nature neuroscience

Oregano, thyme and clove-derived flavors and skin sensitizers activate specific TRP channels

Haoxing Xu[1–3], Markus Delling[1–4], Janice C Jun[1–4] & David E Clapham[1–3]

Carvacrol, eugenol and thymol are major components of plants such as oregano, savory, clove and thyme. When applied to the tongue, these flavors elicit a warm sensation. They are also known to be skin sensitizers and allergens. The transient receptor potential channel (TRPV3) is a warm-sensitive Ca^{2+}-permeable cation channel highly expressed in the skin, tongue and nose. Here we show that TRPV3 is strongly activated and sensitized by carvacrol, thymol and eugenol. Tongue and skin epithelial cells respond to carvacrol and eugenol with an increase in intracellular Ca^{2+} levels. We also show that this TRPV3 activity is strongly potentiated by phospholipase C–linked, G protein–coupled receptor stimulation. In addition, carvacrol activates and rapidly desensitizes TRPA1, which may explain the pungency of oregano. Our results support a role for temperature-sensitive TRP channels in chemesthesis in oral and nasal epithelium and suggest that TRPV3 may be a molecular target of plant-derived skin sensitizers.

Capsaicin (the pungent ingredient in chili peppers) produces the psychophysical sensation of 'hot' or 'burning' by acting on TRPV1 (ref. 1), whereas menthol activates TRPM8 to produce a 'cooling' sensation[2,3]. Oregano, savory, thyme and clove, widely used as spices, are perceived as flavors by activating receptor mechanisms for other physical senses (such as touch and temperature) in the tongue and nose[4]. These chemesthetic mechanisms, along with specific gustatory and olfactory receptors, contribute to taste and smell[4]. Oregano, savory and thyme have an aromatic odor, are 'bitter', 'pungent' or 'sharp' and evoke a sense of warmth[5], but the underlying molecular and cellular mechanisms for these flavors are not known. These plant-derived flavors have also long been known to cause skin sensitization[4,6,7]. Here we report that TRPV3, a warm-sensitive ion channel that is highly expressed in the skin, tongue and nose, is the target for the flavor actions of these plant derivatives as well as for skin sensitization.

RESULTS

TRPV3 in the palate, tongue and nasal epithelium

We observed TRPV3-specific *in situ* hybridization in the mouth and nose of prenatal (E16) and adult mice, with the strongest signals in the epithelium of the palate, tongue and nose (**Fig. 1**). Consistent with expression in primate tongue[8], TRPV3 staining in the mouse tongue was largely restricted to the epithelial layers facing the oral cavity. TRPV3 staining was more prevalent in the dorsal than in the ventral epithelium. A sharp border was apparent above the basal lamina of the epithelial layer; the thin vascular layer of connective tissue beneath the epithelium (lamina propria) was completely devoid of TRPV3 staining (**Fig. 1i,j**). Antibody staining by two distinct-epitope, TRPV3-specific antibodies (**Supplementary Fig. 1** online) stained primarily the epithelial layers of the dorsal tongue and palate with little or no staining in the lamina propria (E16), consistent with the *in situ* hybridization results. We observed little or no keratinization at this stage and detected similar patterns of TRPV3 staining in tongue and palate sections of juvenile mice (postnatal day 4, **Fig. 2a**). In adult mouse tongue, we observed TRPV3 immunoreactivity in the epithelia of filiform papillae (**Fig. 2a,b**) as well as in the epithelia surrounding taste buds of circumvallate papillae (**Supplementary Fig. 2** online). As the epithelium thickens with age, TRPV3 immunoreactivity decreases in the surface keratinized layer. We saw sparse TRPV3 immunoreactivity in the stratum corneum, the outermost layer of the epithelium that is completely keratinized and devoid of nuclei[9] (**Fig. 2**). TRPV3 was also expressed in the base of the epithelium, albeit less so than in the more superficial region of the stratum granulosum below the cornified superficial layer. It is likely that the more basal distribution of the *in situ* markers was attributable to higher amounts of TRPV3 mRNA than protein in the newer basal cells, and higher amounts of TRPV3 protein than mRNA upon epithelial migration and differentiation. Human tongue immunoreactivity patterns were similar to those in mouse (**Fig. 2c**). We also detected TRPV3 protein expression in the tongue epithelia by western blotting in rat (**Fig. 2d**). TRPV3 mRNA and protein were also highly expressed in the epithelial lining of the nasal cavities (**Figs. 1** and **2e,f**; see ref. 10).

Carvacrol activates TRPV3 and tongue epithelial cells

In addition to their gustatory and olfactory properties, oregano and thyme evoke a sense of 'warmth'[5]. Carvacrol (**Supplementary Fig. 3** online), the major ingredient of oregano, and thymol, a lesser component of oregano but an important constituent of thyme, are both

[1]Howard Hughes Medical Institute. [2]Department of Cardiology, Children's Hospital and [3]Department of Neurobiology, Harvard Medical School, 320 Longwood Avenue, Boston, Massachusetts 02115, USA. [4]These authors contributed equally to this work. Correspondence should be addressed to D.E.C. (dclapham@enders.tch.harvard.edu).

Received 30 January; accepted 28 March; published online 16 April 2006; doi:10.1038/nn1692

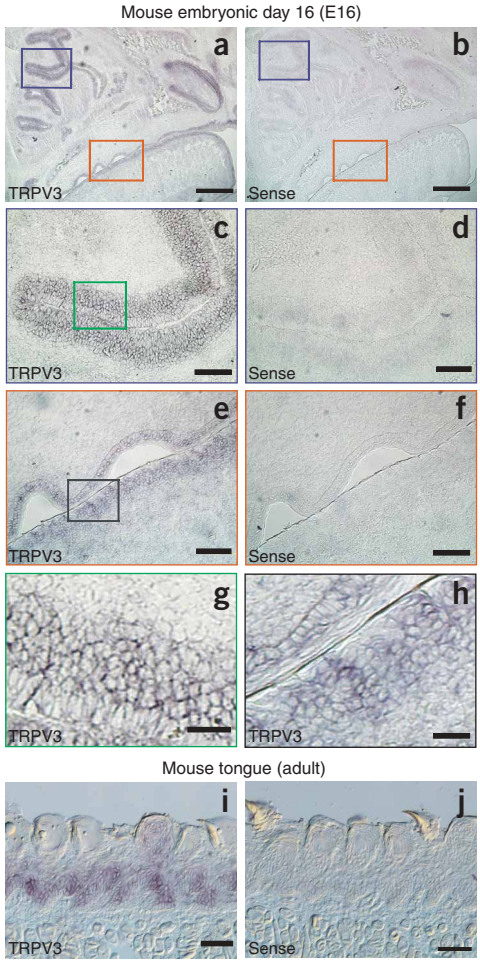

Figure 1 TRPV3 mRNA is highly expressed in the tongue and nasal epithelium. (**a**) *In situ* hybridization of mTRPV3 in head regions (sagittal view) of E16 mice. There was little or no staining in sense controls (**b**). Areas of nasal and oral cavities (rectangles) are expanded in **c**–**h**. Scale bar = 500 μm (**a**,**b**), 100 μm (**c**–**f**) and 25 μm (**g**,**h**). (**i**,**j**) TRPV3 in the basal epithelial layer of mouse tongue as shown by *in situ* hybridization. Bar = 50 μm.

known to evoke a sense of warmth and sensitize skin. The expression of TRPV3 in primate tongue[8], nose, and skin[10] suggests that TRPV3 might mediate chemesthetic sense and the skin-sensitizing actions of carvacrol and thymol. Carvacrol (500 μM) induced a substantial increase in intracellular calcium concentration ($[Ca^{2+}]_i$) in mTRPV3-expressing HEK293 cells (**Fig. 3**), but not in nontransfected cells, in cells transfected with rat TRPV2 (rTRPV2) (**Fig. 3b**,**d**) or in cells transfected with mouse TRPV4 (mTRPV4). In a subset (~60%, 76/129 cells) of isolated epithelial cells from rat tongue, carvacrol induced a small increase in $[Ca^{2+}]_i$ (**Fig. 3e**) that was strongly enhanced in the presence of 200 μM 2APB (an agonist of TRPV1, TRPV2 and TRPV3; refs. 11,12). The carvacrol and 2APB-induced $[Ca^{2+}]_i$ increase was blocked by ruthenium red (10 μM) or by removal of external Ca^{2+} (**Supplementary Fig. 4** online). In the presence of 200 μM 2APB, camphor (an agonist for TRPV1 and TRPV3; refs. 8,13), also induced a large $[Ca^{2+}]_i$ increase in the same cells. Neither the TRPV1 agonist capsaicin (1 μM), the TRPM8 agonist menthol (1 mM, **Supplementary Fig. 4**) nor the TRPA1 agonist mustard oil (50 μM, **Fig. 3e**) induced any significant response. Taken together, the evidence suggests the carvacrol-induced $[Ca^{2+}]_i$ increase in the tongue epithelial cells is mediated by TRPV3.

Consistent with Ca^{2+} imaging, carvacrol (500 μM) evoked a slowly developing TRPV3-mediated current (I_{TRPV3}) in mTRPV3-expressing HEK293 cells (**Fig. 4a**,**b**) but did not activate I_{TRPV1}, I_{TRPV2} or I_{TRPV4} (**Supplementary Fig. 5** online). Like heat-activated[8,10,14], camphor-activated[13], and 2APB-activated[11,12] TRPV3-mediated currents, carvacrol-activated I_{TRPV3} currents were reversible and became sensitized with repeated challenges (**Fig. 4a**,**b**). Thymol (500 μM, **Supplementary Fig. 3**), the main component of thyme, also activated I_{TRPV3} in mTRPV3-expressing cells, and repeated application of thymol sensitized I_{TRPV3} (**Fig. 4c**).

Vanillin, the active ingredient of vanilla, weakly activated a small outwardly rectifying current in TRPV3-expressing cells (**Fig. 4d**). Ethyl-vanillin, a more potent synthetic vanillin[15] that evokes a 'warm' sensation in the tongue, activated I_{TRPV3} much more potently than vanillin (**Fig. 4d**). As a common feature of carvacrol, thymol, and vanillin is their phenol group (**Supplementary Fig. 3**), we tested several other phenol-containing compounds. Eugenol, another vanilloid-like compound, also activated and sensitized TRPV3, as described below. However, neither guaiacol (2 mM), zingerone (2 mM) nor 4-acetamidophenol (2 mM) activated mTRPV3.

Activation of human TRPV3

Human tongue immunoreactivity patterns were similar to those in mouse (**Fig. 2c**). Heterologously expressed human TRPV3 (hTRPV3) was also activated and sensitized by carvacrol and thymol in a dose-dependent manner (**Fig. 4e**,**f**). At concentrations higher than 1 mM, I_{hTRPV3} was higher (but often did not wash out completely), and its *I*/*V* relation became more linear (I_2 mode[16]). Thus, carvacrol activates TRPV3 at lower concentrations than required for activation by camphor (3–10 mM; see ref. 17). This concentration range is similar to that for menthol activation of TRPM8 (0.1–1 mM; refs. 2,3) but higher than that for capsaicin activation of TRPV1 (0.1–1 μM; ref. 1). The unique sensitizing property of TRPV3 suggests that the efficacy and/or potency of carvacrol can be gradually increased with repeated or prolonged stimulation. The effect of carvacrol on hTRPV3 was further potentiated at physiological temperatures. Carvacrol (500 μM) potentiated heat-activated I_{hTRPV3} ($T = 38 \pm 1$ °C; -11 ± 2 pA/pF) to -247 ± 33 pA/pF ($n = 5$; –60 mV; **Fig. 4g**). This current density was 13-fold higher than that evoked by 500 μM carvacrol applied at ~22 °C in a separate group of cells (-19 ± 10 pA/pF, $n = 9$, $P < 0.001$). The large enhancement of the carvacrol-activated current in the presence of elevated temperatures was larger than the summed responses of either stimulus alone, suggesting that the effect of carvacrol is much more potent at physiological temperatures.

Carvacrol also activates TRPA1

Oregano not only produces a 'warm' sensation but is also described as pungent[5], suggesting that carvacrol may activate other TRP channels. TRPV1 was insensitive to 1 mM carvacrol (**Supplementary Fig. 5**). Consistent with Ca^{2+} imaging results, 1 mM carvacrol did not induce any noticeable current in rTRPV2-expressing cells during voltage ramps (–100 mV to +100 mV), although robust activation of doubly rectifying I_{rTRPV2} was elicited by bath perfusion of 300 μM diphenyl boronic anhydride (DPBA) (a 2APB–like agonist of TRPV1–TRPV3[16]) solution to the same cells (**Supplementary Fig. 5** online). Carvacrol (1 mM) did not activate heterologously expressed mTRPV4, another 'warm' sensing channel, or TRPM8, the menthol-activated 'cool' sensing channel (**Supplementary Fig. 5**).

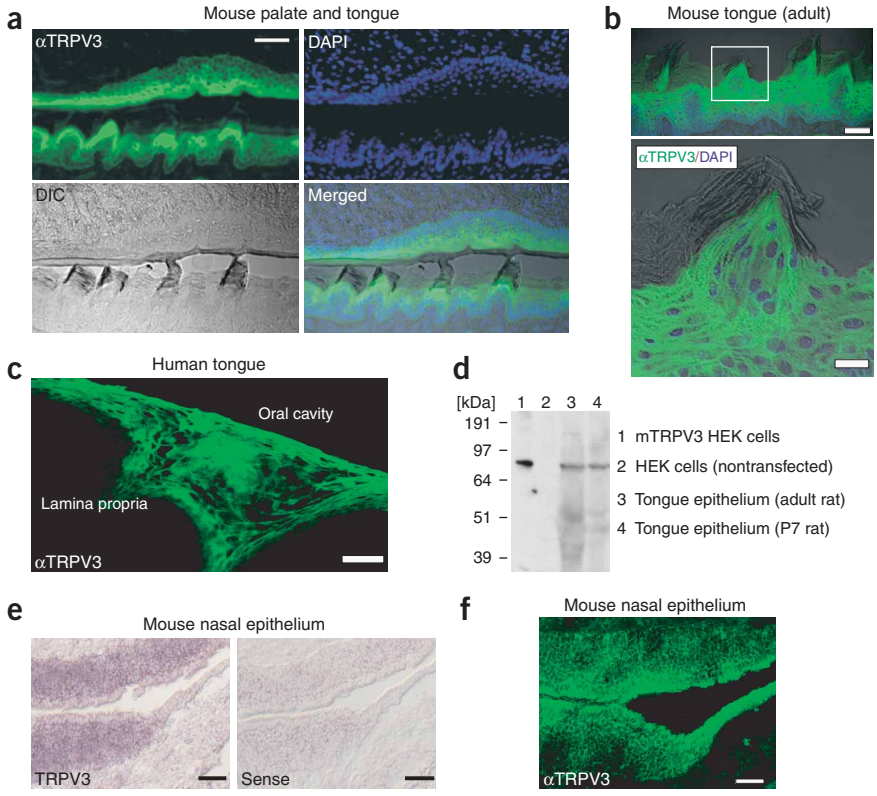

Figure 2 TRPV3 protein is highly expressed in the tongue and nasal epithelium. (**a**) TRPV3 immunoreactivity in the palate and tongue epithelium of P4 mice. Nuclei stained with DAPI. Bar = 50 μm. (**b**) TRPV3 immunoreactivity was not seen in keratinized epithelial cells that had lost their nuclei. Bar = 50 μm (top) and 20 μm (bottom). (**c**) TRPV3 immunoreactivity in tongue epithelium, but not lamina propria, of fetal human tissues. (**d**) Western blotting of rat epithelial lysates and control HEK293 cells lacking, or expressing, TRPV3. (**e**) Localization of TRPV3 in the mouse nasal epithelium, as shown by *in situ* hybridization. (**f**) TRPV3 localization in mouse nasal epithelium, as shown by TRPV3-specific antibodies. The surface epithelial cells facing the nasal cavity were most strongly stained.

TRPA1 is expressed in trigeminal ganglion neurons and is activated by several pungent compounds, including mustard oil[18] and garlic[19,20]. In HEK293 cells expressing the rat isoform of TRPA1, we observed constitutive currents that declined to a steady state ~10–100 s after break-in. As expected[18], the current was activated by 50 μM mustard oil (allyl isothiocyanate). Carvacrol (250 μM) robustly activated I_{TRPA1}, and the current quickly desensitized in the continuous presence of agonist (**Fig. 5**). Repeated stimulation resulted in much smaller responses (tachyphylaxis). Tongue epithelial cells did not respond to the TRPA1 agonist mustard oil (**Fig. 3e**). As TRPA1 is blocked, rather than activated by camphor[17], TRPV3 rather than TRPA1 may account for the carvacrol- or camphor-induced $[Ca^{2+}]_i$ increase in tongue epithelial cells (**Supplementary Fig. 4**). Although our data suggest that activation of TRPV3 may mediate the 'warmth' of oregano, TRPA1 in the free nerve endings of tongue and nose[19,20] may signal the pungency of oregano in a mechanism similar to that of mustard oil and garlic[18–20].

Eugenol activates TRPV3 and sensitizes skin

Chemical allergens and irritants induce cutaneous inflammation and skin sensitization by activating keratinocytes that release cytokines and adhesion molecules[21–23]. Before immunological cells are recruited, the release of cytokines from keratinocytes initiates the essential antigen- and hapten-independent phase of skin inflammation[22]. TRPV3 and TRPV4 are two Ca^{2+}-permeable cation channels highly expressed in epidermal keratinocytes (**Fig. 6a**). Mouse TRPV3-specific immunoreactivity colocalized with cytokeratin, a molecular marker for keratinocytes (**Fig. 6b**). TRPV3 was also functionally expressed in mouse 308 keratinocytes (**Supplementary Fig. 6**). These results generally agree with previous reports of the expression of TRPV3 in keratinocytes[8,10,13,24,25].

Cytokine production and release is often dependent on an increase in $[Ca^{2+}]_i$ (ref. 26). We tested the hypothesis that some chemical irritants, allergens or skin sensitizers act directly on TRPV3 or TRPV4 channels to induce Ca^{2+} entry. We screened numerous sensitizers,

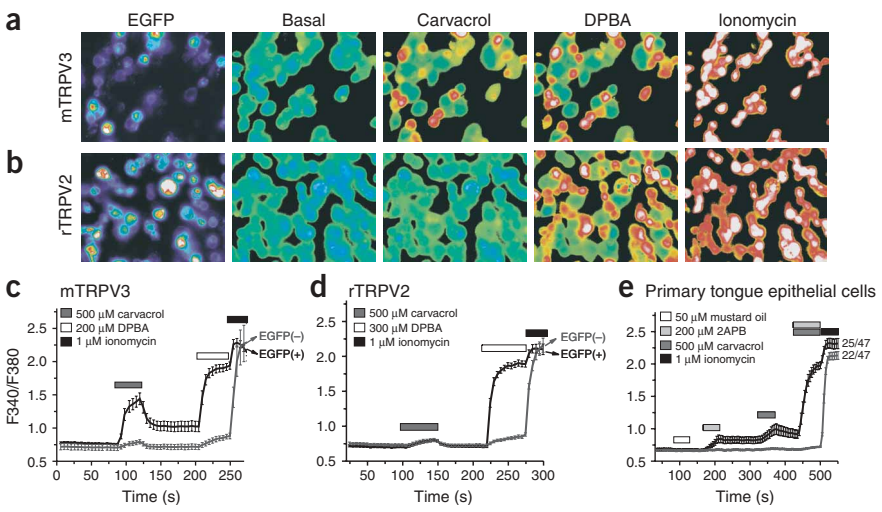

Figure 3 Carvacrol increases $[Ca^{2+}]_i$ in TRPV3-expressing HEK293 cells and tongue epithelial cells. (**a**,**c**) Carvacrol increased $[Ca^{2+}]_i$ (F340/F380; HEK293T cells transfected with mTRPV3-EGFP) in mTRPV3-expressing cells. TRPV3-negative cells exhibited negligible increases. All cells responded to the Ca^{2+} ionophore ionomycin (1 μM). (**c**) Average changes of Fura-2 ratios induced by carvacrol and other reagents in TRPV3-positive cells (EGFP(+)) and TRPV3-negative cells (EGFP(–), gray). (**b**,**d**) Carvacrol (500 μM) did not increase $[Ca^{2+}]_i$ in rTRPV2-positive cells, which responded robustly to 300 μM DPBA. (**e**) Rat tongue epithelial cell $[Ca^{2+}]_i$ response to carvacrol and 2APB. Average data from responding (25 of 47 cells) and nonresponding cells (22 of 47 cells in the same coverslip).

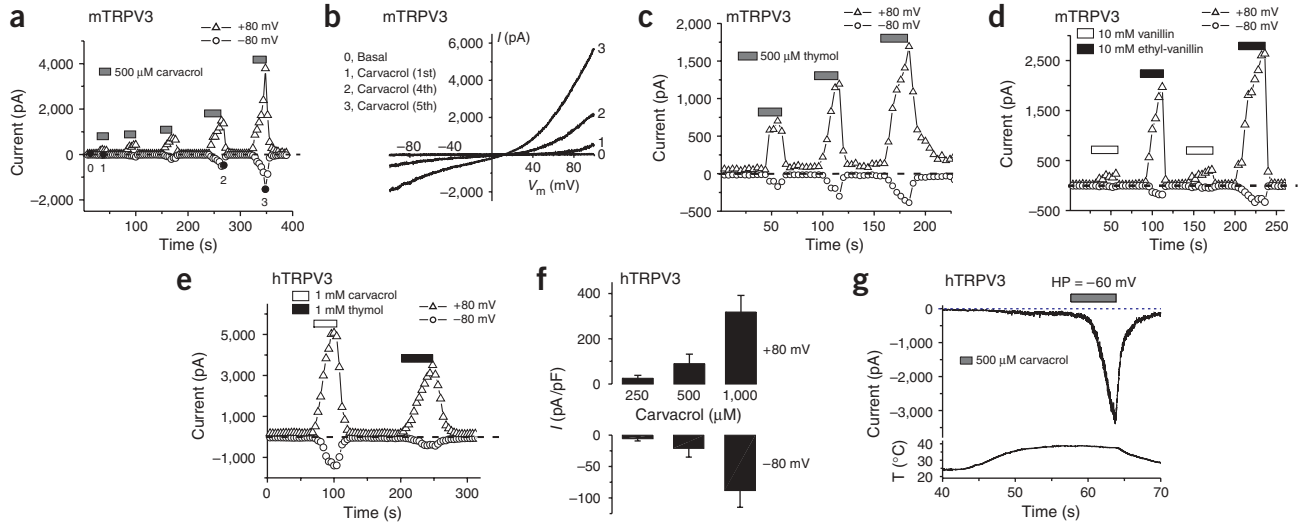

Figure 4 TRPV3 is activated by carvacrol, thymol, vanillin and ethyl-vanillin and sensitized by warmth. (**a**) Repeated applications of carvacrol (500 μM) activated and sensitized mTRPV3-mediated current (I_{TRPV3}). Currents in response to 400-ms voltage ramps from –100 to + 100 mV applied every 4 s. Holding potential = –60 mV. Each symbol represents the current amplitude at +80 mV (triangles) and –80 mV (circles). Dashed line = zero current. (**b**) Representative ramp currents. *I/V* relations were recorded at times denoted by filled circles in **a**. (**c**) Repeated application of thymol (500 μM) sensitized I_{mTRPV3}. (**d**) Ethyl-vanillin is roughly five to ten times more potent than vanillin. (**e**) Human TRPV3 (hTRPV3)-expressing cells responded strongly to carvacrol (1 mM) and thymol (1 mM). (**f**) Average I_{hTRPV3} inward current densities elicited by carvacrol at –80 mV were 250 μM; –6 ± 4 pA/pF, 500 μM; –21 ± 14 pA/pF and 1 mM; –88 ± 27 pA/pF ($n = 8$). Outward currents at +80 mV were, respectively, 25 ± 13 pA/pF, 89 ± 42 pA/pF and 318 ± 74 pA/pF ($n = 8$). (**g**) Increasing bath temperature to 38 °C alone activated a small inward current of ~–100 pA at –60 mV. Application of 500 μM carvacrol to the same cell in the continued presence of 38 °C solution dramatically enhanced the current to ~–3,300 pA.

including 1-chloro-2,4-dinitrobenzene (DNCB), methylsalicylate, cyclohexanol, eucalyptol and eugenol[7,23,27] in HEK293 cells expressing mTRPV3-EGFP or other related TRP-EGFP fusion proteins.

Eugenol, the principal active ingredient of clove, is used in dentistry as a topical analgesic. It is also an allergen affecting dental workers[28] at 0.1–10 mM concentrations[27]. DPBA[16] induced an increase in $[Ca^{2+}]_i$ in almost all mTRPV3-expressing cells (**Fig. 6c** and **Supplementary Fig. 7** online). Eugenol activated TRPV3 but not the closely related TRPV2 or TRPV4 channels (**Fig. 6d** and **Supplementary Fig. 7**). As positive controls, mTRPV4 and rTRPV2 responded robustly to distinct agonists (1 μM 4-αPDD and 300 μM DPBA, respectively; **Supplementary Fig. 7**). The eugenol-induced TRPV3-mediated $[Ca^{2+}]_i$ increase was absent in Ca^{2+}-free solution. In keratinocytes (308), eugenol induced a substantial increase in $[Ca^{2+}]_i$ (**Fig. 6e**) and significantly increased the release of interleukin (IL)-1α (**Fig. 6f**; see also ref. 7).

Consistent with Ca^{2+} imaging results, eugenol (2 mM) evoked a slowly developing, outwardly rectifying current (I_{TRPV3}) in mTRPV3-expressing (**Fig. 7a**,**b**) and hTRPV3-expressing HEK293 cells. Repeated applications of eugenol gradually sensitized the eugenol-activated TRPV3 current. In contrast, we did not observe any eugenol-evoked currents in cells transfected with vector alone, TRPV2 (**Supplementary Fig. 7**) or TRPV4. Ruthenium red (10 μM), the voltage-dependent blocker of TRPV1–TRPV4 channels, inhibited eugenol-activated inward I_{TRPV3}. Although the naive response of eugenol-induced I_{TRPV3} was relatively small, eugenol (3 mM) markedly sensitized heat-activated and DPBA-evoked currents (**Fig. 7c**,**d**), increasing them ~26-fold, from 34 ± 17 pA/pF to 899 ± 144 pA/pF (measured at +80 mV, $n = 4$). The DPBA-evoked inward current (measured at –80 mV) also increased ~47-fold, from –3.3 ± 0.8 pA/pF to –154 ± 41 pA/pF ($n = 4$). Thus, eugenol also activates and potently sensitizes TRPV3. Consistent with the observed increase in $[Ca^{2+}]_i$, eugenol also evoked TRPV3-like currents in mouse 308 (**Fig. 7e**,**f**) and primary keratinocytes (data not shown).

As reported previously[29], rTRPV1-transfected cells demonstrated small eugenol-evoked currents that underwent acute desensitization and tachyphylaxis. Also, in our hands, rTRPV1 was weakly activated by eugenol (**Supplementary Fig. 5**) but was insensitive to 1 mM carvacrol (potency of activation: capsaicin > eugenol >> carvacrol for TRPV1; carvacrol > eugenol >> capsaicin for TRPV3), with longer acyl chain–containing compounds preferentially activating TRPV1. These results suggest that TRPV1 and TRPV3 may serve as related but complementary chemosensory mechanisms for vanilloid-like molecules. Capsazepine, a competitive antagonist of TRPV1, has been shown to completely inhibit eugenol-activated TRPV1-mediated current[29], suggesting that eugenol acts at the capsaicin binding site. However, eugenol-activated I_{TRPV3} was not inhibited by 10 μM

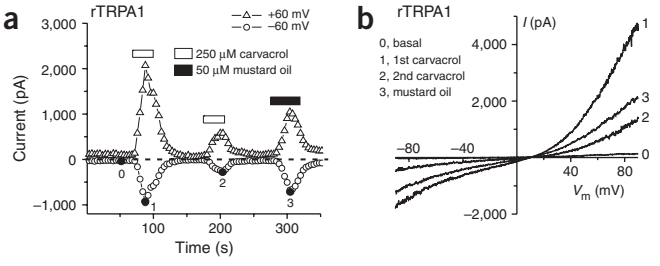

Figure 5 Carvacrol also activates TRPA1. (**a**,**b**) Carvacrol induced robust activation and subsequent desensitization of I_{TRPA1}. Large currents were elicited by carvacrol (250 μM) in an rTRPA1-expressing cell. Carvacrol-evoked I_{TRPA1} underwent acute desensitization and tachyphylaxis. The same cell also responded to mustard oil (allyl isothiocyanate, 50 μM), a known TRPA1 agonist. (**b**) Representative currents in response to voltage ramps.

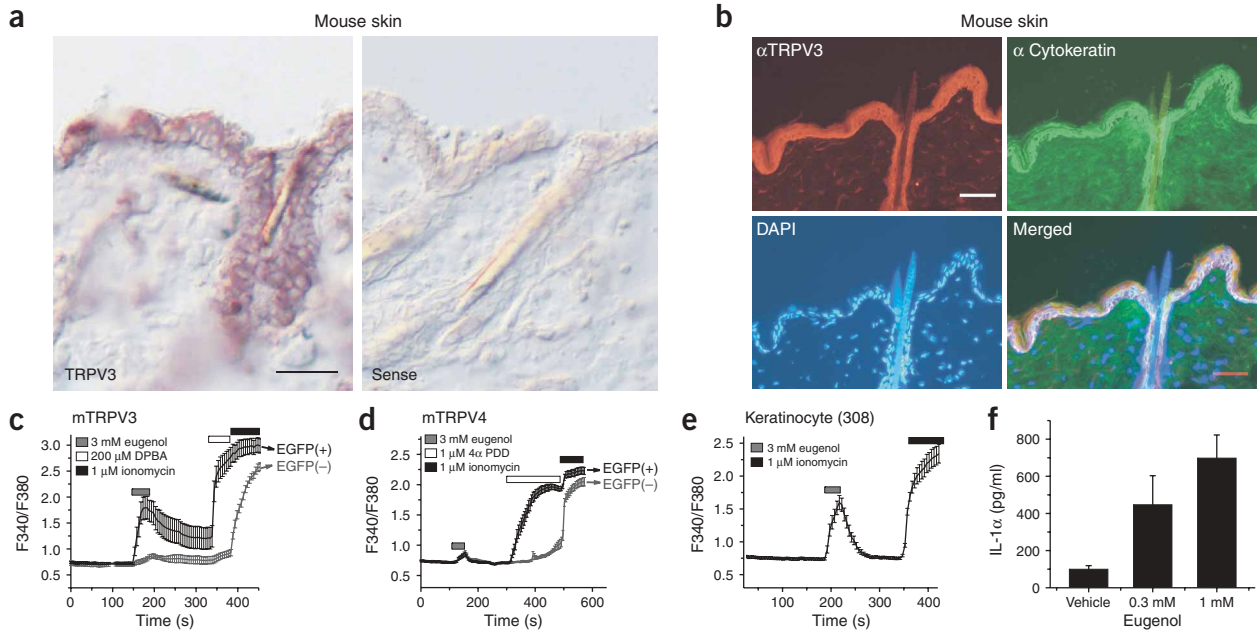

Figure 6 Eugenol activates TRPV3 and induces IL-1α release in mouse keratinocytes. (**a**) TRPV3 localization to mouse skin epidermis by *in situ* hybridization. (**b**) TRPV3 immunoreactivity in mouse skin epidermis. Bar = 50 μm. (**c**) Average changes in $[Ca^{2+}]_i$ induced by eugenol and DPBA in TRPV3-expressing and nontransfected cells, respectively. Eugenol (3 mM) increased $[Ca^{2+}]_i$ in most mTRPV3 (EGFP-positive) cells with negligible effects on mTRPV3-negative cells (gray). DPBA (200 μM) increased $[Ca^{2+}]_i$ in almost all mTRPV3-positive cells. (**d**) Average changes in $[Ca^{2+}]_i$ induced by eugenol and 4α-PDD in TRPV4-expressing and nontransfected cells, respectively. (**e**) Eugenol increased $[Ca^{2+}]_i$ in mouse keratinocytes (308). (**f**) Eugenol increased IL-1α release (as shown by ELISA) from 308 keratinocytes. Cytokine release was assayed at physiological temperatures, accounting for the lower eugenol concentration required for IL-1α release.

capsazepine. TRPV3 lacks a structural determinant for capsaicin binding[30] and is insensitive to capsazepine and capsaicin[8]. Thus, TRPV3 might be an alternate vanilloid receptor with substrate specificity distinct from TRPV1.

PLC-coupled receptor potentiation of TRPV3

Notably, many G protein–coupled receptors (GPCRs) for tastants and odorants activate phospholipase Cβ (PLC) via G protein (Gq/11)–linked receptors[31–33]. As TRPV3 is expressed throughout the oral and nasal epithelium, it may also be expressed in taste buds and solitary chemoreceptor cells[34]. Indeed, specific taste and odorant GPCRs for carvacrol, thymol or vanillin may exist[33]. Moreover, activation of neighboring free nerve endings by other flavors such as capsaicin, activation of epithelial cells by other stimuli such as temperature or food texture, or activation of taste cells by tastants[35] would result in the release of a variety of compounds, which could modulate the response of nasal and oral epithelial cells. As an initial test for these hypotheses, we assessed whether stimulation of PLC-coupled receptors enhanced the ability of carvacrol or other TRPV3 agonists to activate TRPV3 channels. In HEK293 cells stably expressing the human muscarinic

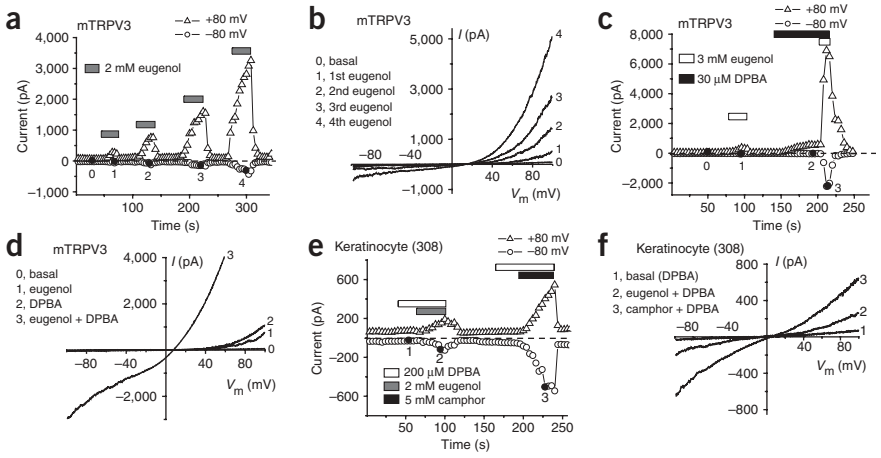

Figure 7 Eugenol activates and sensitizes TRPV3 in HEK293 cells and activates TRPV3-like current in the mouse keratinocytes. (**a**,**b**) Repeated applications of eugenol (2 mM) activated and sensitized I_{mTRPV3}. Amplitudes of eugenol-induced I_{TRPV3} in response to second applications were increased roughly threefold in both inward and outward directions (from –6.3 ± 1.5 to –17.6 ± 5.0 pA/pF, and from 49 ± 14 to 126 ± 40 pA/pF at –80 and +80 mV, respectively; $n = 11$). (**c**) Eugenol markedly sensitized I_{mTRPV3} induced by a low DPBA concentration (30 μM). Application of both eugenol (3 mM) and DPBA (30 μM) evoked a large, weakly rectifying current in the same cell. (**d**) Representative ramp currents activated by DPBA alone, or together with eugenol. (**e**) Activation of TRPV3-like currents in 308 keratinocytes by eugenol and camphor in the presence of DPBA. (**f**) Representative ramp currents activated by eugenol or camphor in 308 keratinocytes.

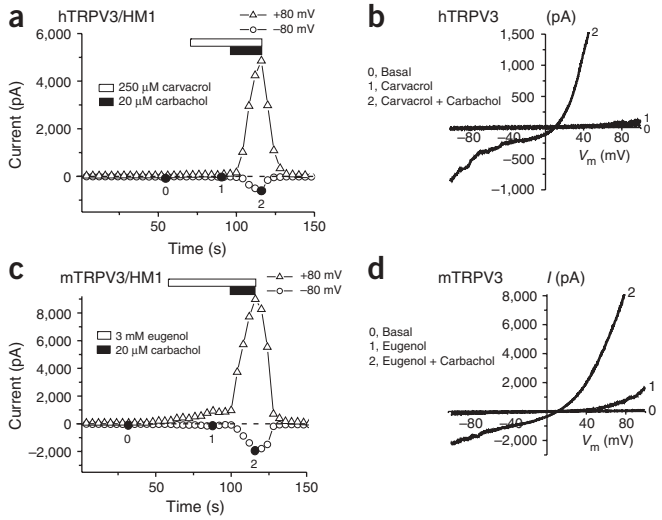

Figure 8 Sensitization of TRPV3 by PLC-coupled receptor stimulation. (**a**,**b**) Potentiation of 250 μM carvacrol–induced I_{hTRPV3} by carbachol (20 μM) stimulation of the human muscarinic type 1 receptor (HM1). (**b**) Representative currents in response to voltage ramps as shown. (**c**) Sensitization of eugenol-activated I_{mTRPV3} resulting from coapplication of carbachol. Inward I_{mTRPV3} induced by 3 mM eugenol was markedly potentiated (~12-fold) by coapplication of 20 μM carbachol to an mTRPV3-expressing HM1 cell. (**d**) Representative currents in response to voltage ramps.

receptor type 1 (HM1) and transiently transfected with mTRPV3, the application of carvacrol (250 μM) slowly activated a relatively small TRPV3 current (**Fig. 8a,b**). However, addition of the muscarinic agonist carbachol (20 μM) in the continued presence of 250 μM carvacrol greatly enhanced I_{TRPV3}, whereas carbachol alone did not induce a current. These results suggest that the effect of carvacrol could be enhanced by coactivation of taste or olfactory GPCRs if colocalized in these cells.

Many proinflammatory agents such as ATP, bradykinin and histamine are released from mast cells and keratinocytes after dermal injury or under irritated conditions. These molecules facilitate inflammatory responses by activating Gq/11-protein coupled receptors coupled to PLCβ[36]. In HM1 cells expressing mTRPV3, the application of 3 mM eugenol slowly activated a relatively small TRPV3 current (**Fig. 8c,d**). However, addition of the muscarinic agonist carbachol (20 μM) in the continued presence of 3 mM eugenol greatly enhanced I_{TRPV3}. The eugenol-evoked outward current (measured at +80 mV) increased roughly eightfold from 75 ± 15 pA/pF to 603 ± 122 pA/pF ($n = 5$). The eugenol-evoked inward current (measured at –80 mV) increased ~12-fold from –8.2 ± 2.6 pA/pF to –95 ± 40 pA/pF ($n = 5$). Carbachol alone did not induce a current. We also observed the potentiation by carbachol after activation with the TRPV3 agonists camphor (5 mM) and 2APB/DPBA (30 μM, **Supplementary Fig. 8**). Bradykinin (500 nM) had similar sensitizing effects on 2APB-induced currents in cells cotransfected with mTRPV3 and the bradykinin type 2 receptor. Similarly, histamine (100 μM) potently sensitized 2APB-induced I_{TRPV3} in cells cotransfected with mTRPV3 and the histamine type 1 receptor (**Supplementary Fig. 8**). We did not observe sensitizing effects of carbachol, histamine and bradykinin in HEK293 cells transfected with TRPV3 lacking these receptors or in cells treated with the PLC inhibitor U73122 (10 μM). We did not see any significant carbachol-induced sensitization in the complete absence of internal (pipette) Ca^{2+} (free $[Ca^{2+}]_i$ <10 nM), where PLC activity is expected to be negligible[37]. Thus, PLC-coupled receptor enhancement of TRPV3 may increase the effectiveness of eugenol in inflamed conditions. The unique sensitizing characteristics of eugenol-induced I_{TRPV3}, together with the properties of receptor modulation, suggest a role of TRPV3 in chemical-induced skin sensitization or inflammation. Eugenol-induced cytokine release from keratinocytes may contribute to these processes.

DISCUSSION

Presumably, animals evolved chemosensory mechanisms in skin, tongue, nose and mouth to test, or to remember, their tolerance of substances before ingestion. In turn, plants evolved chemicals to repel animals to avoid being eaten or attract them in order to spread their seeds. Indeed, the seed-bearing organs of plants, their fruits, are often endowed with substances that attract animals. In contrast, many leaves and other plant parts contain noxious substances that induce pain or allergic reactions. Here we have shown that TRP channels are chemesthetic receptors in oral and nasal epithelium and that TRPV3 may be a molecular target for plant-derived skin sensitizers.

Evidence is growing for significant humoral communication between epithelial cells in skin and between these cells and nerves. Lipophilic carvacrol and thymol can penetrate the keratinized, non-sensory layers to target TRPV3 in underlying keratinocytes. As keratinocytes respond to both thermal and mechanical stimulation and release ATP to signal sensory neurons[25,38], similar transduction mechanisms may relay the chemosensory information of carvacrol and thymol in oral and nasal epithelia to trigeminal nerves. Lingual epithelial cells directly respond to a number of chemicals and irritants and release cytokines and neuropeptides[9] that will affect other epithelial cells, taste receptor cells and free nerve endings. Thus, a variety of TRP channels (such as TRPV1, TRPV3, TRPA1, TRPM5 and TRPM8) may contribute to aspects of taste and may modify or initiate signals that integrate with other signals from the G protein–linked olfactory and taste receptors, either within the epithelium, between epithelia and specialized cells, or at the level of neuronal input to the CNS. PLC-coupled regulation of TRP channels may provide one mechanism for such integration. Although it may not be possible to use genetically altered TRP mice to sort out the subtleties of flavor recognition for substances like carvacrol, they may be useful in tests of skin sensitization, teratogenicity and carcinogenicity. We speculate that activation and receptor modulation of TRPV3 may provide a platform and peripheral mechanism for cross-modal interactions between chemesthesis, smell, taste, temperature and touch. Future studies will also explore how activation of TRPV3 by carvacrol or thymol leads to warmth perception in the brain.

METHODS

***In situ* hybridization.** *In situ* hybridization was performed using two mouse *TRPV3* cRNA probes labeled with digoxin essentially as described[8]. The first probe (mV3-5) contained 821 bp (covering amino acid residues 29–302) and was amplified with the primers 5′-GACAGACTCGAGGCCAGCAGATCTC ACCC-3′ and 5′-ACTGTCGCGGCCGCGTGCAGGATGTTGTTTCC-3′. The second probe (mV3-C) contained 1,051 bp (covering amino acid residues 430–776) and was amplified with the primers 5′-AGCCTCCTCGAGCGCTG CTACACACGAAATGG-3′, and 5′-TCATCAGCGGCCGCGAGGGTGGTTTTG CTATTGC-3′. Both probes were cloned into the pBluescript II SK (+) vector. The sense and antisense probes for both constructs were transcribed with T7 and T3 primers, respectively.

Immunocytochemistry and immunohistochemistry. An N-terminal glutathione-*S*-transferase (GST) fusion protein (residues 1–166) and a C-terminal GST fusion protein (residues 684–791) were cloned into the pGEX-4T1 vector

and expressed in *Escherichia coli* BL21 bacteria. Fusion proteins were purified on a GST column. Antisera of both GST fusion proteins were obtained from rabbits (Chemicon) and were affinity purified with an AminoLink Kit (Pierce) after passage through an immobilized GST column. Additional antisera to TRPV3 were raised against an N-terminal peptide (CMNAHSKEMVPLMGK-RITAPG) and a C-terminal peptide (CNSICFTLYAFDELDEFPETSV) and were affinity purified on a Sulfolink (Pierce) column. Cytokeratin 8.60 antibody was purchased from Sigma, and gusducin antibody from Santa Cruz Biotechnology.

Heterologous expression of TRP channels in HEK293 cells. Rat *Trpv1*, rat *Trpv2*, mouse *Trpv3* and mouse *Trpv4* were cloned into enhanced green fluorescence protein (EGFP, BD Biosciences Clontech) vectors to generate N- or C-terminal fusion proteins. Human *TRPV3* was subcloned into an EGFP-containing vector (pTracer-CMV2, Invitrogen) and other constructs were cloned into pcDNA3 and cotransfected with pTracer-CMV2 for expression. HEK293 cells and HM1 cells (stably expressing the human muscarinic type 1 (HM1) receptor) were transfected using the Lipofectamine 2000 (Invitrogen) transfection reagent (6–8 h). Transfected cells were cultured at 37 °C, plated onto glass coverslips and used for electrophysiological recordings after 24 h. Transfected cells were identified by GFP fluorescence.

Mouse 308 keratinocytes. Mouse 308 keratinocytes were provided by S.H. Yuspa (US National Cancer Institute). The cell line is a papilloma-derived keratinocyte cell line from adult BALB/c mouse skin[24,39]. Cells were grown in DMEM medium containing 8% FBS.

Preparation of mouse keratinocytes: Mice (postnatal day 1–7) were killed[13,24], and the skin of the back (approximately 1 cm^2) was removed and placed in a Petri dish containing PBS solution (with antibiotics). Subcutaneous tissue was gently scraped off, and samples were transferred to 0.25% trypsin (Invitrogen) or dispase II (Roche) solutions and incubated at 32 °C for 1–2 h. The epidermis was scraped into minimal essential medium (S-MEM, Invitrogen-GIBCO) solution, transferred to a sterile container, and stirred at 100 rpm for 20 min. The solution was filtered and centrifuged for 10 min at 160*g*. The cells were pipetted gently and plated onto coverslips using serum-free keratinocyte medium at least 24 h before use.

Preparation of tongue epithelial cells. Five- to six-week-old male Sprague-Dawley rats (Charles River Laboratories) were killed and their tongues immediately removed[40]. Tongues were placed in a Petri dish containing Keratinocyte Medium (KM; Gibco) supplied with EGF and bovine pituitary extract. Approximately one milliliter of a mixture of collagenase (2 mg/ml, Sigma) and elastase (1 mg/ml, Sigma) dissolved in KM medium was injected with a 27G needle submucosally at three to four locations in the tongue and was incubated at 22 °C for 30 min. The epithelium was gently peeled away from the muscle layer with forceps, minced into 1- to 4-mm^2 pieces using surgical blades, and digested with 3 mg/ml dispase (Roche) in KM medium at 37 °C for 15 min. To remove clumps, disaggregated cells were passed through a 70 μm nylon cell strainer (Falcon). Cells were spun down and suspended in KM medium. Cells were plated on Matrigel (BD Bioscience)-coated coverslips and assayed 24 h after isolation. Human tongue tissues were purchased from US Biological.

IL-1α ELISA. IL-1α determination was performed using a Quantikine kit for mouse IL-1α (R&D Systems). Near-confluent keratinocytes were placed in KM medium containing test compounds and vehicle (DMSO). After 18 h, supernatants were collected for IL-1α quantification.

Electrophysiology. For most recordings from EGFP-positive HEK293 cells, the pipette solution contained (in mM) 147 Cs, 120 methane sulfonate, 8 NaCl, 4.1 $CaCl_2$, 10 EGTA, 2 magnesium ATP and 20 HEPES (pH 7.2; free $[Ca^{2+}]_i$ ~120 nM using MaxChelator. For all receptor-stimulation experiments, the pipette solution contained (in mM): 147 Cs, 120 methane sulfonate, 8 NaCl, 4.6 $CaCl_2$, 10 EGTA, 2 magnesium ATP, 0.3 sodium GTP and 20 HEPES (pH 7.2; free $[Ca^{2+}]_i$ ~140 nM). The standard extracellular (modified Tyrode's) solution contained (in mM) 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 20 HEPES (pH 7.4). For recordings from mouse keratinocytes, we sometimes used low-[Cl–] external solutions that contained (in mM) 150 sodium gluconate, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 20 HEPES (pH 7.4). For experiments addressing the temperature activation of TRPV3 in HEK293 cells, the perfusate was heated using a Warner TC-325B temperature controller and an SH-27B solution heater as previously described[8]. Temperature was recorded with a thermistor placed <0.5 mm from the cell. All other experiments were conducted at room temperature (~21–23 °C). Cells were voltage clamped using an Axopatch 200B amplifier, controlled by a Digidata 1320 and pClamp 9.0 software (Axon Instruments). Recordings were acquired at 5 kHz and filtered at 2 kHz (–3 dB, 4-pole Bessel). Capacity current was reduced as much as possible using the amplifier circuitry; series resistance compensation was 60–85%. Voltage ramps (400 ms) from –100 to +100 mV were applied every 4 s from a holding potential of –60 mV. All recordings were analyzed with pCLAMP9 and Origin 7 (OriginLab).

Ca^{2+} imaging. HEK293 cells, keratinocytes and rat tongue epithelial cells were loaded with 5 μM Fura-2 AM in culture medium at 37 °C for 60 min. Cells were then washed in modified Tyrode's solution for 10–30 min. Fluorescence at different excitation wavelengths was recorded on a MetaFluor imaging system (Universal Imaging Corporation). For HEK293 cell recordings, GFP fluorescence was monitored with an excitation wavelength of 440 nm (F440). Fura-2 ratios (F340/F380) recorded changes in $[Ca^{2+}]_i$ upon stimulation. Ionomycin (1 μM) was added at the conclusion of all experiments to induce a maximal response for comparison. Calcium imaging experiments were repeated four to seven times, and representative traces are shown in the figures.

Reagents: Eugenol, thymol, carvacrol, vanillin, ethyl-vanillin and camphor were prepared in stock solutions of 2–3 M (in DMSO). Carvacrol, eugenol, thymol, vanillin, ethyl-vanillin, camphor, capsaicin, capsazepine, menthol, mustard oil (allyl isothiocyanate), 4-αPDD, carbachol, guaiacol, 2APB and DPBA were supplied by Sigma.

Data analysis. Group data are presented as mean ± s.e.m. Statistical comparisons were made using analysis of variance (ANOVA). P value < 0.05 was considered statistically significant.

URLs. MaxChelator is available at http://www.stanford.edu/~cpatton/webmaxc/webmaxcS.htm.

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

This work was supported by the Sandler Program for Ashma Research and the Howard Hughes Medical Institute. We thank S.H. Yuspa (National Cancer Institute) for providing us with mouse 308 keratinocytes. We thank D. Julius for rat *TRPV1*, *TRPV2*, *TRPM8* and *TRPA1* constructs. M. Caterina and D. Ren provided the mouse *TRPV3* and *TRPV4* constructs, respectively. We are grateful to U. Berger and H. Wang for assistance. We thank S. Cibulsky, N. Blair, B. Carter, W. Pu, S. Ikeda, C. Medler, S. Roper and members of the Clapham laboratory for discussion and helpful comments.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Neuroscience* website for details).

Published online at http://www.nature.com/natureneuroscience
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Caterina, M.J. *et al.* The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389**, 816–824 (1997).
2. Peier, A.M. *et al.* A TRP channel that senses cold stimuli and menthol. *Cell* **108**, 705–715 (2002).
3. McKemy, D.D., Neuhausser, W.M. & Julius, D. Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature* **416**, 52–58 (2002).
4. Bryant, B. & Silver, W.L. in *The Neurobiology of Taste and Smell* (eds. Finger, T.E., Silver, W.L. & Restrepo, D.) (Wiley-Liss, New York, 2000).
5. US Food and Drug Administration. Spices - definitions〈http://www.fda.gov/ora/compliance_ref/cpg/cpgfod/cpg525-750.html〉.
6. Scholl, I. & Jensen-Jarolim, E. Allergenic potency of spices: hot, medium hot, or very hot. *Int. Arch. Allergy Immunol.* **135**, 247–261 (2004).
7. Corsini, E., Primavera, A., Marinovich, M. & Galli, C.L. Selective induction of cell-associated interleukin-1alpha in murine keratinocytes by chemical allergens. *Toxicology* **129**, 193–200 (1998).
8. Xu, H. *et al.* TRPV3 is a calcium-permeable temperature-sensitive cation channel. *Nature* **418**, 181–186 (2002).

9. Finger, T.E. & Simon, S.A. in The neurobiology of taste and smell (ed. Finger, T. E., Silver, W.L. & Restrepo, D.) (Wiley-Liss, New York, 2000).
10. Peier, A.M. *et al.* A heat-sensitive TRP channel expressed in keratinocytes. *Science* **296**, 2046–2049 (2002).
11. Hu, H.Z. *et al.* 2-aminoethoxydiphenyl borate is a common activator of TRPV1, TRPV2, and TRPV3. *J. Biol. Chem.* **279**, 35741–35748 (2004).
12. Chung, M.K., Lee, H., Mizuno, A., Suzuki, M. & Caterina, M.J. 2-aminoethoxydiphenyl borate activates and sensitizes the heat-gated ion channel TRPV3. *J. Neurosci.* **24**, 5177–5182 (2004).
13. Moqrich, A. *et al.* Impaired thermosensation in mice lacking TRPV3, a heat and camphor sensor in the skin. *Science* **307**, 1468–1472 (2005).
14. Smith, G.D. *et al.* TRPV3 is a temperature-sensitive vanilloid receptor-like protein. *Nature* **418**, 186–190 (2002).
15. Belitz, H.-D., Grosch, W. & Schieberle, P. in *Food Chemistry* (eds. Belitz, H.-D., Grosch, W. & Schieberle, P.) (Springer, Berlin, 2004).
16. Chung, M.K., Guler, A.D. & Caterina, M.J. Biphasic currents evoked by chemical or thermal activation of the heat-gated ion channel, TRPV3. *J. Biol. Chem.* **280**, 15928–15941 (2005).
17. Xu, H., Blair, N.T. & Clapham, D.E. Camphor activates and strongly desensitizes the transient receptor potential vanilloid subtype 1 channel in a vanilloid-independent mechanism. *J. Neurosci.* **25**, 8924–8937 (2005).
18. Jordt, S.E. *et al.* Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. *Nature* **427**, 260–265 (2004).
19. Macpherson, L.J. *et al.* The pungency of garlic: Activation of TRPA1 and TRPV1 in response to allicin. *Curr. Biol.* **15**, 929–934 (2005).
20. Bautista, D.M. *et al.* Pungent products from garlic activate the sensory ion channel TRPA1. *Proc. Natl. Acad. Sci. USA* **102**, 12248–12252 (2005).
21. Corsini, E. & Galli, C.L. Cytokines and irritant contact dermatitis. *Toxicol. Lett.* **102–103**, 277–282 (1998).
22. Barker, J.N., Mitra, R.S., Griffiths, C.E., Dixit, V.M. & Nickoloff, B.J. Keratinocytes as initiators of inflammation. *Lancet* **337**, 211–214 (1991).
23. Effendy, I., Loffler, H. & Maibach, H.I. Epidermal cytokines in murine cutaneous irritant responses. *J. Appl. Toxicol.* **20**, 335–341 (2000).
24. Chung, M.K., Lee, H., Mizuno, A., Suzuki, M. & Caterina, M.J. TRPV3 and TRPV4 mediate warmth-evoked currents in primary mouse keratinocytes. *J. Biol. Chem.* **279**, 21569–21575 (2004).
25. Lee, H. & Caterina, M.J. TRPV channels as thermosensory receptors in epithelial cells. *Pflugers Arch.* **451**, 160–167 (2005).
26. Lewis, R.S. Calcium oscillations in T-cells: mechanisms and consequences for gene expression. *Biochem. Soc. Trans.* **31**, 925–929 (2003).
27. Lee, M.H. *et al.* Eugenol inhibits calcium currents in dental afferent neurons. *J. Dent. Res.* **84**, 848–851 (2005).
28. Berova, N., Stransky, L. & Krasteva, M. Studies on contact dermatitis in stomatological staff. *Dermatol. Monatsschr.* **176**, 15–18 (1990).
29. Yang, B.H. *et al.* Activation of vanilloid receptor 1 (VR1) by eugenol. *J. Dent. Res.* **82**, 781–785 (2003).
30. Jordt, S.E. & Julius, D. Molecular basis for species-specific sensitivity to "hot" chili peppers. *Cell* **108**, 421–430 (2002).
31. Kinnamon, S.C. & Margolskee, R.F. Mechanisms of taste transduction. *Curr. Opin. Neurobiol.* **6**, 506–513 (1996).
32. Zhang, Y. *et al.* Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signaling pathways. *Cell* **112**, 293–301 (2003).
33. Katada, S., Hirokawa, T., Oka, Y., Suwa, M. & Touhara, K. Structural basis for a broad but selective ligand spectrum of a mouse olfactory receptor: mapping the odorant-binding site. *J. Neurosci.* **25**, 1806–1815 (2005).
34. Finger, T.E. *et al.* Solitary chemoreceptor cells in the nasal cavity serve as sentinels of respiration. *Proc. Natl. Acad. Sci. USA* **100**, 8981–8986 (2003).
35. Huang, Y.J., Maruyama, Y., Lu, K.S., Pereira, E. & Roper, S.D. Mouse taste buds release serotonin in response to taste stimuli. *Chem. Senses* **30** (Suppl.), i39–i40 (2005).
36. Wang, H. & Woolf, C.J. Pain TRPs. *Neuron* **46**, 9–12 (2005).
37. Horowitz, L.F. *et al.* Phospholipase C in living cells: activation, inhibition, Ca^{2+} requirement, and regulation of m current. *J. Gen. Physiol.* **126**, 243–262 (2005).
38. Koizumi, S., Fujishita, K., Inoue, K., Shigemoto-Mogami, Y. & Tsuda, M. Ca^{2+} waves in keratinocytes are transmitted to sensory neurons: the involvement of extracellular ATP and P2Y2 receptor activation. *Biochem. J.* **380**, 329–338 (2004).
39. Yuspa, S.H., Kulesz-Martin, M., Ben, T. & Hennings, H. Transformation of epidermal cells in culture. *J. Invest. Dermatol.* **81**, 162s–168s (1983).
40. Gilbertson, T.A., Roper, S.D. & Kinnamon, S.C. Proton currents through amiloride-sensitive Na^+ channels in isolated hamster taste cells: enhancement by vasopressin and cAMP. *Neuron* **10**, 931–942 (1993).