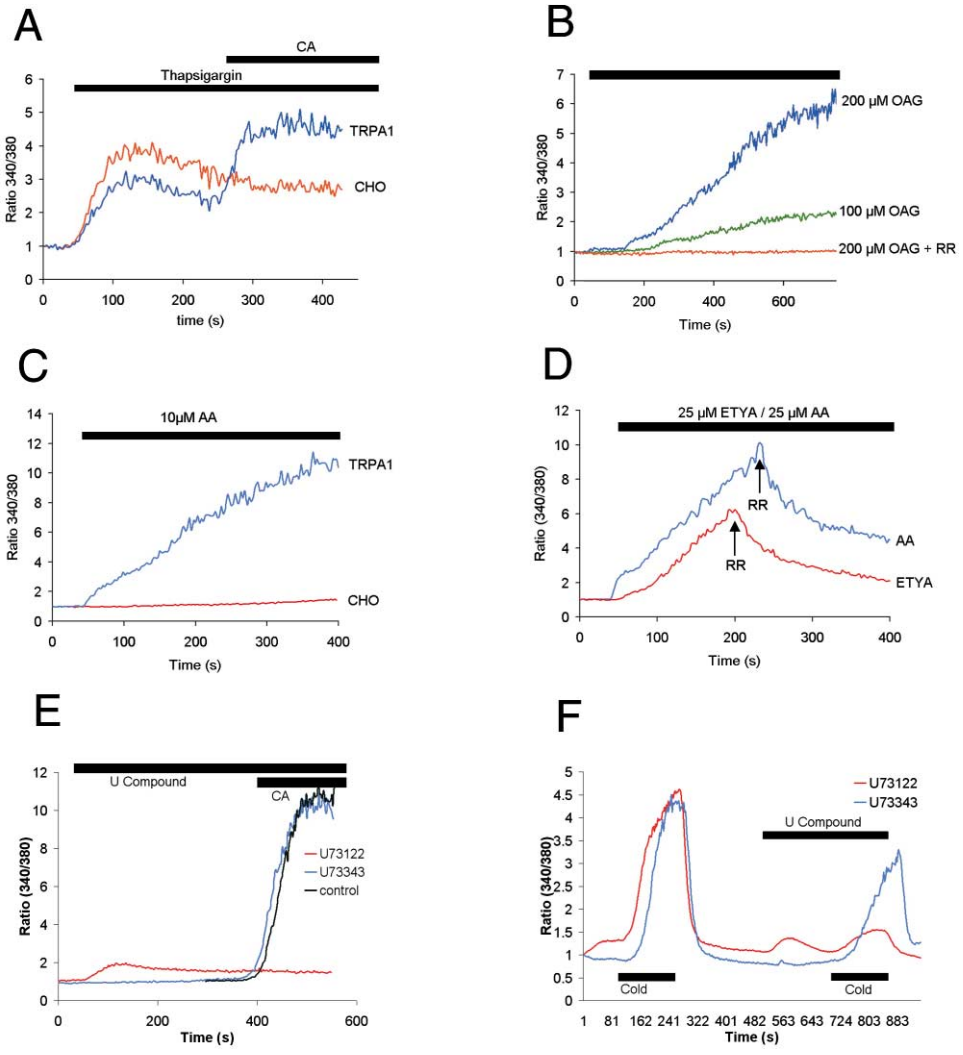



Figure 2. Phospholipase C Pathway and TRPA1 Activation

(A) Effect of thapsigargin on TRPA1-expressing CHO cells. Ratiometric calcium imaging of cells transiently transfected with hTRPA1 and YFP. The responses of TRPA1-positive and control cells on the same coverslip to thapsigargin (1 μM) and cinnamaldehyde (100 μM) were directly compared. TRPA1-positive cells were identified by YFP fluorescence.

(B–F) Ratiometric calcium imaging of CHO cells stably transfected with mTRPA1. OAG, AA, and ETYA activate mTRPA1-expressing cells, and ruthenium red (10 μM) blocks the activation by these compounds (B–D). U-73122 (a specific PLC inhibitor) (10 μM) but not U-73343 (an inactive close analog) strongly downregulates mTRPA1 responses to 50 μM cinnamaldehyde (E) and cold (F). Traces represent average fluorescent ratios of ~40 cells.

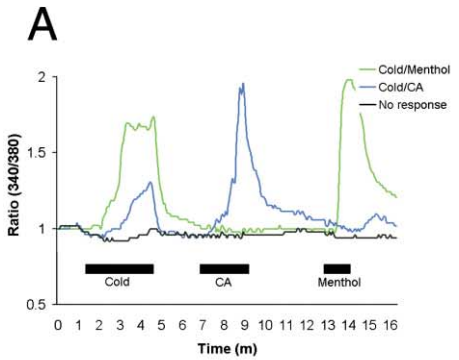
We next tested if specific inhibition of PLC by U-73122 would affect TRPA1 activation by bradykinin. In calcium imaging studies, BK responses in B2R/TRPA1-expressing CHO cells and B2R-expressing cells were indeed inhibited by 10 μM of U-73122 but not by U-73343 (a similar but inactive analog) (Supplemental Figure 2A at http://www.neuron.org/cgi/content/full/41/6/849/DC1 and data not shown). We then tested if PLC inhibition of TRPA1 was stimulus specific. U-73122 inhibited TRPA1 activation by cinnamaldehyde and strongly downregulated TRPA1 activation by cold (Figures 2E and 2F). We further tested the role of PLC inhibition on TRPM8. U-73122 strongly dowregulated the cold- and menthol-induced responses of TRPM8 (Supplemental Figures S2B and S2C). Preincubation of U-73122 was necessary to observe a block of the menthol response, suggesting that this compound is not acting as an ion channel blocker (Supplemental Figure S2D). In some experiments, U-73122 caused a delayed and mild rise in calcium levels independent of the application of any stimulus. This response has been previously described and did not interfere with the interpretations of our experiments (Estacion et al., 2001).

### Cinnamaldehyde and Bradykinin Activate TRPA1-like DRG Neurons in Culture

We have previously shown that two distinct populations of cold-responding neurons are present in cultured DRGs (Nealen et al., 2003; Story et al., 2003). One population is activated by mild cool temperatures and responds to menthol. The other population is activated by colder temperatures and responds to capsaicin but

not to menthol. In vivo, TRPA1 is expressed in a subset of TRPV1 neurons but is not coexpressed with TRPM8. Therefore, we hypothesized that TRPM8 and TRPA1 mark the two cold populations, respectively. To further test this hypothesis and to find out if the pungent compounds described above activate TRPA1 specifically, we performed calcium imaging of adult rat DRG neurons in response to cold, menthol, cinnamaldehyde, allyl isothiocyanate, and bradykinin. Cinnamaldehyde activated 39% of cold-activated DRG neurons but only 1% of cold-insensitive neurons (Figures 3A and 3D). To a large extent, cinnamaldehyde and menthol activated mutually exclusive populations of cold-responsive neurons, as our model would predict. Allyl isothiocyanate appeared less specific, as it activated 63% of the cold-responsive population (including a large overlap with menthol) and 12% of the cold-insensitive population (Figures 3B and 3D). We have used 1.6 times the EC50 values of cinnamaldehyde and allyl isothiocyanate to enable us to directly compare DRG response profiles to these compounds (Figures 1A and 1B). Furthermore, raising the concentration of cinnamaldehyde to 200 μM did not show any dramatic shift in response profiles (data not shown). The numbers shown in Figure 3D reflect pooled numbers from cultures in the presence of 1 and 100 ng/ml of NGF. There were no significant differences in the profiles of cinnamaldehyde and allyl isothiocyanate between the two culture conditions.

In addition to the pungent sensory compounds, we have shown that BK can activate TRPA1. To demonstrate if this interaction could be physiologically relevant, we tested if BK- and cinnamaldehyde-responsive profiles overlap in cultured DRG neurons. BK activated 14% (19/138) of total DRG neurons, and 78% (7/9) of cinnamaldehyde-responsive neurons (Figure 3C and data not shown). These results suggest that a majority of TRPA1-expressing neurons also express bradykinin receptors.

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| | Cinnamaldehyde | Allyl Isothiocyanate |
|---|---|---|
| Total neurons | 3.3% (18/545) | 15.4% (88/570) |
| Cold insensitive neurons | 1.0% (5/512) | 11.7% (62/529) |
| Cold sensitive neurons | 39.4% (13/33) | 63.4% (26/41) |
| Cold/menthol insensitive | 90.9% (10/11) | 70% (7/10) |
| Cold/menthol sensitive | 13.6% (3/22) | 61.3% (19/31) |

Figure 3. Cinnamaldehyde, Allyl Isothiocyanate, and Bradykinin Activate Cold-Sensitive Cultured DRG Neurons

(A and B) Ratiometric calcium imaging of representative DRG neuron responses to cold (9°C), 100 μM CA (A), 33 μM AI (B), and 250 μM menthol.

(C) Representative DRG neuron responses to 5 μM BK, 100 μM CA, and cold (9°C).

(D) Cinnamaldehyde and allyl isothiocyanate response profiles

## Cinnamaldehyde Causes Nociceptive Behavior in Mice

To provide evidence for the role of TRPA1 in pain signaling, we performed intraplantar injections of mice with cinnamaldehyde (the most-specific TRPA1 agonist) and recorded nociceptive behavior. We used two concentrations of cinnamaldehyde injections: 5 mM and 16.4 mM (6.6 and 21.7 μg). To identify a negative control more meaningful than vehicle injections, we screened for cinnamaldehyde-like compounds that did not activate TRPA1 in heterologous expression systems. Cinnamic acid, a close analog of cinnamaldehyde, did not activate mTRPA1 even in millimolar concentrations (Figure 4A).

Both concentrations of cinnamaldehyde induced licking and shaking of the injected hindpaw during the 5 min assayed postinjection, a behavior not observed in vehicle- or cinnamic acid-injected mice (Figure 4B and data not shown). As expected, *TRPV1*$^{-/-}$ mice responded to cinnamaldehyde injections similarly to wild-type mice (Figure 4C). To provide further evidence of the role of TRPA1 in pain sensation, we investigated if cinnamaldehyde injections could lead to hyperalgesia, an increased response to pain due to sensitization often caused by inflammation or injury. Thirty minutes after cinnamaldehyde injection (acute responses do not last more than 10 min), a noxious heat stimulus was applied to the paw, and the latency of the withdrawal response was measured. Paw withdrawal latency was significantly lowered in the injected compared to the control paws ($p = 0.009$) (Figure 4D). In contrast, no significant difference could be observed between the latencies of the two paws when vehicle was injected (data not

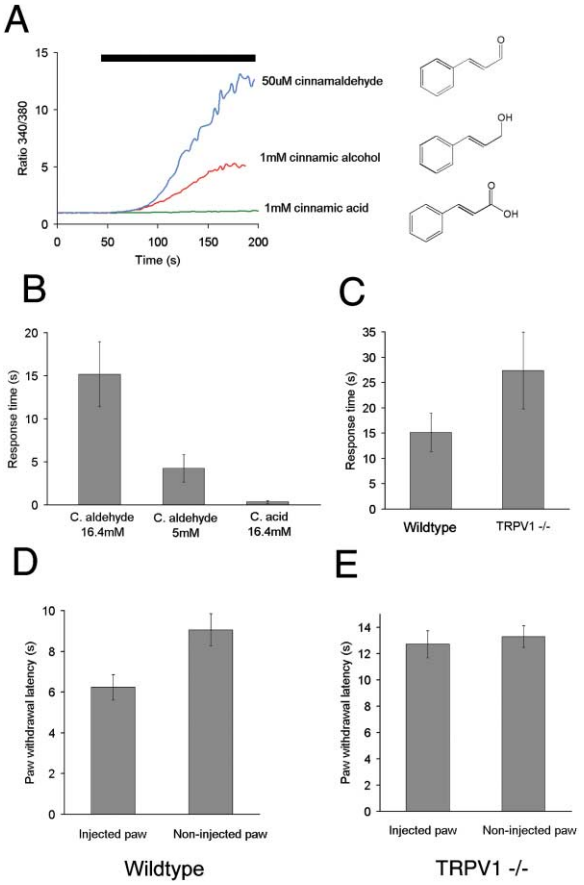


Figure 4. Cinnamaldehyde Elicits Nociceptive Behavior in Mice

(A) Calcium imaging experiments demonstrate that cinnamic acid, a close analog of cinnamaldehyde, does not activate mTRPA1-expressing CHO cells.

(B) Wild-type C57Bl6/J mice are injected intraplantarly in the hindpaw with cinnamaldehyde or cinnamic acid at the indicated concentrations. Acute nociceptive behavior (paw licking and shaking) is recorded over a period of 5 min ($n = 10$).

(C) *TRPV1*$^{-/-}$ mice show robust nociceptive response to 16.4 mM of cinnamaldehyde ($n = 10$). The responses do not significantly differ from wild-type mice ($p = 0.21$).

(D and E) Wild-type C57Bl6/J (D) and *TRPV1*$^{-/-}$ (E) mice were injected with 16.4 mM cinnamaldehyde, and the response latency to radiant heat was measured 30 min after injection in both hindpaws ($n = 20$). Wild-type mice show a significant decrease in withdrawal latency in the injected paw ($p = 0.009$).

shown). Hyperalgesia to heat is thought to involve sensitization of TRPV1 (Chuang et al., 2001; Tominaga et al., 2001). Despite the robust acute pain behavior of cinnamaldehyde-injected *TRPV1*$^{-/-}$ mice, heat hyperalgesia was absent in these mice (Figure 4E). Therefore, the acute nociceptive response of cinnamaldehyde is independent of TRPV1, while the heat hyperalgesia is mediated through TRPV1.

## Discussion

We have previously shown that TRPA1 (ANKTM1) is a noxious cold-activated ion channel specifically expressed in a subset of TRPV1-expressing nociceptive neurons (Story et al., 2003). Its physiological profile and expression pattern suggested that TRPA1 marks polymodal nociceptors. Here, we show that TRPA1 is activated by a variety of noxious molecules. Furthermore, our studies suggest that activation of TRPA1 is an important component of pain sensation that signals the noxious, burning element of cold.

### TRPA1 Is Activated by Cold

We have extended our previous studies on cold activation of TRPA1. Here, we show that human as well as mouse TRPA1 is activated by noxious cold temperatures. We also show that cinnamaldehyde, a specific TRPA1 activator in vitro, predominantly excites cold-sensitive DRG neurons in culture. Moreover, the response profiles of menthol and cinnamaldehyde accurately reflect the mutually exclusive expression of the two cold-activated ion channels TRPM8 and TRPA1, respectively. Indeed, cinnamaldehyde- and menthol-responding neurons account for almost all cold-responsive neurons in culture (32/33). A recent report failed to reproduce the cold activation of TRPA1 (Jordt et al., 2004). There are many possible explanations for the negative results. Since cinnamaldehyde and mustard oil are more robust activators of TRPA1 compared to cold, it is possible that the discrepancy is a matter of assay sensitivity.

### TRPA1 Is Activated by Cinnamaldehyde and Other Sensory Compounds

A variety of pungent compounds—oils of cinnamon, mustard, wintergreen, ginger, and clove—activate TRPA1. Cinnamaldehyde is the main constituent of cinnamon oil (~70%) and is extensively used for flavoring purposes in foods, chewing gums, and toothpastes. Allyl isothyocianate (mustard oil) is one of the active ingredients in horseradish and wasabi. Methyl Salicylate (wintergreen oil) is used commonly in products such as mouthwash and as a counterirritant in topical analgesic ointments. We tested the specificity of these TRPA1-activating compounds against other thermoTRPs. Cinnamaldehyde and allyl isothyocianate activate only TRPA1. Furthermore, cinnamaldehyde preferentially activates a subset of cold-activated cultured adult DRG neurons that have a TRPA1-like profile. Mustard oil activates this same population in addition to a larger cold-insensitive group of neurons. Jordt et al. (2004) also observed a comparatively large percent of newborn trigeminal neurons responding to mustard oil, although no correlation with cold response was observed. Our results suggest that mustard oil activates TRPA1 and perhaps another unidentified ion channel.

When orally administered at ~15 mM (0.2%) to human subjects, cinnamaldehyde is perceived to have a burning and tingling sensory quality (Cliff and Heymann, 1992; Prescott and Swain-Campbell, 2000). Cinnamaldehyde activates TRPA1-expressing CHO cells in micromolar concentrations, and TRPA1 is expressed in trigeminal neurons that project to the tongue. Therefore, TRPA1 could indeed be responsible for the burning sensory quality of cinnameldehyde. Traditionally, the gustatory and olfactory systems are thought to account for the perception of oral flavorings. The extended list of sensory compounds that activate thermoTRPs provides

molecular evidence that the trigeminal system also plays an important role in taste perception.

It is not possible to collect precise information regarding the sensory quality of compounds from rodents; however, we have used intraplantar injection of cinnamaldehyde in mice to show that cinnamaldehyde causes nociceptive response behavior and thermal hyperalgesia. The hyperalgesia could be mediated by the release of neuropeptides such as substance P and CGRP upon TRPA1 activation (Julius and Basbaum, 2001). Alternatively, TRPA1 activation could directly sensitize TRPV1 responses. Although we did not observe cross-sensitization of TRPA1 and TRPV1 in CHO cells, the situation in vivo could differ (data not shown).

### TRPA1 Is Activated by the Inflammatory Peptide Bradykinin

The activation of many TRP ion channels is linked to G protein-coupled receptor (GPCR) signaling (Clapham, 2003). Here, we show that TRPA1 can be activated by BK, an inflammatory signal involved in nociception that acts through its GPCR. BK directly excites nociceptive DRG neurons and causes hyperalgesia (Levine et al., 1993; Couture et al., 2001). Mechanisms of BK-induced hyperalgesia are well studied; however, the identity of the ion channels acutely activated by BK is not known. TRPV1 is required for BK-induced thermal hyperalgesia in vivo (Chuang et al., 2001; Premkumar and Ahern, 2000). Although BK signaling has been shown to sensitize TRPV1 responses to low pH and capsaicin, direct activation of TRPV1 by BK has not been clearly demonstrated (Chuang et al., 2001; Liang et al., 2001). In agreement with this, the acute BK responses in TRPV1 knockout mice are not significantly altered (Kollarik and Undem, 2003). Our electrophysiological data suggests that TRPA1 is coupled to the activation of the B2R receptor. We further show that the majority of cinnamaldehyde-responding neurons are also activated by BK in adult DRG cultures, suggesting that TRPA1 is an endogenous component of BK-induced excitation of polymodal nociceptors.

### Role of Phospholipase C in TRPA1 Activation

The activation of TRPA1 by BK raises important questions about the gating mechanism of this ion channel. BK is known to activate the PLC pathway, a known modulator of TRP channels (Minke, 2001). One of the consequences of PLC activation is breakdown of phosphatidylinositol-4,5-bisphosphate (PIP2) into DAG and inositol triphosphate (IP3). IP3 then releases calcium from internal stores, while DAG activates protein kinase C and can also be converted to PUFAs such as AA by DAG lipase. PLC activity plays a major role in the regulation and activation of many TRP channels. For example, PIP2 degradation by PLC has been proposed to release TRPV1 from inactivation, DAG can activate TRPC3 and TRPC6, and TRPV4 can be activated by arachidonic acid (Clapham, 2003; Minke, 2001). Our finding that OAG, a membrane-permeable DAG analog, can activate TRPA1-expressing cells suggests a potential mechanism of how BK signaling is coupled to TRPA1 activity. We also find that AA can activate TRPA1-expressing cells. However, the exact pathway of DAG- and AA-induced activation of TRPA1 is not clear, as both of these compounds can act indirectly by activating PLC and other targets (Hardie, 2003). In contrast, the release of calcium from stores through the application of thapsigargin does not activate TRPA1, disagreeing with results from Jordt et al. (2004). The activation of other TRP channels by store depletion have remained controversial, as outcomes appear to depend on complex variables, including the type of heterologous expression systems used (Clapham 2003). Nevertheless, our results suggest that calcium release alone is not sufficient to activate TRPA1.

We further show that activation of TRPA1 and TRPM8 by cold and a variety of compounds are severely attenuated by a specific PLC inhibitor. Cinnamaldehyde, menthol, and cold do not cause a release of calcium from cells not expressing TRPM8 or TRPA1, and therefore these stimuli do not directly activate PLC. Furthermore, it is unlikely that menthol and cinnamaldehyde simply induce TRP channel gating by activating a downstream component of PLC (such as DAG synthesis), since this would fail to explain why TRPA1 is activated by cinnamaldehyde but not by menthol. Instead, our data suggests that basal PLC activity is required for the proper function of these channels, perhaps by keeping them in a state that is primed for activation. In addition, our data suggests that robust PLC activation—as in the case for bradykinin signaling—might be sufficient to gate TRPA1, perhaps via DAG or AA. The dependence of both cold-activated TRP channels on PLC signaling is especially remarkable. Although U-73122 is known to be a specific PLC inhibitor, the possibility that this compound also acts via a PLC-independent pathway cannot be dismissed at this point.

### Coding of TRPA1-Expressing Neurons

The study of the pharmacological compounds capsaicin and menthol has suggested that activation of TRPV1 leads to a burning hot sensation, while TRPM8 feels cool. Our finding that the activation of TRPA1 by cinnamaldehyde and a variety of sensory compounds with a painful, burning sensory quality has important implications on the coding of TRPA1-expressing neurons. It is formally possible that TRPA1 activation by cinnamaldehyde and cold feel qualitatively different. We instead propose that TRPA1 activation by cold would convey a paradoxical burning sensation. Burning is usually associated with heat; however, both noxious cold and mechanical stimuli have a burning component. Our model suggests that TRPA1 neurons signal the painful, burning component of a noxious stimulus, and whether the same stimulus is also hot, cold, or otherwise is interpreted depending on the activity of other sensory neurons. For example, TRPM8 activation would feel cool, while coactivation of TRPM8 and TRPA1 would feel burning cold. An elegant set of physiological experiments on veins innervated by polymodal nociceptors lends strong support for such a model. Intravenous cooling of a cutaneous vein segment of the hand causes sensations of both cold and pain (Arndt and Klement, 1991; Klement and Arndt, 1991). Local intravenous anesthesia blocks the pain but not the coldness of the stimulus, while cutaneous anesthesia blocks the coldness but not the pain.

Thus, the polymodal afferents that innervate the veins convey pain upon cold stimulation, while coactivated cutaneous thermoreceptors are responsible for sensing the coldness. Whether TRPA1 neurons project to the veins is not yet known.

## Experimental Procedures

### Cell Culture and Gene Expression

#### *TRPV1, TRPM8, and TRPA1*

CHO cells stably expressing rat TRPV1, mouse TRPM8, and mouse TRPA1 have been previously described (Peier et al., 2002; Story et al., 2003). TRPA1 cells were grown in the presence of 5 μM ruthenium red (Fluka), and tetracycline (0.5 μg/ml) was added to the media 16–24 hr before experiments to induce expression. A 3.3 Kb fragment of the human homolog of TRPA1 (GeneBank-Y10601) was amplified from the human cell line WI38 cDNA using primers hANK F1 (5′-GTCAATGAAGTGCAGCCTGAG) and hANK R2 (5′−CCTCA GACCTTCAGTGAGGCTTCTA) and subcloned into the pOX vector for expression in *Xenopus* oocytes.

#### *TRPV2 and TRPV4*

CHO cells were transiently transfected using Fugene according to manufacturer's protocol. Cells were transfected with the expression plasmids pcDNA3.1(+) or pCDNA5/FRT (Invitrogen) containing rat TRPV2 or rat TRPV4, respectively. Expression plasmids were cotransfected with a green fluorescent reporter plasmid at a 4:1 ratio. Cells were transfected 1–2 days before experiments. GFP-positive cells were used for analysis.

#### *Bradykinin Receptor2*

A human B2R was cloned into pcDNA3.1+ containing a YFP marker (Invitrogen). B2R was transiently transfected in mTRPA1-CHO cells or naive CHO cells.

### Intracellular Calcium Imaging in CHO Cells and DRGs

Calcium imaging experiments were performed as described (Peier et al., 2002; Story et al., 2003). For DRGs, cold was applied for 4–5 min or until the maximum/stable coldness (~9°C) was achieved.

### Intracellular Calcium Measurements Using a Fluorometric Imaging Plate Reader

mTRPA1-CHO cells were seeded at 6000 cells/well into black-walled clear-base 384-well plates and were grown for 2 days. Cells were induced for mTRPA1 expression as described and loaded with Fluo-3 according to protocol (Molecular Probes). The plates were then placed into a FLIPR (Molecular Devices, UK) to monitor cell fluorescence (EX = 488 nM; EM = 540 nM) before and after compound addition (Sullivan et al., 1999).

### Compounds

Arachidonic acid, bradykinin, menthol, trans-cinnamaldehyde, trans-cinnamic acid, cinnamyl alcohol, eugenol, iospulegol, methyl salicylate, allyl isothiocyanate, trans-p-menthane-3,8-diol, and capsaicin were purchased from Sigma-Aldrich; methyl lactate, L-carvone, WS23 (Acros), and cis-p-menthane-diol were from ABCR (Germany); WS3 was from Millenium Chemicals; and [6]-gingerol, OAG, ETYA, U-73122, and U-73343 were from Biomol.

### Electrophysiology

Electrophysiological recordings were performed as described (Story et al., 2003). For two-electrode voltage clamp experiments with *Xenopus* oocytes, the recording solution consisted of 80 mM NaCl, 4 mM KCl, 1 mM MgCl, 100 μM CaCl, and 5 mM HEPES (pH 7.5). For whole-cell recording of CHO cells, currents were measured and stored using a MultiClamp 700A amplifier (filtered at 5 kHz) interfaced to a Digidata 1322A data acquisition system with pClamp 9.0 software (Axon Instruments). The voltage clamp at −60 mV or the voltage ramp protocol was used for the experiments as follows: the ramp protocol with the sampling rate of 400 μs was initiated by a voltage step to −60 mV for 1.6 s followed by a 0.8 s linear ramp from −80 mV to +80 mV and returning to the 1.6 s step of −60 mV. The regular pipet solution in the whole-cell experiments was (in mM) CsCl, 140; EGTA, 5; HEPES, 10; MgATP, 2; NaGTP, 0.2; titrated to pH 7.4 with CsOH. The external solution for cold or chemical applications was (in mM) NaCl, 140; KCl, 5; HEPES, 10; $CaCl_2$, 2; $MgCl_2$, 1; titrated to pH 7.4 with NaOH.

### Behavioral Assays of Nociception

Male C57Bl6/J and TRPV1 null mice (B6.129S4-*Trpv1*$^{tm1Jul}$/J—backcrossed to C57BL/6 for at least five generations; Jackson Laboratories) of 8–10 weeks age were used. Animals were acclimated for 30 min in individual plexiglass chambers prior to injections (10 μl in phosphate-buffered saline/0.5% Tween 80). The pH of cinnamic acid was adjusted to neutral by using NaOH. Cinnamaldehyde solutions were briefly warmed to 65°C to facilitate dissolving. Thermal hyperalgesia was tested using a Hargreaves infrared source (UGO Basile 7370 Plantar Test, IR20). Latencies were recorded three times with at least a 1 min interval between heat applications. The results were averaged.

## Acknowledgments

We thank Van Lee and Amber Murray for technical assistance; and Nancy Hong, Tim Jegla, Andrea Peier, and Lisa Stowers for valuable input. Sponsored in part by grants from NINDS (NS42822 and NS046303). S.R.E. was partially supported by a Swiss National Science Foundation fellowship. A.P. is a Damon Runyon Scholar.

Received: January 27, 2004
Revised: February 18, 2004
Accepted: March 4, 2004
Published: March 24, 2004

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