

## More than cool: Promiscuous relationships of menthol and other sensory compounds

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Several temperature-activated transient receptor potential (thermoTRP) ion channels are the molecular receptors of natural compounds that evoke thermal and pain sensations. Menthol, popularly known for its cooling effect, activates TRPM8—a cold-activated thermoTRP ion channel. However, human physiological studies demonstrate a paradoxical role of menthol in modulation of warm sensation, and here, we show that menthol also activates heat-activated TRPV3. We further show that menthol inhibits TRPA1, potentially explaining the use of menthol as an analgesic. Similar to menthol, both camphor and cinnamaldehyde (initially reported to be specific activators of TRPV3 and TRPA1, respectively) also modulate other thermoTRPs. Therefore, we find that many “sensory compounds” presumed to be specific have a promiscuous relationship with thermoTRPs.

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### Introduction

A subset of the transient receptor potential (TRP) ion channels is activated by temperature (dubbed thermoTRPs) and a variety of plant-derived sensory chemicals. Members of the Vanilloid subfamily are activated by heat (TRPV1–4) and by sensory compounds that elicit sensations of heat or warmth (TRPV1 and TRPV3). TRPV1 is activated by capsaicin (active

component of chili peppers) and camphor (aromatic botanical derived from the *Cinnamomum camphora* tree) in addition to heat ( $\geq 43^\circ\text{C}$ ) (Caterina et al., 1997) (Xu et al., 2005). Warmth-activated ( $\geq 33^\circ\text{C}$ ) TRPV3 is also activated by camphor (Peier et al., 2002b; Xu et al., 2002; Moqrich et al., 2005). TRPM8 and TRPA1, members of the melastatin and ankyrin repeat subfamilies, respectively, are both activated by a range of cooling temperatures (TRPM8  $< 25^\circ\text{C}$ , TRPA1  $< 17^\circ\text{C}$ ) (McKemy et al., 2002; Peier et al., 2002a; Story et al., 2003). Natural compounds that evoke cooling sensations such as mint-derived menthol activate TRPM8, whereas pungent compounds derived from garlic, cinnamon, and mustard seeds activate TRPA1 (McKemy et al., 2002; Peier et al., 2002a; Bandell et al., 2004; Jordt et al., 2004; Bautista et al., 2005; Macpherson et al., 2005). All of these channels are expressed within cells essential to cutaneous thermal and pain sensation. TRPV1, TRPM8 and TRPA1 are predominantly expressed within sensory neurons of the trigeminal and dorsal root ganglia (DRG), while TRPV3 is expressed in keratinocytes (skin cells) (Patapoutian et al., 2003).

These sensory compounds have been of great utility in furthering our understanding of the function of thermoTRP channels in sensory systems. For instance, in vitro pharmacological studies of cultured DRG neurons show that menthol, capsaicin and cinnamon oil (cinnamaldehyde) activate functionally distinct (and temperature-sensitive) subsets of neurons, similar to the in vivo expression patterns of TRPM8, TRPV1 and TRPA1 (Story et al., 2003; Bandell et al., 2004). These compounds have also proven to be highly useful tools in mouse behavioral and genetic studies, providing confirmation about their in vivo sensory effects and the physiology of the thermoTRPs (and neurons) they activate (Caterina et al., 2000; Davis et al., 2000; Moqrich et al., 2005). In fact, capsaicin sensitivity has been used to study the physiology of nociceptive neurons even before the discovery of TRPV1 (Winter et al., 1995). Overall, studies of sensory compounds have contributed evidence to a simple model of sensory coding:

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activation of thermoTRPs account directly for sensations evoked—e.g. capsaicin's sensory effects are explained by TRPV1 activation; and menthol's, via TRPM8 activation.

Menthol enhances cooling sensations at room temperature, but at temperatures above 37°C, it enhances sensations of warmth (Green, 1985). Therefore, we challenged menthol's specificity by reexamining its activity on thermoTRPs other than TRPM8. Surprisingly, we find that menthol activates TRPV3 and inhibits TRPA1. We also extended these studies to other sensory compounds and find that most have a promiscuous relationship with thermoTRPs, activating and inhibiting multiple ion channels. Indeed, we find that only capsaicin specifically “coupled” to a single channel. Many of the relationships we find are likely to be relevant as these chemicals are used at considerably high concentrations in over-the-counter products.

## Results

### *Menthol activates TRPV3 and inhibits TRPA1*

A curious effect of cooling menthol is its paradoxical enhancement of perceived warmth intensity in tests of human subjects. Solutions containing 0.02% menthol (equivalent to ~1.3 mM) feel significantly warmer than water alone at temperatures above 37°C (Green, 1985). In addition, in a recent psychophysical study, 10% of subjects reported spontaneous sensations of warmth after application of 40% menthol (equivalent to 2.56 M) (Hatem et al., 2006). We sought to identify mechanism(s) for the warming effect of menthol by challenging the specificity of menthol activation of cold-sensing TRPM8 and testing whether it could activate the recently identified warm- and heat-sensing thermoTRPs. We performed whole-cell patch clamp electrophysiology and calcium imaging experiments on stable cell lines and CHO cells transiently transfected with the thermoTRPs. We tested menthol concentrations ranging from 0.5–2 mM. These concentrations approximate those used in human psychophysical studies and are considerably lower than those used in over-the-counter products (~500 mM) (Yosipovitch et al., 1996; Namer et al., 2005). Menthol, which activates TRPM8 with an  $EC_{50}$  of ~30  $\mu$ M, did not activate TRPV1, TRPA1, or naïve CHO cells at higher concentrations (0.5–2 mM) in calcium imaging studies (data not shown) (Peier et al., 2002a). However, in CHO cells expressing TRPV3, 0.5 mM menthol evoked TRPV3 currents, which increased in magnitude with 2 mM (Fig. 1A, and data not shown). TRPV3 currents with repeated application of 0.5 mM menthol were sensitizing, consistent with the nature of repeated heat and camphor activation of TRPV3 (Fig. 1A; Moqrich et al., 2005). Since TRPV3 is expressed in skin keratinocytes, we tested whether menthol could activate native TRPV3 in primary cultured keratinocytes in calcium imaging assays (Fig. 1B). Application of 1 mM menthol activated wild-type mouse keratinocytes. As we have shown before, application of 1 mM camphor with 50  $\mu$ M 2-aminoethoxydiphenyl borate (2-APB) also activated wild-type keratinocytes (Moqrich et al., 2005). Neither menthol nor camphor plus 2APB was able to activate primary keratinocytes cultured from TRPV3 knockout animals (Moqrich et al., 2005). Application of 100  $\mu$ M ATP serves as a positive control for both wild type and knockout keratinocytes.

TRPM8 (and TRPV1) is voltage-activated, and menthol shifts the voltage sensitivity of TRPM8 to more physiological levels

(Brauchi et al., 2004; Voets et al., 2004). Recently, it was shown that TRPV3 can also be voltage-activated, at least in some active states (Chung et al., 2005). We tested if menthol modulates voltage sensitivity of TRPV3. At concentrations as low as 1 mM, menthol reversibly increased TRPV3 whole-cell currents in a voltage-dependent manner (Fig. 1A, right and bottom panels). While a leftward shift in voltage dependence may contribute to TRPV3 activation by menthol, further studies will be required to address the mechanism of activation. Although poor solubility of menthol at concentrations >20 mM precluded maximal activation of TRPV3 (Fig. 1C, left panel), the data indicate an estimated  $EC_{50}$  for menthol of ~20 mM (19.8 mM 95% CI: 11.3–35.0 mM). This is a crude estimate, as menthol insolubility at high concentrations prevented us from obtaining a full response curve. However, these results were corroborated in whole-cell voltage clamp studies (Fig. 1C, right panel). These data suggest menthol activation of TRPV3 could contribute to menthol-induced sensitization to warm stimuli (see Discussion).

Menthol is marketed in the formulation of many topical pain treatments as a counterirritant and analgesic, but the mechanisms of its anti-pain actions are unclear. The cooling sensation of menthol could distract us from pain; alternatively, it could block the activity of a pain-sensing molecule. We considered whether the analgesic effects of menthol could act via inactivation of the pain-sensing thermoTRPs. We performed calcium imaging and electrophysiological studies on CHO cells expressing TRPA1 and TRPV1. Menthol at concentrations up to 2 mM did not inhibit the heat or capsaicin-evoked activity of TRPV1 (data not shown). Solutions of higher concentrations of menthol (5 mM) induced increased fluorescence ratios (indicative of calcium influx and/or release from intracellular stores) in naïve CHO cells. 5 mM menthol also attenuated CHO cell responses to 10  $\mu$ M ATP, suggesting non-specific effects and disruption of calcium signaling by menthol at this high concentration (data not shown). Human Embryonic Kidney (HEK) cells used in FLIPR determinations of menthol  $EC_{50}$  on TRPV3 and TRPM8 were more resistant to activation by high concentrations of menthol (Fig. 1C). However, modest menthol concentrations (<1 mM) strongly inhibited calcium influx via TRPA1 in response to both cold and cinnamaldehyde in calcium imaging and electrophysiology studies (Figs. 1D and E and data not shown). The  $IC_{50}$  for menthol inhibition of TRPA1 activated by 75  $\mu$ M cinnamaldehyde was 68  $\mu$ M (95% CI: 35.6–128.5  $\mu$ M) (Fig. 1F). These experiments clearly show that menthol's sensory effects are not limited to TRPM8 activation. TRPV3 activation and TRPA1 inhibition by submillimolar menthol is likely to be relevant, as these chemicals are often used in balms at concentrations that are orders of magnitude greater.

Given that menthol effects TRPV3 and TRPA1 in addition to TRPM8, we tested whether camphor, cinnamaldehyde and capsaicin might also modulate the activity of more than one thermoTRP receptor. Therefore, we extended our studies and performed a “4 × 4” analysis of the specificity of four sensory compounds on four thermoTRPs. We employed a screen for activating or inhibiting relationships of varying concentrations of camphor, cinnamaldehyde and capsaicin on four thermoTRPs—TRPV3, TRPA1, TRPM8 and TRPV1 (thermoTRPs known to be activated by sensory compounds) (Table 1). For each compound-channel pairing, we investigated both agonistic and antagonistic effects. We used the same criteria to evaluate the specificity of the effect on thermoTRPs: high compound concentrations should not induce calcium release/influx nor disrupt native ATP responses in

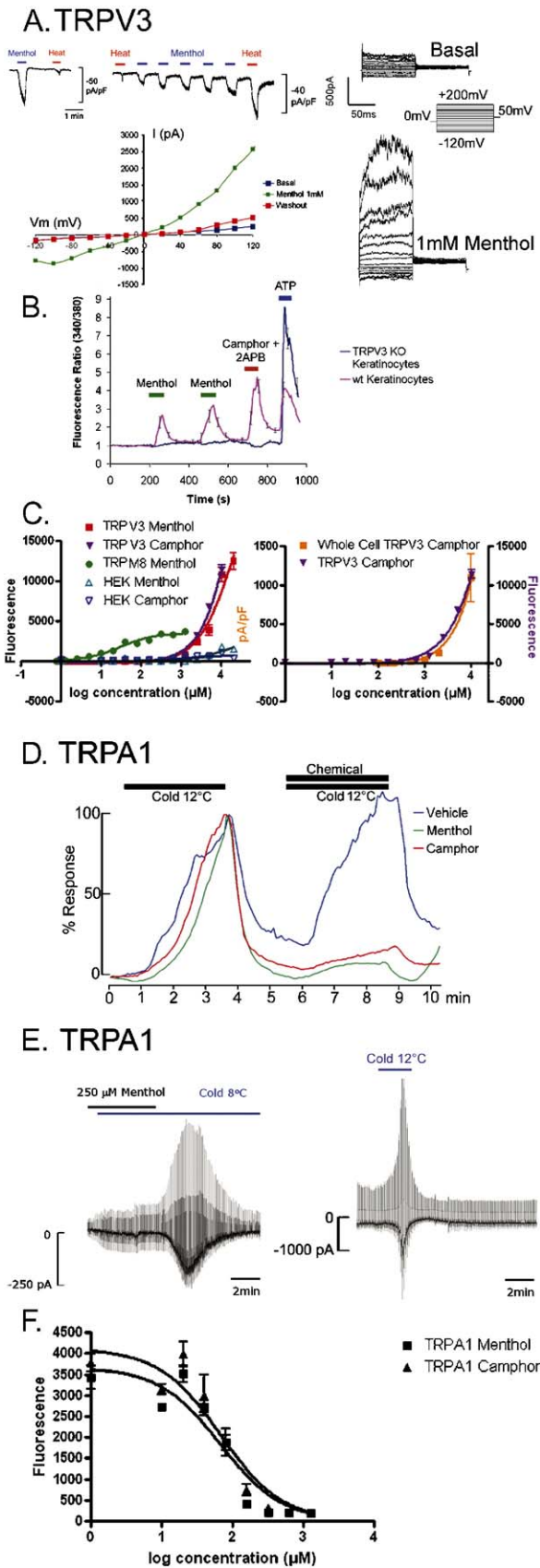


Table 1

Summary of analyses of promiscuity among thermoTRPs and sensory compounds

	Sensory compounds			
	Menthol	Camphor	Cinnamaldehyde	Capsaicin
TRPV1	∅	4.5 mM <sup>a</sup> (+)	∅	32 nM (+)
TRPV3	20 mM <sup>a</sup> (+)	40 mM <sup>a</sup> (+)	ND <sup>b</sup> (+)	∅
TRPM8	30 μM (+)	∅	1.5 mM (-)	∅
TRPA1	68 μM (-)	68 μM (-)	9.5 μM (+)	∅

Each of the four compounds was screened for activating and inhibiting relationships to the four thermoTRPs shown. Concentrations tested in CHO and/or HEK cells ranged from 0.75 mM–20.48 mM menthol, 10 mM–10.24 mM camphor, 10 nM–1 mM capsaicin, and 1 mM–20.48 mM cinnamaldehyde. If determined, the EC<sub>50</sub> or IC<sub>50</sub> value determined by FLIPR is included in the table. (+) activation. (-) inhibition. ∅ No effect. ND, not determined.

<sup>a</sup> Saturation point of dose response curve was not met, EC<sub>50</sub> values are estimates based on the slope of the initial curve.

<sup>b</sup> Background activation of CHO and HEK cells to concentrations of cinnamaldehyde >10 mM prevented an EC<sub>50</sub> determination.

calcium imaging experiments using non-transfected CHO cells. In addition, solutions containing ethanol or DMSO were used as negative controls to rule out vehicle effects. The effects of compounds on ion channel activity were tested in calcium imaging and verified in electrophysiological experiments.

*Camphor inhibits TRPA1 and activates TRPV1*

Application of camphor on human skin leads to sensitization to heat (and to a lesser extent cold) and like menthol, camphor is an ingredient of many topical analgesics (Green, 1990). We recently reported that camphor activates TRPV3 (Moqrich et al., 2005). Similar to the situation for menthol, we reasoned that camphor might also block TRPA1 or TRPV1. Calcium imaging studies using 0.25–2 mM camphor showed no inhibition of TRPV1 or activation/inhibition of TRPM8 (data not shown, see below). However, 0.25 mM camphor inhibited cold- and cinnamaldehyde-induced TRPA1 responses (Figs. 1D and F). The IC<sub>50</sub> for camphor

Fig. 1. Menthol activates TRPV3 and inhibits TRPA1. (A) 2 mM menthol elicits large currents in whole-cell recordings of TRPV3-expressing cells (*n* = 8, average current density = 192 ± 69 pA/pF). 0.5 mM menthol applications sensitize TRPV3 responses to heat. The last heat response increased to 525 ± 54% (*n* = 9) relative to the first. Right panel: steady-state current–voltage relationship of a TRPV3-expressing cell, before and during application of 1 mM menthol (*n* = 5). Voltage step protocol performed as shown. Bottom panel: IV curve of a TRPV3 expressing cell, before, during, and after application of 1 mM menthol. (B) 1 mM menthol activates wild-type but not TRPV3 knockout cultured keratinocytes in calcium imaging experiments. Lines are an average of 75 TRPV3 null and 29 wild-type keratinocytes. Error bars are ±SEM. Colored bars indicate addition of compound (1 mM menthol, 1 mM camphor + 50 μM 2-APB, 100 μM ATP). (C) Camphor and menthol dose response, determined by FLIPR, of TRPV3 and TRPM8 transiently transfected and untransfected HEK cells. (D) 0.25 mM menthol or camphor inhibits cold-induced activation of TRPA1-expressing CHO cells in calcium imaging experiments (traces represent normalized 340/380 ratio responses of ~20 cells). (E) Whole-cell electrophysiological recording of menthol inhibition of TRPA1 cold response (left panel). Right panel—example of a TRPA1 cold response without menthol. (F) Inhibition dose response curve of menthol and camphor, determined by FLIPR, of CHO cells stably expressing mTRPA1, activated by 75 μM cinnamaldehyde.

inhibition of TRPA1 activation by 75  $\mu\text{M}$  cinnamaldehyde was 68  $\mu\text{M}$  (95% CI: 36.8–127.0  $\mu\text{M}$ ) (Fig. 1F), similar to the concentration dependence of menthol. To validate heterologous expression studies, we tested the inhibitory effects of camphor on cinnamaldehyde responses in cultured DRG neurons as described (Bandell et al., 2004). Menthol was not tested as a blocker in these studies to avoid confounds produced by menthol sensitivity of TRPM8-expressing DRG neurons. Application of 2 mM camphor during constant 0.2 mM cinnamaldehyde perfusion strongly inhibited cinnamaldehyde-induced intracellular calcium responses in DRG neurons (183 total cells, 9/10 cinnamaldehyde-sensitive cells blocked, data not shown). High concentrations of camphor (10 mM) were recently reported to inhibit basal currents of TRPA1 but block of agonist-activation of TRPA1 was not reported (Xu et al., 2005). Here, we show that relatively low concentrations of both camphor and menthol block activation of TRPA1 induced by multiple stimuli.

In our published report on TRPV3 activation by camphor, we screened for specificity in relation to other thermoTRPs by using calcium imaging and 1–2 mM concentrations (Moqrich et al., 2005). We did not test concentrations above 2 mM camphor as background calcium influx/release was observed in naïve CHO cells. Utilizing this technique and these concentrations, we found camphor to be a specific activator of TRPV3. However, a recent study showed that TRPV1 exhibited small responses to 1 mM camphor, becoming more robust with concentrations up to 10 mM in electrophysiological recordings from HEK cells (Xu et al., 2005). As with high concentrations of menthol, in FLIPR studies, background activation of HEK cells by camphor was minimal (Fig. 1C). Although a maximal response of TRPV1 to camphor was not achieved because of insolubility at high concentrations, the  $\text{EC}_{50}$  value based on the initial concentration dependence curve is 4.55 mM (95% CI: 1.39–14.92 mM) (Table 1). We revisited the camphor–TRPV1 relationship using electrophysiological recordings of TRPV1-expressing CHO cells and found that 2 mM camphor activated TRPV1, albeit to a lesser extent than 100 nM capsaicin (Fig. 2A).

We also revisited camphor sensitivity of cultured DRG neurons using whole-cell recordings. Similar to our previous findings, we did not observe sensitizing TRPV3-like currents in response to 5 mM camphor (Moqrich et al., 2005). However, similar to the report by Xu et al., a small and strongly desensitizing current was observed in a subset of capsaicin-sensitive neurons, presumably via activation of TRPV1. Importantly, DRG neurons derived from both wild-type and TRPV3 knockout mice revealed similar responses to camphor (Figs. 2B–D). This is consistent with the hypothesis that camphor-induced small desensitizing currents are mediated by TRPV1. In electrophysiological studies, the number of camphor-sensitive neurons observed was dependent on the sequence of agonist application: for wild-type DRG neurons, 24% of capsaicin-sensitive neurons showed currents in response to camphor pulses when applied after a capsaicin application (Fig. 2C, top); while 71% were sensitive when camphor was applied prior to capsaicin (Fig. 2C, bottom). In TRPV3 knockout DRG neurons, 25% of capsaicin-sensitive neurons were also responsive to camphor, similar to wild type (Fig. 2C, right). 5 mM camphor failed to activate all capsaicin-sensitive neurons because it is a weak agonist compared to capsaicin, and because camphor-induced currents are subject to desensitization when applied after an initial capsaicin application (protocol used in our previous study). In calcium imaging studies of cultured DRG neurons, 85% and 82% of

camphor-sensitive neurons also responded to capsaicin in wild type and TRPV3 knockout mice, respectively (Fig. 2D). In this protocol, 5 mM camphor was applied first, allowed to wash out for 5 min, and then 100  $\mu\text{M}$  capsaicin was applied. Using this protocol, 24 wild-type and 22 TRPV3 knockout neurons responded to camphor, but not to capsaicin. This could represent activation of a receptor distinct from TRPV1 and TRPV3. Alternatively, it might be due to camphor's desensitization of some TRPV1-expressing neurons despite a relatively long period of camphor washout. Psychophysical experiments have revealed that camphor, as opposed to capsaicin, is not pungent, reinforcing a role of camphor in desensitization rather than activation of TRPV1 (Xu et al., 2005).

#### *Impact of cinnamaldehyde and capsaicin on thermoTRPs*

Solutions containing 0.2–10% (~1.5–75 mM) cinnamaldehyde induce burning sensations in humans (Prescott and Swain-Campbell, 2000; Namer et al., 2005). In our previously published report on cinnamaldehyde activation of TRPA1, we screened for activation of thermoTRPs (TRPV1, TRPM8, TRPV2, TRPV4) using concentrations of up to 0.6 mM cinnamaldehyde (Bandell et al., 2004). The higher cinnamaldehyde concentrations used in human studies might contribute to the overall sensations of pain via activation (or inhibition) of thermoTRPs in addition to TRPA1. TRPV1 was not activated by cinnamaldehyde concentrations of up to 2 mM. We were unable to test higher concentrations of cinnamaldehyde on TRPV1, as the amount of ethanol required to dissolve such amounts of cinnamaldehyde can alone activate TRPV1 (data not shown) (Trevisani et al., 2002). Interestingly, cinnamaldehyde (0.5–5 mM) activates TRPV3 (Fig. 3A). Although responses to 0.5 mM cinnamaldehyde were small, the effect of higher concentrations (5 mM) was specific to TRPV3. Although a dose response curve shows increasing TRPV3 activity to increasing concentrations of cinnamaldehyde, background fluorescence of untransfected HEK and CHO cells also increased, precluding an  $\text{EC}_{50}$  determination (data not shown). Up to 5 mM, concentrations of cinnamaldehyde did not activate non-transfected CHO cells, nor did it diminish native responses to 10  $\mu\text{M}$  ATP (data not shown). Vehicle (ethanol) concentrations ranging from 0.5–1.0% also had no effect on TRPV3 or naïve CHO cells.

Intriguingly, cinnamaldehyde had the opposite effect on TRPM8. Both cold and menthol responses were attenuated by cinnamaldehyde in a concentration-dependent manner with 5 mM concentrations causing complete block (Figs. 3B and C). 5 mM cinnamaldehyde contains 0.5% ethanol. Ethanol is known to modulate TRPM8 by inhibiting responses to menthol in a concentration-dependent manner (TRPM8 currents in response to 100  $\mu\text{M}$  menthol are reported to be reduced ~50% by 0.5% ethanol (Weil et al., 2005). However, TRPM8 activated by cold (or menthol) was not inhibited by solutions containing 0.5% ethanol in the present study (Fig. 3C). FLIPR experiments revealed cinnamaldehyde blocked the menthol-induced activation of TRPM8 with an  $\text{IC}_{50}$  of 1.5 mM (95% CI: 0.8–2.8 mM) (Fig. 3D; green symbols). Menthol and cold activate TRPM8 by shifting the voltage dependence of activation to more negative potentials (Voets et al., 2004; Nilius et al., 2005). Since activators of TRPM8 shift the  $V_{1/2}$  to the left, we tested whether inhibitors of the channel could shift the  $V_{1/2}$  for channel activation to the right, effectively making the channel more difficult to open at physiological voltages. Indeed, application of 1 mM cinnamaldehyde at 16°C,

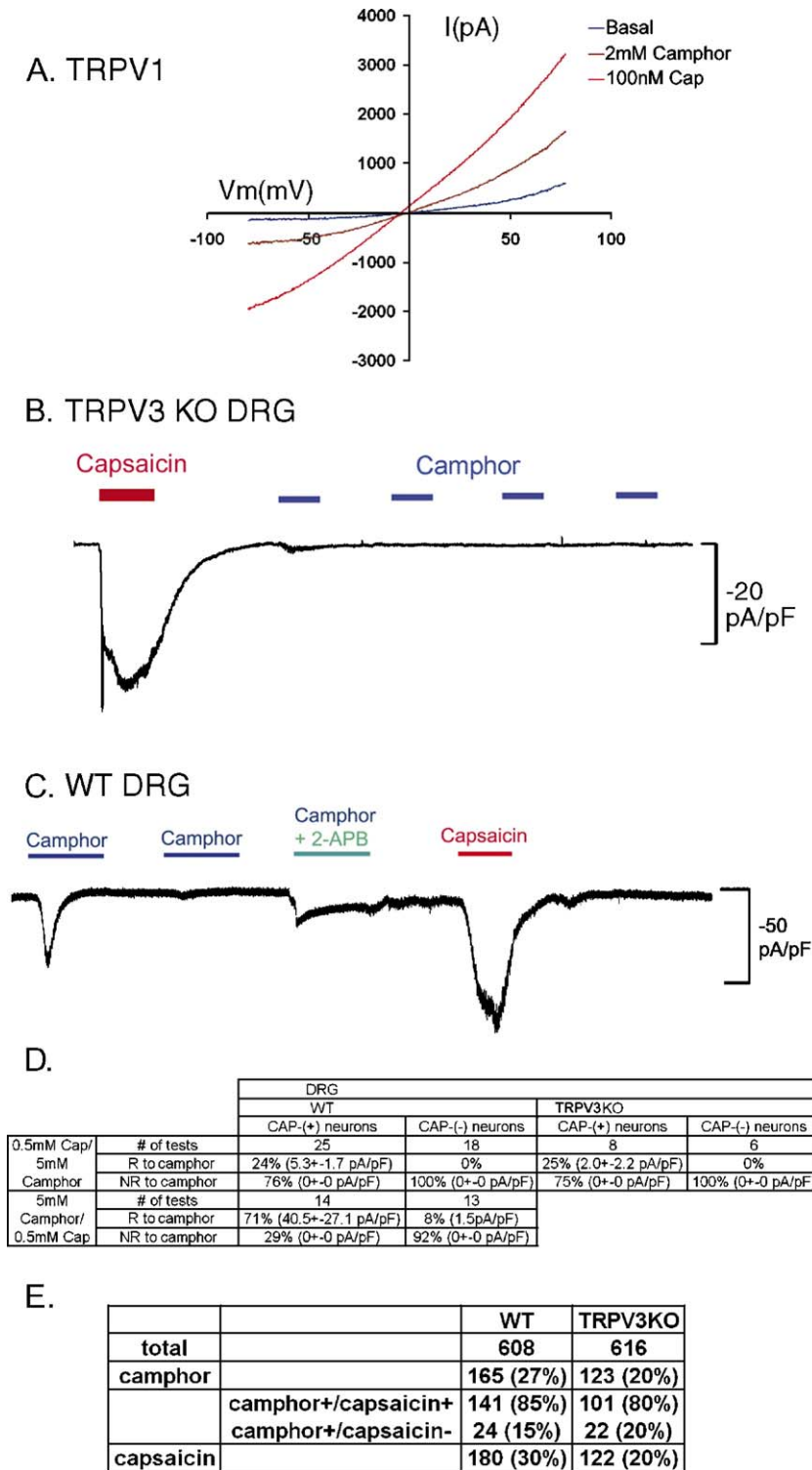
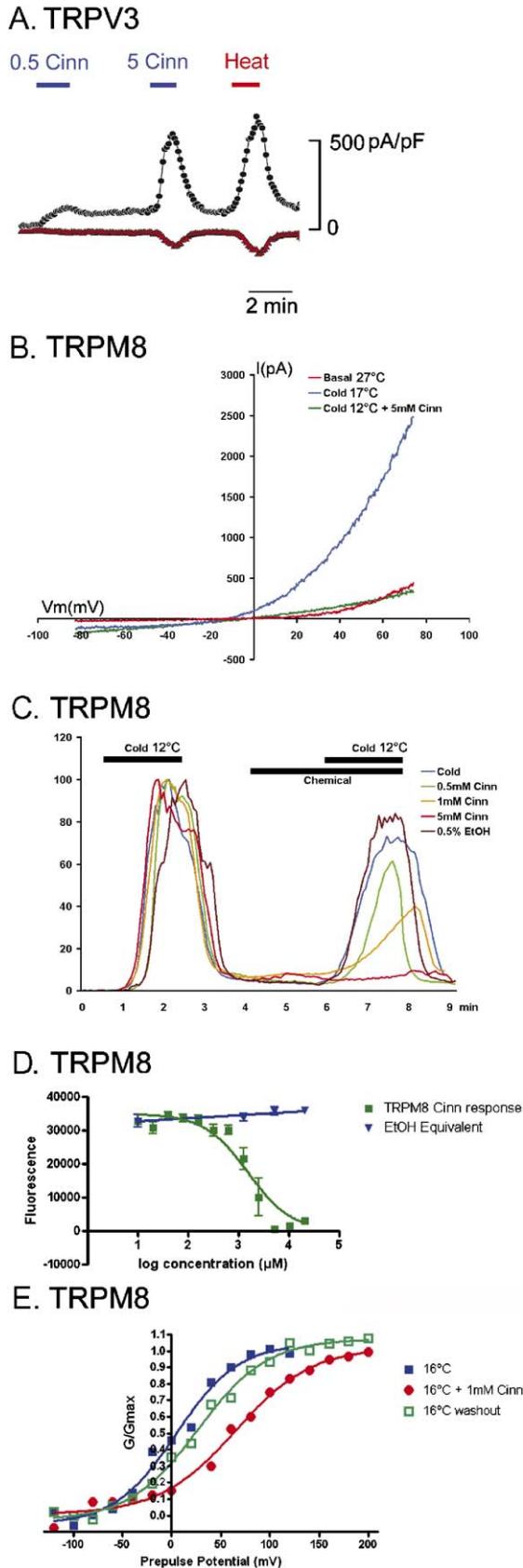


Fig. 2. Camphor activates TRPV1 and a subset of capsaicin-sensitive DRG neurons. (A) Voltage ramp protocol showing 2 mM camphor activates TRPV1-expressing cells. 2 mM camphor elicits outward and some inward currents, although the response to 100 nM capsaicin is larger by comparison ( $n = 3$ ). (B) Whole-cell currents in a TRPV3 knockout DRG neuron recorded in response to 0.5 mM capsaicin followed by recurrent application of 5 mM camphor. A subset of capsaicin-responsive neurons show a small, desensitized response to 5 mM camphor. Camphor consistently elicits much smaller currents relative to 0.5  $\mu$ M capsaicin in the same cell. (C) Whole-cell currents in a wild-type DRG neuron in response to repeated application of 5 mM camphor, 5 mM camphor with 50  $\mu$ M 2-APB, and 0.5 mM capsaicin. (D) Responses of wild type and TRPV3 knockout DRG neurons to capsaicin and recurrent camphor application using whole-cell electrophysiology. The profile of responses when a capsaicin pulse is followed by multiple camphor pulses as in panel B is compared with those when the camphor pulses precede that of a capsaicin pulse as in panel C. Abbreviations: R: response; NR: no response. Populations are shown as percentages of total neurons tested, and average current densities elicited by the first pulse of camphor are included in parentheses  $\pm$ SEM in pA/pF. (E) Tally of responses of wild type and TRPV3 knockout DRG neurons to application of 5 mM camphor followed by 100  $\mu$ M capsaicin assayed by calcium imaging.



shifted the  $V_{1/2}$  of TRPM8 from +5 mV to +65 mV (Fig 3E,  $n = 3$ ). The effect was partially reversible. Steady-state currents evoked at 28°C were also consistent with a rightward shift in voltage dependence ( $n = 2$ , data not shown). These studies show that cinnamaldehyde activates TRPV3 and inhibits TRPM8, in addition to activating TRPA1.

Perhaps the most specific of sensory compounds is thought to be capsaicin. Nociceptive behavior induced by intraplantar injection of 0.3–0.6 mM capsaicin is abolished in TRPV1 knockout mice, suggesting it is the sole capsaicin receptor (Caterina et al., 2000; Davis et al., 2000). We find that concentrations of up to 0.5 mM capsaicin are specific to TRPV1. Higher concentrations of capsaicin (1 mM) appeared to inhibit TRPV3 (camphor and heat responses) and TRPM8 (cold and menthol responses) and activate TRPA1. However, 1 mM capsaicin also induced large fluorescence increases in non-transfected CHO cells and diminished ATP responses, so it is unlikely the effects on these other thermoTRPs were specific (data not shown).

## Discussion

This study highlights a complex, tangled relationship of sensory compounds to thermoTRPs, and raises questions on the evolution of this relationship. We began by reexamining the specificity of TRPM8 activation by menthol, a natural compound with paradoxical sensory effects. Although the sensation of menthol is primarily one of “cool,” it also heightens warmth sensation (Green, 1985). Here, we show that the complexity of menthol sensations may be explained via receptor-based mechanisms. We demonstrate that menthol acts on a total of three thermoTRPs: cool-activated TRPM8, warm-activated TRPV3 and TRPA1, a thermoTRP activated by noxious cold and pungent/burning compounds. At warm temperatures menthol might be interpreted as warm based on its sensitizing effect on TRPV3, while at cooler temperatures, its activation of TRPM8 dominates its sensory quality. Although this seems to be the most parsimonious explanation of the data, it is a difficult model to prove. First, the effects of menthol on warming in human subjects have been examined in only a few studies with small sample size (Green, 1985; Hatem et al., 2006). Furthermore, menthol may activate other pathways to produce this warm sensation. Finally, millimolar amounts of menthol are required to activate TRPV3. Although similar and higher concentrations of menthol are used in over the counter medication, penetration issues

Fig. 3. Cinnamaldehyde activates TRPV3 and inhibits TRPM8. (A) 5 mM cinnamaldehyde and 37°C heat elicit TRPV3 currents. Whole-cell currents in TRPV3-expressing cells were recorded at two voltages every 4 s (+60 mV, filled circle; –60 mV, red triangle). ( $n = 5$ ) (B) Cold activation of TRPM8 is inhibited by 5 mM cinnamaldehyde. Large outward currents evoked by perfusion of cold extracellular solution are blocked to basal current levels by addition of 5 mM cinnamaldehyde. (C) TRPM8 cold responses are blocked by cinnamaldehyde in a dose-dependent manner independent of vehicle effects in calcium imaging experiments. Each trace represents average fluorescence ratio increases of 100 cells. Two cold pulses were delivered by lowering the perfusate temperature from 25°C to 12°C over 2 min. (D) Inhibition dose response curve of cinnamaldehyde on CHO cells stably expressing TRPM8, activated with 75 μM menthol. TRPM8 responses to 75 μM menthol with ethanol at equivalent concentrations are shown as vehicle controls. (E) Tail current analysis of cells expressing TRPM8 at 16°C before, during, and after application of 1 mM cinnamaldehyde.

could limit actual concentration (but see below). On the other hand, submillimolar menthol is able to block TRPA1. The  $IC_{50}$  for menthol inhibition of TRPA1 is 68  $\mu$ M, a relatively low concentration compared to the concentrations used in pain rubs and not qualitatively different from menthol's ability to activate TRPM8 ( $EC_{50}$  30  $\mu$ M). Therefore, menthol's analgesic action could be based on TRPM8 activation, and/or inhibition of pain-sensing TRPA1.

To further investigate thermoTRP-mediated mechanisms of the complexity of sensory compounds, we performed a "4 × 4" analysis of the specificity of menthol, camphor, cinnamaldehyde and capsaicin against TRPV3, TRPA1, TRPV1 and TRPM8 (in order of our analyses, Table 1). For each compound-channel pairing, we investigated both agonistic and antagonistic effects with complete concentration dependence, when possible. For all pairing relationships (32 in total), we found only one instance of selectivity—in all cases, with the exception of capsaicin activation of TRPV1, the thermoTRPs were either activated or inhibited by more than one of the compounds we studied. Menthol, camphor and cinnamaldehyde each activated/inhibited a total of three of the four thermoTRPs tested (Table 1). TRPV3 and TRPA1 appeared to be the most promiscuous thermoTRPs, being activated or inhibited by menthol, camphor and cinnamaldehyde. Block of TRPA1 by menthol (a TRPM8 agonist), and TRPM8 by cinnamaldehyde (a TRPA1 agonist) revealed a reversed agonist/antagonist relationship between these two cold-activated thermoTRPs. Camphor activated TRPV1 but inhibited TRPA1 at notably disparate concentrations—4.5 mM ( $EC_{50}$ ) to activate vs. 68  $\mu$ M ( $IC_{50}$ ) to block, respectively (Xu et al., 2005).

The concentrations of sensory compounds we have utilized to study specificity are likely to be relevant—they are well below or within range of the concentrations used in studies of human subjects and in over-the-counter pain rubs and creams. It is also likely that the sensory compounds at these concentrations could penetrate the skin to act on the thermoTRP receptors. Transdermal absorption has been studied to determine the efficacy and safety of delivery by patches, which contain high concentrations of several sensory compounds (and drugs). Low but detectable levels of menthol and camphor were detected in the plasma of human subjects who applied such patches to their skin (Martin et al., 2004). In addition, 2% (~100 mM) menthol acts as a penetration enhancer in drug delivery studies, suggesting it not only permeates the epidermis well but also acts to increase the accessibility of other molecules (Yener et al., 2003; Kanikkannan et al., 2004). However, actual concentrations of these sensory compounds at keratinocytes and nerve endings could not be calculated at this time. It is therefore possible that some of the sensory compound-thermoTRP relationships we have discovered are relevant (e.g., 68  $\mu$ M  $IC_{50}$  of camphor and menthol to TRPA1) while others are not. Nevertheless, all the findings are important as pharmacological tools. The overall promiscuity of sensory compounds shown here suggests caution when using such molecules as specific pharmacological agents.

Mechanistically, little is known about how sensory compounds activate thermoTRPs. With the exception of capsaicin and more recently menthol, none of the structural elements required for activation of thermoTRPs by the compounds tested in this study are known (Jordt and Julius, 2002; Bandell et al., 2006). Further study is needed to determine the mechanisms of "cross-talk" among these sensory compounds and thermoTRPs. It is notable that the concentration dependence of menthol and camphor to

either activate TRPV3 (each in the mid-mM range) or to block TRPA1 (each ~70  $\mu$ M) are similar. These compounds may interact at the same binding sites to directly activate TRPV3 or competitively inhibit TRPA1. This may also explain the reversed agonist/antagonist relationship of menthol and cinnamaldehyde on TRPM8 and TRPA1 as well as the ability of cinnamaldehyde to activate TRPV3. We show here that cinnamaldehyde can shift the  $V_{1/2}$  of TRPM8 to the right, opposing the leftward shift of voltage due to cold temperatures. While voltage modulation by temperature and compounds is well documented for TRPV1 and TRPM8, and may be true for TRPV3, a compound/temperature/voltage relationship with TRPA1 has yet to be investigated (Brauchi et al., 2004; Voets et al., 2004; Chung et al., 2005; Xu et al., 2005). Therefore, voltage modulation could be the central mechanism explaining the activity of these sensory compounds on thermoTRPs. Alternatively, it is possible that the effect of menthol and other sensory compounds on thermoTRPs is indirect, through modification of membrane structure, binding to other receptors, or activating second messengers.

## Experimental methods

### CHO cell, DRG neuron, and primary keratinocyte culture

Chinese Hamster Ovary (CHO) cells were transiently transfected with plasmids encoding rTRPV1 (rat), mTRPA1 (mouse), mTRPM8, and mTRPV3 using FuGene transfection reagent (Roche). Cells were co-transfected with a pcDNA3-based (Invitrogen) YFP marker plasmid. Negative control cells were transfected with YFP alone. Stable cell lines expressing mTRPM8, mTRPA1 and rTRPV1 were used in some studies as previously described (Story et al., 2003). Adult mouse DRG culture was carried out according to our previous protocol (Bandell et al., 2004). Primary culture of newborn mouse keratinocytes was performed according to our previous protocol (Moqrich et al., 2005). For the menthol experiments on primary keratinocytes, warm calcium imaging buffer (33°C) was perfused over the cells. 1 mM menthol was applied in the warm using 2 × 1 min pulses separated by 3 min of buffer washout. As a positive control for TRPV3 expression, cells were then cooled to 26°C, then 1 mM camphor + 50  $\mu$ M 2-APB was applied for 1 min which produces a maximal TRPV3 response. After 2 min of washout, 100  $\mu$ M ATP was applied at 26°C for 1 min.

### Calcium imaging and electrophysiology

After 24–72 h, transiently transfected YFP-expressing CHO cells were selected for calcium imaging or electrophysiology experiments. Intracellular calcium imaging experiments were performed by washing cells three times with calcium imaging buffer (1× Hanks' Balanced Salt Solution supplemented with 10 mM HEPES), then loaded with ratiometric calcium indicator dye Fura-2 (Molecular Probes) up to 1 h, according to product information. Cells were washed three times and incubated at room temperature for 15–30 min prior to imaging. For whole-cell recordings, a voltage ramp protocol was used with a sampling interval of 400  $\mu$ s/channel (2.5 kHz), holding at –60 mV for 250 ms, then ramping from –80 mV to +80 mV over 325 ms, returning to –60 mV for 250 ms after the ramp. Ramps were taken with an inter-sweep interval of 3 s. For some DRG neuron experiments, a voltage clamp (at –60 mV) was used. Unless otherwise indicated, recordings were performed at room temperature. The regular pipette solution in the whole-cell experiments was (in mM) CsCl, 140; EGTA, 5; HEPES, 10; MgATP, 1; titrated to pH 7.4 with CsOH. For TRPM8, the extracellular solution contained (in mM) NaCl 140; EGTA, 5; HEPES, 10; and MgCl<sub>2</sub>, 2. This extracellular solution was the same for other experiments with the following exceptions: for TRPV3, 5 mM KCl was added, for TRPV1, 2 mM CaCl<sub>2</sub> and 5 mM KCl was added, and for

TRPA1, 0.5 mM CaCl<sub>2</sub> was added to the extracellular solution. For determination of steady-state IV and V<sub>1/2</sub> of TRPM8, cells were challenged with a family of 100-ms duration voltage steps stepping from –120 mV to +200 mV in 20-mV intervals, followed by a step to +50 mV for 100 ms to measure tail currents. The holding potential was 0 mV. An inter-sweep interval of 10 s was used to ensure complete channel closure between sweeps. V<sub>1/2</sub> determinations were obtained using the Boltzmann function to fit G/G<sub>max</sub> vs. voltage curves derived from tail current analyses.

#### FLIPR (fluorescence imaging plate reader)

For FLIPR (Molecular Devices) determination of EC<sub>50</sub> dose response curves, transiently transfected HEK cells were used for rTRPV1, mTRPV3, and mTRPM8 (FuGene—Roche). mTRPM8 stable CHO and Tet-inducible CHO cell lines expressing mTRPA1 were also used for FLIPR determinations of IC<sub>50</sub>. Cells were washed with assay buffer (1× Hanks' Balanced Salt Solution supplemented with 20 mM HEPES and 2.5 mM probenecid) and then loaded with Fluo-3 (Molecular Probes) dye according to protocol. Concentration dependence was obtained from 7 to 12 point determinations. Compounds menthol and cinnamaldehyde were dissolved in ethanol as 4 M stocks and serially diluted (1:2) in assay buffer from 40.96 mM in polypropylene 384-well compound plates to 2-fold the final concentration (20 μM to 40.96 mM). Cinnamaldehyde and menthol were soluble in assay buffer at 40.96 mM after gentle heating. Camphor was dissolved in ethanol as a 2 M stock. Camphor was sparingly soluble at 20.48 mM after heating. The maximum final ethanol concentration was 0.5% for all compounds. Vehicle controls and untransfected CHO/HEK cells were included on the plate. For determination of the IC<sub>50</sub> for compounds on TRPM8 and TRPA1, cells were challenged on line with inhibitor for 1 min prior to the application of activator compound and inhibitor, keeping the concentration of inhibitor constant. Cinnamaldehyde (75 μM) and menthol (75 μM) (final concentration) were used to activate TRPA1 and TRPM8, respectively. Fluorescence readings were acquired at 1 Hz for 1 min prior to inhibitor addition for baseline, 1 min after inhibitor addition (secondary baseline), and 3 min after agonist addition. Dose response curves were fit using PRISM (GraphPad) software using a nonlinear regression model. EC<sub>50</sub>, IC<sub>50</sub> and 95% confidence intervals (95% CI) were calculated from these curves.

#### Compounds

Menthol, camphor, cinnamaldehyde, 2-APB, and ATP were purchased from Sigma-Aldrich. Capsaicin was purchased from Fluka. Stock solutions for menthol, camphor and cinnamaldehyde were made using ethanol; capsaicin and ATP were dissolved with DMSO. All compounds were diluted with test solutions before use.

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