Role and Mechanism of Transient Receptor Potential (TRP) Channels in Gustatory and Chemesthetic Sensations Associated with Dietary Molecules

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« La nature est un livre sublime que nous ne devons pas nous lasser de lire »
Camille Flammarion

A mes chers parents

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Abstract

"Chemosensory perception", the perception of food chemicals, relies on the complex cooperation of gustatory, olfactory and somatosensory detection systems. In the oral cavity, the taste buds and the lingual branch of the trigeminal nerve are the organs that respond to ingested dietary molecules. Remarkable advances in the understanding of the physiology of these systems have been achieved by identification of key proteins directly implicated in the detection machinery. Among them, several members of the Transient Receptor Potential (TRP) superfamily of ion channels play critical roles in sensory physiology, with contribution in taste, touch, thermosensation and nociception (perception of harmful stimuli). However, even though important key players have been identified, much less is known about their exact role and the specific tuning of their response.

In the framework of this Ph.D thesis, I focussed on several dietary molecules possessing complex perceptions and studied their sensory targets. To achieve this, *in vitro* and *in vivo* approaches were combined. In a first step, the goal was to answer a simple question: is metallic taste a unique sensation, as are sweet or bitter modalities? To address this issue, the sensory pathways of prototypical metallic tastants being divalent salts of metal cations and artificial sweeteners were evaluated. It was aimed at determining if these molecules could stimulate both somatosensory neurons projecting in the oral cavity and taste buds contained in the papillae. The screening for receptor candidates contained in sensory neurons revealed that these molecules were stimulating TRPV1, the capsaicin (from red hot chilli peppers) receptor. Calcium transients were observed in dissociated sensory neurons from the Dorsal Root Ganglia (DRG) expressing TRPV1 and by using calcium and voltage imaging techniques, a selective stimulation when heterologously expressed in HEK 293 cells was also showed. To elucidate the pathways involved in the perception of sweeteners and metal cations, taste preference assays were performed using mice lacking TRPV1 and TRPM5, the latter being a key channel involved in downstream signalling of sweet, umami and bitter tastes. The results revealed that preference profiles were modified when these two channels were lacking. Direct stimulation of TRPM5 with metallic stimuli did not result in channel opening consistent with a downstream role of this channel in taste cells after activation of the taste GPCR. Remarkably, it was observed that wild type mice displayed strong preference for two metallic stimuli, $FeSO_4$ and $ZnSO_4$ and this preference was absent in mice lacking TRPM5. It was further showed that suppression of T1R3, the receptor of sweet and umami stimuli in taste cells also leads to indifference in the mice, suggesting that these metals induce pleasantness possibly through one of these pathways. Collectively, these data showed that artificial sweeteners and divalent salts elicit biphasic responses in mice, with an hedonic phase involving T1R3-TRPM5 pathways and an aversive phase recruiting TRPM5 and TRPV1 pathways.

In addition, it was aimed at obtaining a better understanding of trigeminal sensory targets stimulated by culinary spices and their activation mechanisms. For this purpose, two spices remarkable for their pungent and tingling attributes were selected: Sichuan pepper and Afromomum Melegueta. The results indicate that the active molecules contained in these spices stimulate DRG neurons and activate TRPV1 and TRPA1, the capsaicin and the cinnamaldehyde (from cinnamon) receptors, respectively. Their stimulation of TRPA1 requires covalent binding at specific intracellular cysteine residues in the N-termini whereas no such interactions were occurring with TRPV1. The results present novel insight into the co-stimulation of TRPA1 and TRPV1 where the two channels, even though sensitive to the same compounds, react via different modes. In addition, by conducting taste preference assays in the TRPV1 KO mice, it was shown that the aversive response is mainly accounted by TRPV1 with a residual aversion that possibly underlies a TRPA1 response.

In summary, these results show that several TRP channels are key elements to detect dietary molecules such as artificial sweeteners, divalent salts and pungent spices. This is achieved by different processes including receptor modulation by the chemical, covalent binding at the receptor and indirect stimulation in the case of store-operated channel such as TRPM5.

Keywords: taste, somatosensation, chemosensory coding, TRP channel, taste GPCR, calcium imaging, membrane voltage imaging, heterologous expression, receptor binding site, DRG neurons, knockout mice, taste preference, artificial sweetener, metal cation, pungent spice.

RESUME

La “perception chémosensorielle”, c’est-à-dire la perception des composés alimentaires, est basée sur la coopération complexe des systèmes de détection gustatif, olfactif et somatosensoriel. Dans la cavité buccale, les bourgeons gustatifs et la branche linguale du nerf trigéminal sont les organes qui ressentent les nutriments ingérés. De remarquables progrès dans la compréhension de ces systèmes physiologiques ont été accomplis grâce à l’identification de protéines clé directement impliquées dans la machinerie sensorielle. Parmi ces protéines, plusieurs membres de la famille de canaux ioniques Transient Receptor Potential (TRP) jouent un rôle critique dans la physiologie sensorielle, avec des contributions dans le goût, le toucher, la thermosensation et la nociception (perception des stimuli dangereux). Cependant, même si d’importants acteurs sont connus, il reste encore beaucoup d’inconnues concernant leur rôle exact ainsi que l’encodage particulier de leur réponse.

Dans le cadre de ce travail de thèse, je me suis concentrée sur plusieurs composés alimentaires possédant des perceptions complexes et ai étudié leurs cibles sensorielles. Pour parvenir à ce résultat, des approches *in vitro* et *in vivo* ont été combinées. Dans un premier temps, le but a été de répondre à une question simple : est-ce que le goût métallique est une sensation unique tout comme le sont les modalités sucrées ou amères? Pour répondre à cette question, les voies sensorielles de molécules au goût typiquement métallique tels que les sels divalents de cations métalliques et les édulcorants artificiels ont été évaluées. J’ai cherché à déterminer si ces molécules avaient la capacité de stimuler à la fois les neurones somatosensoriels qui projettent dans la cavité buccale ainsi que les bourgeons du goût contenus dans les papilles gustatives. Le « screening » pour des récepteurs candidats contenus dans les neurones sensoriels a révélé que ces molécules stimulaient TRPV1, le récepteur de la capsaicine (piment rouge). TRPV1 est à la fois activé par ces molécules en imagerie calcique dans les neurones dissociés provenant du ganglion spinal et grâce à l’utilisation de techniques d’imagerie mesurant le calcium cytosolique et le changement de voltage membranaire, une stimulation spécifique a été observée quand le canal est exprimé de manière hétérologue dans des cellules HEK293. Pour élucider les mécanismes de perception des métaux et des édulcorants artificiels, des tests de préférence sensoriels ont été réalisé en utilisant des souris déficientes pour TRPV1 et TRPM5, ce dernier étant un canal clé impliqué dans la cascade de transmission du signal pour les modalités sucrées, umami et amères. Ces résultats révèlent que les profils de préférences sont modifiés en l’absence de ces canaux. L’activation directe de TRPM5 par les stimuli métalliques ne provoque pas d’ouverture du canal, en accord avec son rôle de messager secondaire après l’activation de la GPCR gustative. En particulier, les souris de « souche sauvage » présentent une forte préférence pour deux stimuli métalliques, $FeSO_4$ et $ZnSO_4$ mais cette préférence est absente chez les souris mutantes pour TRPM5. J’ai ensuite montré que la suppression de T1R3, le récepteur du sucré et de l’umami dans les cellules gustatives conduit à l’indifférence chez ces souris, suggérant ainsi que ces métaux induisent des sensations plaisantes par l’une de ces voies. Ces résultats montrent que les édulcorants artificiels et les sels divalents produisent des réponses biphasiques chez la souris, avec une phase hédonique recrutant la voie T1R3-TRPM5 et une phase aversive impliquant TRPM5 et TRPV1.

Aussi dans le cadre de cette thèse, j’ai cherché à obtenir une meilleure compréhension des cibles sensorielles trigéminales stimulées par les épices culinaires et leurs mécanismes d’activation. Pour cela, deux épices ont été sélectionnées pour leurs remarquables attributs fortement piquant et picotant qui sont le poivre de Sichuan et l’Afromomum Melegueta. Ces résultats indiquent que les molécules actives contenues dans ces épices stimulent les neurones dissociés du ganglion spinal et activent TRPV1 et TRPA1, respectivement les récepteurs de la capsaicine et du cinnamaldehyde (cannelle). Leur activation sur TRPA1 requiert une liaison covalente avec des cystéines intracellulaires spécifiques dans le N-terminal alors qu’aucune intéraction de ce type n’a lieu avec TRPV1. Ces résultats présentent un nouvel aperçu de la co-stimulation de TRPA1 et TRPV1 ou les deux canaux, malgré leur sensibilité aux mêmes composés, réagissent par des modes différents. De plus, en conduisant des tests de préférence gustative sur les souris TRPV1 mutantes, il a été observé que l’aversion pour ces molécules était principalement due à TRPV1 avec une aversion résiduelle, sous-jacente d’une réponse de TRPA1.

En résumé, ces résultats montrent que plusieurs canaux TRP sont des éléments clé pour détecter les composés alimentaires tels que les édulcorants artificiels, les sels divalents et les épices piquantes. Cette détection est accomplie par différents procédés incluant la modulation du récepteur par le composé, la liaison covalente avec le récepteur et la stimulation indirecte dans le cas de canaux commandés par les réserves calciques tels que TRPM5.

Mots clés: goût, sensation somatique, TRP chémosensoriel, GPCR gustative, imagerie calcique, imagerie de voltage membranaire, expression hétérologue, site de liaison du récepteur, neurones du ganglion spinal, souris knockout, préférence gustative, édulcorant artificiel, cation métallique, épice piquante.

Aim of the Study and Outline

The hedonic value of food nutrients is evaluated in the mouth by our chemical senses, known as the gustatory and trigeminal systems, which work in concert to detect chemicals in the mouth. Gustatory information is processed by the taste buds contained in the papillae and somatosensory cues are transmitted by the lingual branch of the trigeminal nerve to the brainstem. The close analysis of the perception process has been achieved by the discovery of taste and trigeminal receptors. It appears that these two pathways have been primarily studied as completely dissociated, suggesting that dietary nutrients are thought to signal through one specific route to mediate their sensory effect. However, the understanding of some sensations such as “metallic taste” may rely on the complex interplay of chemical and tactile cues.

The main goal of this thesis is to understand the molecular mechanisms underlying “metallic taste” at the peripheral level by screening for gustatory and somatosensory receptors. Another goal was to assess the receptors activated by two burning and tingling pungent spices and to characterize their activation mechanisms. To achieve these objectives, I focussed on TRP channels which play a critical role in sensory physiology. The primary approach consisted in studying by calcium and voltage imaging the channels stimulated by these molecules both heterologously expressed and the native channels in DRG neurons. To determine the role of these channels in perception, taste preference assays in mice lacking TRPV1, TRPM5 and T1R3 sensory receptors were used.

Chapter 1 introduces the current knowledge on chemosensory perception from the periphery to the brain. The anatomy and information coding of both gustatory and somatosensory systems are described. A particular attention is directed to the receptors implicated and the current findings on their activation mechanisms. In the last part, the interactions between gustatory and somatosensory systems are briefly examined.

Chapter 2 presents the results obtained during the Ph.D thesis. These results are presented as publications and are divided in two sections.

A first section is dedicated to the molecular mechanisms underlying the multisensory attributes of metal salts and artificial sweeteners and comprises three studies. The first study, published in the *American Journal of Physiology Regulatory Integrative Comparative Physiology*, (Riera CE et al., 2007) describes the agonistic activity of artificial sweeteners and metallic tasting salts at the capsaicin TRPV1 receptors. When heterologously expressed in HEK293 cells and endogenously contained in

DRG neurons, TRPV1 channels are specifically modulated by these stimuli. Sweeteners activate TRPV1 in a dose-dependent manner and like capsaicin, sensitize TRPV1 to heat and protons.

The second study, submitted to the *Journal of Neuroscience*, investigates the gustatory and somatosensory pathways followed by complex tasting divalent salts. Using two different and complementary taste preference assays, the responses of wild type and TRPM5, T1R3 and TRPV1 mice were compared. This study revealed that ferrous and zinc salts elicited biphasic responses whereas magnesium and copper were mainly aversive. In this study, we raise a sensory model to explain the contribution of the cited elements, but also pH and post-ingestive factors in the obtention of the biphasic response profiles and the comprehension of “metallic taste”.

A third study, published in *Biochemical and Biophysical Research Communications*, reports the behavioral results obtained with mice lacking TRPV1 and T1R3 when presented to artificial sweeteners in a taste preference paradigm. The results show that suppression of T1R3 not only abolishes preference to sweeteners but reveals strong aversive components. TRPV1 suppression decreases the aversive response to the sweeteners. Point mutations of key residues involved in proton sensing do not alter sweeteners responses when expressing TRPV1 in HEK293 cells whereas mutation of the Tyr 511 responding to capsaicin abolishes reponses to cyclamate and acesulfame-K but has no effect on saccharin and aspartame.

The second section presents the study submitted to the *British Journal of Pharmacology* which addresses the effects of molecules contained in Sichuan and Melegueta peppers on DRG neurons. It is seen that these molecules target TRPV1 and TRPA1 receptors. A structure-activity approach dissects which structural elements in the tested compounds are key for the channels stimulation. Site-directed mutations of cysteines residues in TRPA1 and TRPV1 shows that these molecules stimulate TRPA1 through cyteines modification which is not the case for TRPV1. In behavioural tests, TRPV1 is shown to be physiologically relevant for spices induced-aversion whereas TRPA1’s function in perception remains elusive.

Finally in chapter 4, we discuss the results obtained in the thesis, in term of novelty, technical limitations and possible further experiments to improve this work. In particular, we evaluate the contribution of our results to the current knowledge of chemosensory physiology and especially their impact on psychophysical data. To conclude, a discussion on further directions to pursue this research in order to enlarge the findings is proposed.

INTRODUCTION

In this chapter, the pathways that conduct food stimuli will be revised from the mouth to the brain. The qualitative appreciation of a food stimulus requires several of our peripheral organs. After visual and olfactory evaluation, the palatability of the stimulus is evaluated by sensory perception comprising taste, texture and temperature in the oral cavity. In reality, despite the critical need for sensory evaluation, the decision to ingest food is accompanied and driven by cultural factors based on social, emotional and cognitive contexts. Obviously, satiety state and post-ingestive factors (reward value and acceptance during ingestion) of the food stimulus will play a crucial role by influencing the amount of food ingested. Taken together, the complex interaction of these factors will drive the feeding behavior.

1. Gustatory perception

1.1. The peripheral gustatory system

It is commonly accepted that taste is composed of five basic modalities with hedonically positive and negative attributes: sweet, bitter, sour, salty and umami. Among the hedonically positive perceptions, sweet taste allows the identification of energy-rich nutrients, umami permits the detection of amino-acids and salty taste guaranties the proper dietary electrolyte balance. Bitter and sour tastes prevent the ingestion of poisoning substances and are more associated with hedonically negative sensations.

As depicted in figure 1, taste stimuli are detected on the tongue by Taste Receptors Cells (TRCs). These cells are assembled into clusters of 50-100 cells forming taste buds. The latter are embedded in stratified epithelia, which are distributed across different papillae of the tongue, palate, epiglottis and oesophagus epithelia (Scott and Verhagen, 2000;Spector and Travers, 2005). At their apical end, TRCs project microvilli through a small opening named the taste pore. The detection of tastants is occurring in the pore through taste receptors expressed in the plasma membrane of the microvilli. Tight junctions, located just below the microvilli, form a protective barrier of the basolateral side of the TRCs (Holland et al., 1989).

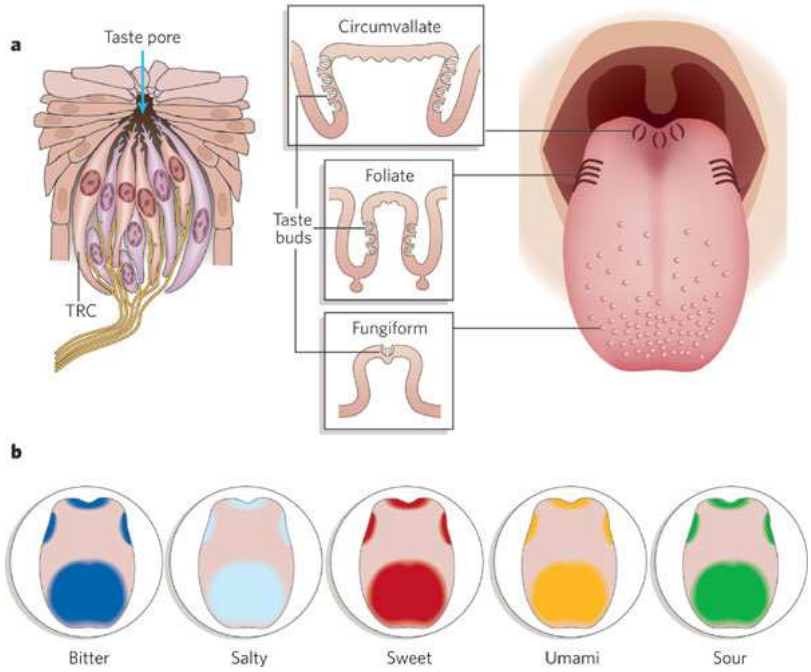

Figure 1. Morphology of Taste Receptor Cells (TRCs), taste buds and papillae. **A**. The tongue contains several types of papillae with fungiform papillae in the anterior part, foliate on the upper lateral sides and circumvallate in the posterior part. The sensory organs in the papillae are the taste buds formed by clusters of TRCs arranged in an onion shaped structure embedded in the epithelium. At the apical end, the taste bud forms a pore where chemical stimuli are detected and at the basolateral it is connected to gustatory afferent fibers. **B**. These papillae even if located in different regions, sense equally all taste modalities. From Chandrashekar *et al*., 2006.

1.2. Transduction pathway for primary tastes

Sweet taste

The human tongue recognises a variety of sweet tastants including natural sugars (typically sucrose), artificial sweeteners (saccharin, sucralose, aspartame, acesulfame-K, cyclamate, neotame, etc…), D-amino acids and sweet proteins (brazzein, monellin, thaumatin) (Nelson et al., 2001;Nelson et al., 2002;Li et al., 2002). These stimuli are detected by two broadly tuned G-protein-coupled receptors (GPCRs), T1R2 and T1R3. By analogy with metabotropic glutamate receptors, it is accepted that these GPCRs will assemble into homomeric or heterodimeric receptor complexes to recognize the ligand (Pin and Acher, 2002) and the binding is predicted to occur in the extracellular N-terminal domain (Kunishima et al., 2000).

T1R2 and T1R3 are found expressed in subsets of TRCs, either TRCs co-expressing T1R2 and T1R3 (T1R2+3 cells) or TRCs containing T1R3 alone (Nelson et al., 2001).

The T1R3 gene has been linked to the Sac locus, which determines threshold differences in the ability of some mouse strains to distinguish sucrose- and saccharin-containing solutions from water (Fuller, 1974;Lush, 1989). Indeed, functional expression studies in heterologous cells revealed that T1R3 combines with T1R2 (T1R2+3) to form a sweet taste receptor that responds to all classes of sweet tastants. These results validated the T1R2+3 heteromer as a sweet receptor, and suggested that T1R2+3 cells are the sweet-sensing TRCs (Figure 2).

Definitive evidence that T1R2+3 is the main sweet receptor was obtained from behavioral and electrophysiological studies of T1R2 and T1R3 deficient mice (Zhao et al., 2003;Damak et al., 2003). Suppression of either receptor leads to a critical loss of sweet responses in these mice.

Interestingly, humans and mice diverge in their ability to taste some sweet stimuli: mice are lacking hedonically positive responses to several artificial sweeteners such as cyclamate or aspartame (Bachmanov et al., 2001).

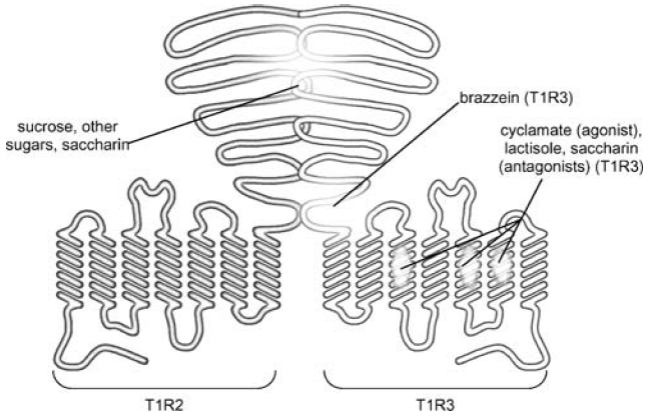

Figure 2. Schematic drawing of the sweet GPCR dimer T1R2+T1R3, showing the multiple ligand binding sites. The GPCRs interact presumably through their N-terminal domain. The N-termini region possess one or several binding pockets for saccharin, sucrose and other sugars. Brazzein and other sweet tasting proteins interact in the N-termini at a binding site near the first transmembrane domain. In the T1R3 subunit, the transmembrane domains 3, 5 and 6 are responsible for the interaction with inhibitory molecules such as cyclamate, high concentrations of saccharin and lactisole. From Roper SD, 2007.

Umami taste

Umami, first described by Ikeda (1908), is the characteristic taste elicited by amino acid evoking savory sensations such as glutamate (MSG) and 5'-ribonucleotides such as IMP (Inosine 5'-monophosphate) and GMP (Guanosine 5'-monophosphate). Umami taste is mainly represented by MSG in humans. Contrary to humans, most mammals are robustly attracted to a broad range of L-amino acids (Zhao et al., 2003;Iwasaki et al., 1985).

As for sweet transduction, two members of the T1R family, T1R1 and T1R3 have been shown to combine and form a broadly tuned amino-acid receptor (Nelson et al., 2002;Nelson et al., 2002). This

was further confirmed by the lack of responses to MSG and L-amino acids in T1R1 and T1R3 knockout mice (Zhao et al., 2003;Zhao et al., 2003;Damak et al., 2003). However, this total absence of umami response was not observed by other laboratories. Damak *et al.* recorded reduced but still appreciable responses to MSG in T1R3 null mice when using behavioral and gustatory nerve responses (Damak et al., 2003). Maruyama *et al* extended these findings by recording responses from individual taste cells from wild type and T1R3 null mice (Maruyama et al., 2006). They have shown that besides a clear diminution of the umami responses in T1R3 null mice, many cells were still sensitive to the stimulus.

This is coherent with the presence of other umami receptors in taste cells. Truncated type 1 and 4 metabotropic glutamate receptors missing most of the N-terminal extracellular domain (taste-mGluR4 and truncated-mGluR1) have been shown to be specifically expressed in taste cells (Chaudhari et al., 1996). Taste-mGluR4 when heterologously expressed in CHO cells responds to MSG in a concentration range corresponding to umami detection threshold (Chaudhari et al., 1996;Chaudhari et al., 2000). Taken together, these findings indicate that umami taste is likely to be transduced by multiple pathways including T1R1+T1R3 dimers as well as metrabotropic glutamate receptors.

Bitter taste

Bitterness is generally an aversive taste and many naturally occurring toxic compounds taste bitter. Thus, this sense is commonly believed to prevent animals from ingesting harmful foodstuff. It is typical elicited by a wide variety of molecules, among them alkaloids (strychnine, caffeine and quinine), amides (denatonium benzoate), amino acids, urea, fatty acids, phenols, amines, inorganic salts of potassium, magnesium and calcium. Also artificial molecules used in drug composition and artificial sweeteners (saccharin, acesulfame K) exhibit bitter taste.

A family of GPCR genes, the Taste Receptors 2 (T2Rs) was identified and proposed to be receptors for bitter tasting chemicals (Adler et al., 2000;Chandrashekar et al., 2000;Matsunami et al., 2000) as illustrated if Figure 3. This gene family comprises 25 and 35 members among humans and mice, respectively (Adler et al., 2000;Chandrashekar et al., 2000;Matsunami et al., 2000). However, only for some of them the bitter ligands have been determined (Chandrashekar et al., 2000;Bufe et al., 2002;Kuhn et al., 2004). The polymorphism of certain T2Rs and the vast repertoire available, account for the species variability in sensitivity to bitter chemicals (Chandrashekar et al., 2000;Kim et al., 2003;Wooding et al., 2006).

Proof that T2Rs are necessary and sufficient for bitter taste came from knockout and misexpression studies in mice (Mueller et al., 2005). Indeed, these researchers first used T2R5 knockout mice to show suppressed responses to cycloheximide, a described ligand of T2R5. Second, they showed that mice engineered to express the human candidate receptors for

phenylthiocarbamide and salicin, two bitter substances that do not elicit any aversive response in mice, became strongly aversive to these stimuli. This demonstrates that T2Rs are necessary and sufficient to detect selective responses to bitter tastants and validated that the T2Rs and T2R-expressing TRCs constitute the unique bitter sensors. It has been shown that clusters of T2Rs are expressed in subsets of the TRCs (Behrens et al., 2006) and this finding might explain why, despite the variety of bitter molecules, the elicited bitter sensation is very similar across compounds despite intensity variations. The same monotonical aversion exists across bitter tasting compounds when presented to mice and rats, suggesting that, in a similar manner than humans, rodents do not discriminate between bitter compounds (Mueller et al., 2005;Zhang et al., 2003;Spector and Kopka, 2002).

Salty taste

The principal stimulus for salty is a common ion, Na^+. Table salt, NaCl is the prototypic salty tastant. Both Na^+ and Cl^- are essential nutrients, vital for maintaining blood volume, blood pressure, regulating body water. The detection threshold for NaCl is 1 to 7 mM in rodents and humans alike (Eylam and Spector, 2002;McMahon et al., 2001). Other anions such as ammonium, lithium and potassium also induce salty taste but have pronounced other tastes in addition. Therefore the component due to Na^+ ions is in mammals the most relevant and the best studied (Lindemann, 1997). Mechanisms for salt detection, especially in humans, still remain poorly understood (Smith and Ossebaard, 1995;Lindemann, 2001). An early attempt to explain salty taste was published in 1984 by Heck et al. (Heck et al., 1984) where it was reported that Na^+ would permeate cation channels in taste cells via amiloride sensitive epithelial sodium channels (ENaCs) on the anterior tongue. ENaC is expressed in epithelial cells of several tissues involved in salt and water reabsorption. In taste buds, a passive Na^+ influx driven by Na^+ concentration gradient would depolarize and thereby excite TRCs (Heck et al., 1984).

The search for ENaC subunits has shown that the α, β and γ subunits are expressed in taste buds (Kretz et al., 1999;Lin and Kinnamon, 1999). However, the effects of amiloride are less significant on the taste of NaCl in humans (Smith and Ossebaard, 1995;Ossebaard et al., 1997), raising the point that ENaC is not the main salt sensor in humans. The ability to taste salt is significantly different in humans and in rodents. One of the difficulties encountered with the study of the role of ENaCs is the difficulty to examine mice lacking one or more of ENaCs subunits. Unfortunately, these knockout mice die shortly after birth, because of respiratory failure (ENaC $\alpha^{-/-}$).

There is evidence of both amiloride sensitive and insensitive salt taste present across mammals, the major mechanism in humans appears to be amiloride insensitive (Halpern, 1998;Feldman et al., 2003;Smith and Ossebaard, 1995). Lyall et al (2004) have identified an amiloride-insensitive salt

pathway mediated by a splice variant of vanilloid receptor channel TRPV1 being TRPV1t, which like TRPV1, is activated by capsaicin and temperature and is modulated by ethanol (Lyall et al., 2004;Lyall et al., 2005b;Lyall et al., 2005a). In addition, upon obtaining whole nerve chorda tympani recordings from rat and wild-type and $TRPV1^{-/-}$ mice that TRPV1t activation is involved in the tonic response produced by salts that were obtained in the presence of inhibitors of ENaCs (e.g. benzamil, amiloride). However, subsequent behavioural studies using mice lacking TRPV1 did not reveal a lack of salt taste (Ruiz et al., 2006) and lack of data on the cloning and functional expression on this splice variant seriously challenge this hypothesis.

The saltiness intensity of Na^+ salts depends on the coupled anions, with NaCl tasting more salty than Na-gluconate at equivalent concentrations (Rehnberg et al., 1993;Simon et al., 1992;Simon, 1992). The substitution of Cl^- with less permeable organic anions reduces Na^+ influx into taste cells which results in a reduced nerve response (Simon, 1992). A larger anion would decrease paracellular anionic conductance, leading to more hyperpolarized cells (Elliott and Simon, 1990;Simon, 1992) and explains why Na^+ salts containing larger anion elicit the same taste response as NaCl at higher Na^+ concentration. Therefore, the anion specific permeability of tight junctions could be involved in salt taste discrimination of different organic anions. In this perspective, Michlig *et al*. have examined members of the claudin family, which are important taste tight junctions constituents and raised the interesting possibility that claudins confer to the epithelium its particular electrical resistance and its specific permeability to solutes and in particular to NaCl (Michlig et al., 2007).

Sour taste

Sourness is mostly aversive unless combined with other tastes such as sweet. It is commonly accepted that this taste is elicited by the acidity of chemicals. Several explanations have been made to rationalize the physiological role of sourness sensing. Sour taste may function to protect against consuming excessive dietary acid and disturbing the body's vital acid-base balance. It has also been hypothesized that it acts as a potential warning against unspoiled food and tissue damage by acids.

The works of Lyall et al. (Lyall et al., 2001) and Richter et al. (Richter et al., 2003) have shown that an acidic taste stimulus being the proton and/or the protonated species (extracellular solution) would lead to intracellular acidification in all taste buds cells. However, Richter et al. showed that Ca^{2+} transients would be observed in only a small subset of these cells. These researchers have shown that these Ca^{2+} transients were originating from a Ca^{2+} influx via voltage gated calcium channels.

As a matter of fact, many ion channels have been proposed to contribute to sour taste detection (Waldmann et al., 1997;Ugawa et al., 1998;Stevens et al., 2001;Lyall et al., 2001). More recently, another candidate sour taste receptor has been evidenced: a member of the transient receptor

potential (TRP) ion channel, the polycystic kidney disease-like ion channel PKD2L1 and its associated partner PKD1L3 (Figure 3). Lopez Jimenez et al (LopezJimenez et al., 2006) first reported the expression of these channels in mouse taste buds. Later, Ishimaru et al confirmed and extended these findings by expressing these channels in HEK293 cells, where acidic stimulation generated an inward current (Ishimaru et al., 2006). Finally, Huang et al (Huang et al., 2006) engineered mutant mice lacking the taste cells where PKD2L1 is expressed and found that mice were lacking responses to sour stimuli. Even though targeted deletion of these ion channels (PKD2L1 and/or PKD1L3) is still missing, these findings strongly implicate these TRP channels as members of an acid-sensing receptor.

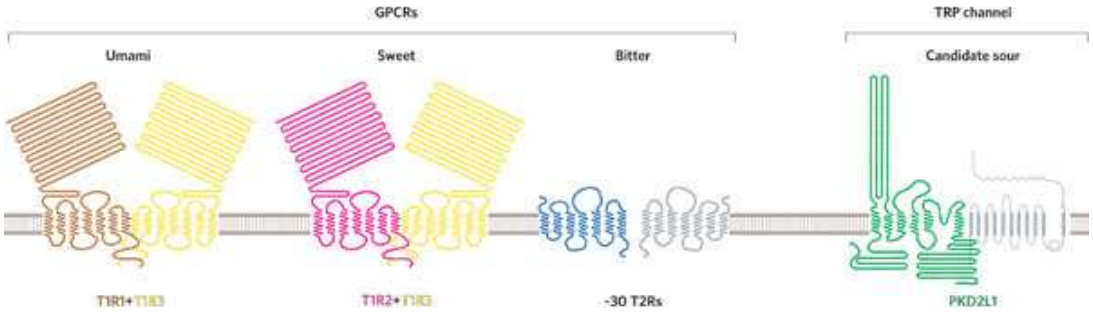

Figure 3. Schematic representation of taste receptors (and candidate receptors) mediating four of the five basic taste modalities. From Chandrashekar *et al*., 2006.

Fat perception, a sixth taste?

In addition to the five recognized classical taste modalities, other stimuli such as dietary fat excite TRCs. Fatty stimuli were initially thought to be sensed through texture and viscosity attributes (Kadohisa et al., 2005;Rolls, 2005). Recent evidence has brought to the fore the presence of a fatty acid transporter CD36 (Laugerette et al., 2005), shown to bind long-chain fatty acid and facilitates their transport into the cells (Kawai and Fushiki, 2003). Suppression of CD36 abolishes preference for these fatty acids in wild-type mice (Gaillard et al., 2008). The transduction mechanisms of fatty-acid detection in TRCs are still at large, in particular the function of GPR40 and GPR120 which are activated by medium- and long-chain free fatty acids (Hirasawa et al., 2008).

1.3. Common downstream signalling elements of sweet, umami and bitter TRCs

Sweet, umami and bitter cells share common transduction mechanisms illustrated in Figure 4. After tastant binding of the GPCR, the latter will activate the heteromeric G protein gustducin

(McLaughlin et al., 1992) or $G\alpha_{i2}$ (Kusakabe et al., 2000) leading to the release of of Gβγ subunits (Zhang et al., 2003;Huang et al., 1999) and the subsequent stimulation of phospholipase Cβ2 (PLCβ2) (Zhang et al., 2003;Huang et al., 1999;Rossler et al., 1998). PLCβ2 would hydrolyse PIP2 to produce the two intracellular messengers inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), and ultimately leads to the gating of the taste-transduction channel, the transient receptor potential (TRP) protein TRPM5 (Zhang et al., 2003; Perez et al., 2002) and subsequent activation of gustatory afferents. Basolaterally expressed TRPM5 channels are gated by the depletion of intracellular Ca^{2+} released from IP3 receptors in the endoplasmic reticulum stimulated by IP3.

This model was further confirmed by impaired sweet, umami and bitter sensing in mice deficient of gustducin (Wong et al., 1996;Ruiz et al., 2003), PLCβ2 (Zhang et al., 2003; Dotson and Spector, 2005) and TRPM5 (Zhang et al., 2003; Damak et al., 2006). On the contrary, salty and sour tastes remain unaltered in these models (Zhang et al., 2003).

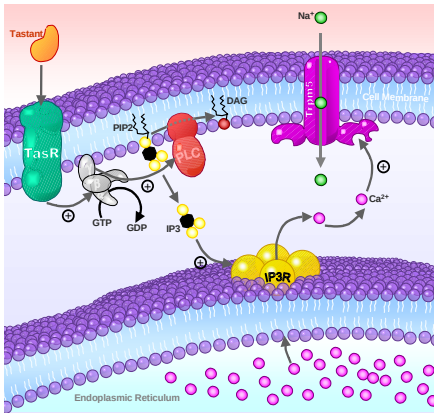

Figure 4. Representation of the signal transduction of sweet, bitter and umami in the TRC. The tastant binding to the TasR will generate a intracellular cascade that include α-gustducin and PLCβ2, which degrades phosphatidylinositol-4,5-bisphosphate (PIP2) to produce diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). IP3 could activate IP3R receptors on the endoplasmic reticulum leading to the depletion of intracellular Ca^{2+} that will gate basolaterally expressed TRPM5 channels. The excitation of TRPM5 channels will conduct into an membrane depolarization that will trigger the release of cell neurotransmitters from synaptic vesicles to excite gustatory afferents. Modified by Cettour-Rose P. from Clapham, 2003.

1.4. Processing of the taste signal in the taste bud

Morphologically different taste cell types have been described from the earliest histological identification of taste cells up through electron microscopical studies, where light cells and dark cells have been identified (Roper, 1989). The taste bud contains four types of cells, named type I, II, III and IV. The assignement of function to cell morphotype has remained unclear until the publication of recent immunostaining studies. Taste GPCRs, G proteins, PLCβ2 and TRPM5 are found in one subtype

of cells (type II), whereas synaptic markers (SNAP-25) and neural cell adhesion molecule (NCAM) were expressed in type III cells, named presynaptic cells (DeFazio et al., 2006;Clapp et al., 2006;Clapp et al., 2004;Yee et al., 2001).

The work of Finger *et al*. (Finger et al., 2005) has demonstrated that ATP is a neurotransmitter involved in communication between the taste bud and gustatory afferent fibers. Mice lacking purinergic receptors P2X2 and P2X3 receptors had markedly reduced gustatory nerve and taste behavioral responses to MSG, sweet and bitter stimuli. P2X receptors have been localized in sensory afferents innervating the taste buds (Finger et al., 2005). Secretion of ATP by type II cell and release of serotonin by type III cells has been measured using biosensor cells (Huang et al., 2005a;Huang et al., 2005b;Huang et al., 2005c). These researchers have shown that ATP secretion from receptor cells triggers excitation of adjacent presynaptic cells and adjacent sensory afferents as depicted in figure 5. ATP is thought to be released by pannexin 1 hemichannels expressed in receptor cells (Huang et al., 2007).

Presynaptic cells express none of the taste GPCRs and consistent with that, do not respond to sweet, bitter and umami stimuli (Huang et al., 2007). The work of Stephen Roper and colleagues has evidenced that sour and salty stimuli are not recorded in receptor cells (type II) but in presynaptic cells (Tomchik et al., 2007). In addition, these cells were broadly tuned and responded to two or more taste qualities whereas type II cells were specifically stimulated by single stimuli. Using calcium imaging on single isolated taste cells and with biosensor cells to identify neurotransmitter release (ATP, serotonin), Huang *et al*. (Huang et al., 2008) showed that presynaptic cells specifically respond to acid taste stimulation and release serotonin. Serotonin's function in taste buds remains puzzling. No taste-behavior abnormality was observed in 5-HT3, a serotonin receptor subtype (Finger et al., 2005). 5-HT1A receptors are expressed on taste cells and serotonin would act as a paracrine transmitter at those receptors (Kaya et al., 2004) but no real evidence exists regarding its exact role.

Therefore, functional distinctions are to be made between type II and III cells, with presynaptic cells integrating messages from receptor cells in a cell to cell communication (Figure 5, pathway 2) but also they are presumably direct sensors of salty and sour stimuli (Figure 5, pathway 3).

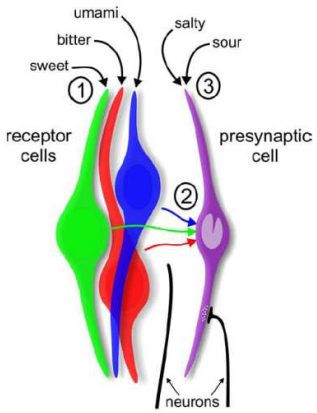

Figure 5. Representation of gustatory processing in the taste bud between the different cell types. Receptor cells (type II) are narrowly tuned to sweet, bitter and umami stimuli (1). Receptor cells excite synaptic cells via ATP release (2). Presynaptic cells are sensors of sour and salty stimuli (3). Presynaptic cells release serotonin possibly as an excitatory afferent gustatory stimulus. From Tomchik et al., 2007.

1.5. Coding at the periphery

A major controversy in taste research has been the coding of the fundamental qualities within TRCs and gustatory afferent fibers. Two theories have been proposed referred as “labelled line” and “across fiber pattern” (Chandrashekar et al., 2006;Simon et al., 2006), illustrated in Figure 6. In the labelled line model, neurons respond to selective cues in the periphery and this information remains segregated in the brain meaning that each taste modality would be detected by a specific cell type and information would be transmitted to afferents individually tuned to one modality. The across fiber pattern theory suggests that TRCs would be broadly tuned across taste modalities and thereby sensory fibers would participate in the detection of all taste signals. In this model, stimulus identity and intensity are specified by a unique combination of activity distributed across neurons. The observation that cells selectively respond to different taste modalities demonstrates that taste information is segregated in the periphery and shows more consistency with a labelled line model of taste coding.

The elegant work of Charles Zuker and colleagues to understand taste coding at the periphery has led to a huge breakthrough in the field. Using RASSL, a κ-opiod receptor activated by spiradoline which is a compound normally undetected by wild type mice, these researchers expressed RASSL in T1R2 cells and parallely in T2R cells: when expressed in sweet cells, mice are attracted by spiradoline whereas in T2R cells, mice avoid the molecule (Zhao, 2003, Mueller 2005). In addition, misexpression of a bitter receptor in sugar taste cells generates mice that are attracted to the bitter compound (Mueller et al., 2005). These experiments show that the activation of different cell types on the tongue is sufficient to drive taste behaviors.

However, evidences in favour of the across fiber pattern theory come from electrophysiological studies. Gilbertson *et al.* (Gilbertson et al., 2001) found that rat TRCs were responding to multiple gustatory stimuli. Yet, this could be rationalized in term of type III cells activation as observed from Stephen Roper and colleagues regarding the broadly tuned response of type III versus type II cells. Other studies showed that both TRCs and peripheral nerves are broadly tuned (Ogura, 2002;Bennick, 2002;Frank et al., 1988). However, non-human primates data indicate that peripheral nerve responses to tastants are almost completely segregated, with specific taste fibers dedicated to primary tastants (Danilova et al., 2002;Danilova and Hellekant, 2004;Hellekant et al., 1998).

Taken together, these experiments argue that TRCs (at least type II cells) are dedicated to sense a specific modality and are in favour of a labelled line at the periphery.

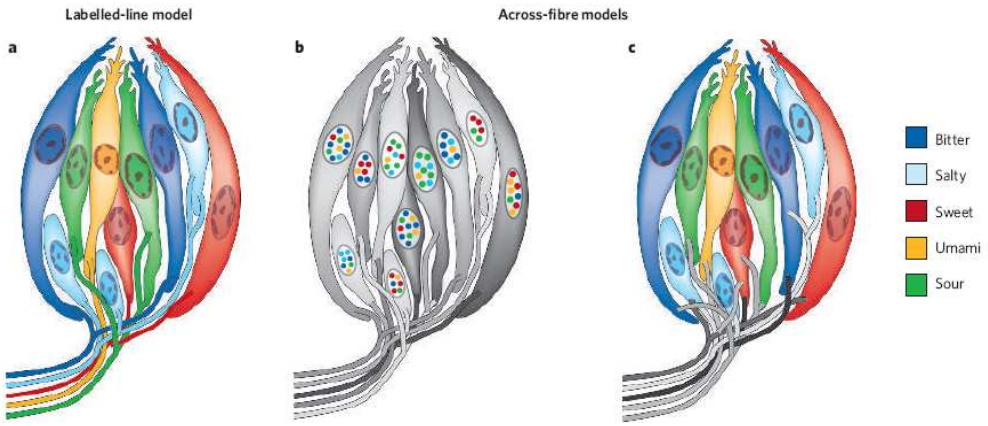

Figure 6. Two theories encode taste qualities at the periphery. From Chandrashekar et al., 2006.

1.6. From the periphery to the brain level

Several nerves are responsible for transmitting gustatory inputs to the rostral nucleus of the solitary tract (rNTS) which is the first relay of the central nervous system (CNS) in the medulla. TRCs are innervated by the *chorda tympani* and *greater superior petrosal* branches of the facial nerve. The role of these nerves is to transmit information about the identity and intensity of the chemical nature of the tastants. The lingual branch of the *glossopharyngeal nerve* and the *superior laryngeal branch* of the vagus nerve innervate TRCs on the epiglottis, oesophagus and posterior tongue. These nerves project to the NTS which also receives inputs from somatosensory fibers relayed from the trigeminal system (Boucher et al., 2003). Additionally, the caudal division of the NTS receives inputs from the visceral (vagal) afferent fibers giving information about the physiological status of the gastrointestinal tract (Travagli et al., 2006;Zhang et al., 1995). Therefore, the NTS integrates the information from gustatory, somatosensory and gastrointestinal signals. Afferent fibers from the NTS propagate into forebrain structures such as the central nucleus of the amygdala, the lateral hypothalamus and the gustatory cortex (GC).

At the level of the NTS, electrophysiological evidences suggest that taste neurons are broadly tuned (Scott et al., 1986), with some topographical segregation between sweet and bitter stimuli with an overlap between bitter, sour and salty stimuli (Lemon and Smith, 2005). These findings suggest that spatial and temporal patterns may encode the different tastes (Lemon and Katz, 2007). At the level of the forebrain, three areas have been implicated in taste detection: the central nucleus of the amygdala, the lateral hypothalamus and the gustatory cortex (Van Der et al., 1984). Electrical stimulation of these areas modulates neuronal responses to tastants in the rNTS (Di Lorenzo and Monroe, 1995;Smith et al., 2005). Using genetic tracing, it has been shown that sweet and bitter taste are processed through segregated neuronal circuitries along the gustatory pathway up to the cortical level (Sugita and Shiba, 2005). This is in disagreement with the evidence that GC neurons recorded in both anesthetized and behaving animals responded to multiple taste modalities (including sweet and bitter) (Hanamori et al., 1998;Stapleton et al., 2006). Spatial neuronal representation of taste in the GC is an important component in the GC neurons firing response. Accolla *et al.* (Accolla et al., 2007) found using intrinsic optical imaging in anesthetised rats that four of the primary taste modalities (sweet, bitter, salty, and sour) are represented by distinctive spatial patterns but that no region was specific to a single modality with clusters observed when tastants of similar hedonic value were presented.

Interestingly, somatosensory and olfactory inputs have been recorded in the GC neurons and in the orbitofrontal cortex (OFC) which contains the secondary taste cortex is defined as a direct target for the GC (Rolls et al., 1999;Stapleton et al., 2006). It seems that the OFC receives convergent somatosensory (temperature and texture) (Rolls et al., 1999), olfactory and taste afferents. Therefore, the detailed neural mechanisms underlying taste integration in the GC and the OFC need further temporal and spatial characterization but studies cited above find a clear multisensory response.

1.7. Post-ingestive factors affect taste perception

As we reported in the opening of this chapter, not only sensory cues participate in the motivation to ingest food but also the metabolic consequences of this ingestion. Experience and memory guide the food choice. To this regard, conditioned taste aversion (CTA) is a well-established technique to study taste preference consisting in associating the ingestion of a pleasant food item together with the injection of a compound that produces gastric malaise (such as LiCl) (GARCIA et al., 1955). Using cortical representation of taste, Accolla *et al.* (Accolla and Carleton, 2008) have shown, using this technique that the spatial pattern of sucrose when associated to CTA, changes to a pattern consistent with an aversive stimulus such as quinine. This shows that gustatory responses in the higher brain are modulated by the animal physiological state. Studies involving primates have shown

that satiety can modulate taste responses in the lateral hypothalamus and in the OFC (Rolls et al., 1986;Rolls et al., 1989). Sensory-specific satiety is observed when the sensory pleasantness of food decreases while the satiation effect increases, with the taste sensitive neural circuits integrate these changes in reward value. However, this is true for a certain food, as the animal might still be motivated to ingest other foods. De Araujo *et al.* (De Araujo et al., 2006) have shown that several taste-sensitive forebrain regions participate in coding in the motivational state to ingest a sucrose solution during alternance of hunger-satiety phases. They also showed tat the TRPM5 null mice, which lack sweet taste transduction, can develop a robust preference for sucrose solutions based solely on caloric content and dopamine release in the ventral striatum arguing that calorie-rich nutrients can directly influence brain reward circuits that control food intake independently of palatability (De Araujo et al., 2008).

2. Somatosensory perception

The detection of temperature and pain stimuli is initiated at the level of primary afferent neurons that terminate as free nerve endings embedded in target tissues such as the dermal and epidermal layers of the skin, the oral and nasal mucosa and joints. The cell bodies of these neurons originate from dorsal root ganglia (DRG) and cranial nerve ganglia such as the trigeminal ganglion (Figure 8). They relay information regarding environmental stimuli to the central nervous system *via* projection to the dorsal horn of the spinal cord. The conduction properties of neurons that respond to stimuli such as heat, cold and mechanical pressure are characteristic of C- and Aδ fibers. These neurons express the receptor tyrosine kinase trkA (Patapoutian and Reichardt, 2001), are dependent on nerve growth factor (NGF) during development and are peptidergic, expressing nociceptive markers such as calcitonin gene-related peptide (CGRP) and substance P (SP). A portion of these neurons express c-ret postnatally, are dependent on glial-derived neurotrophic factor (Snider and McMahon, 1998) and are non-peptidergic.

Among these nociceptors, some are specialized to detect a unique noxious modality, whereas others are polymodal nociceptors that respond to painful levels of heat, cold and mechanical stimuli. During the last ten years, several temperature sensitive TRP channels (ThermoTRPs) expressed in sensory neurons or keratinocytes (epithelial cells) have been identified as detectors of temperature and noxious chemical stimuli.

2.1. The notion of chemesthesis

Described by Barry Green (Rentmeister-Bryant and Green, 1997;Green et al., 2005), chemesthesis is defined as the chemical sensibility of the skin and mucus membranes. Chemesthetic sensations arise when chemical compounds activate receptors associated with other senses than

gustatory perception that mediate pain, touch, and thermal perception. Because these receptors are present in all types of skin, chemesthetic sensations can be aroused from anywhere on the body's surface as well as from mucosal surfaces in the nose, mouth, eyes, etc. Mucus membranes are generally more sensitive to chemesthetic stimuli because they lack the barrier function of cornified skin. The burn from chili pepper, the coolness and burning from menthol in mouthwashes and topical analgesic creams, and the stinging or tingling of carbonation in the nose and mouth are all examples of chemesthetic sensations.

2.2. The Somatosensory organs

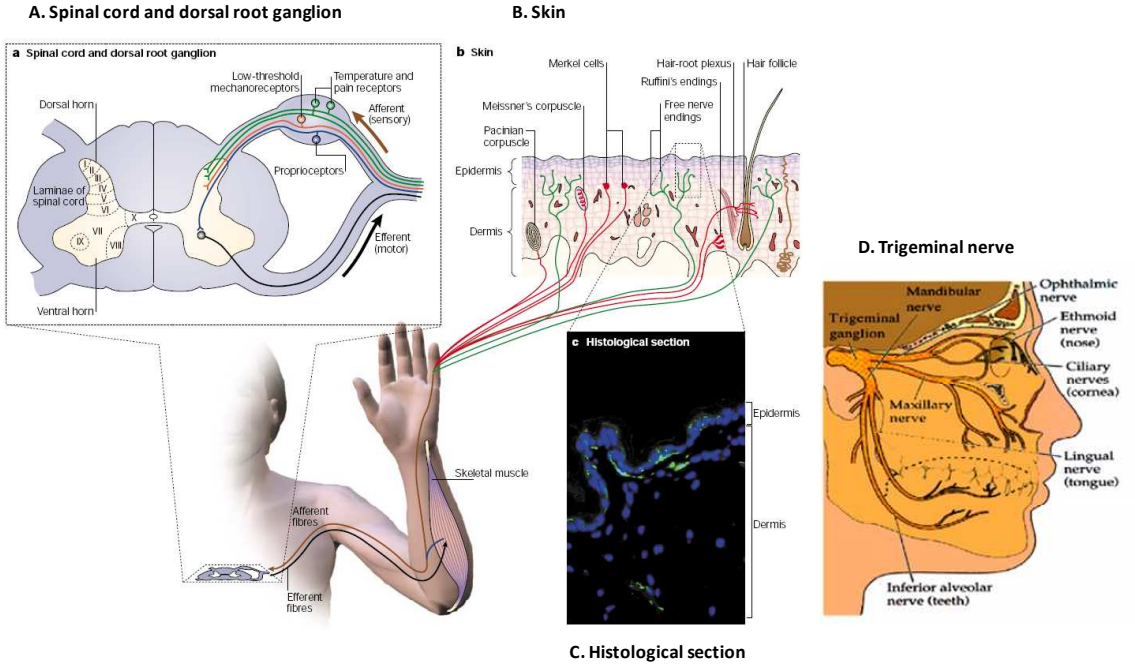

Figure 7. Anatomical and functional organization of somatosensation. **A**. Spinal nerves formed by the joining of afferent (sensory) and efferent (motor) roots provide peripheral innervation to skin, skeletal muscle, viscera and glands. Arrows denote the direction of incoming sensory and outgoing motor impulses. The cell bodies of motor neurons are located within the ventral horn (laminae VII–IX) of the spinal cord. Cell bodies of sensory neurons are located in the dorsal root ganglia (DRG). Within the DRG there are subclasses of sensory neurons known as proprioceptive (blue), low-threshold mechanosensitive (red) and temperature- and pain-sensing neurons (green). These neurons project centrally to dorsal horn interneurons (laminae I–VI of the spinal cord) and peripherally to target tissues. Proprioceptive neurons (blue fibres) project to specialized structures within target tissues such as muscle, and sense muscle stretch. **B**. Low-threshold mechanosensitive neurons (red fibres) project to end organs that transmit mechanical stimuli. Five types of mechanosensitive assemblies have been described and are illustrated in the figure. Temperature and pain sensing neurons (green) do not project to specialized end organs; instead they terminate as free nerve endings in all layers of the skin, and near blood vessels and hair follicles. **C**. Section of skin showing free nerve endings (green fibres) stained with the pan-neuronal marker PGP9.5. The nuclei of skin cells are stained (blue) with 4,6-diamidino-2-phenylindole (DAPI). Free nerve endings are found in both the epidermal and dermal layers. **D**. Representation of the trigeminal nerve (fifth cranial nerve), one of the largest nerves in the head that forms three major branches: the lingual nerve, the ethmoid nerve (nose) and the ophthalmic nerve. These fibers are responsible for sending

impulses of touch, pain, pressure, and temperature to the brain from the face, oral cavity, nose, jaw, gums, forehead, and around the eyes. Modified from Patapoutian *et al*., 2003.

2.3. Structural features of thermoTRPs

To date 28 mammalian TRP channels are known (Clapham, 2003;Venkatachalam and Montell, 2007). Nine TRP channels have been shown to be activated or strongly modulated by distinct temperatures and six of these (TRPV1-4, TRPM8 and TRPA1) are thought to have a role in temperature sensation and chemesthesis (Figure 8, table 1). TRP channels are part of the larger superfamily of voltage-gated like (VGL) ion channels and are generally assumed to be similar in global structure (Yu and Catterall, 2004). The TRP superfamily is divided into seven subfamilies: the five group 1 TRPs sharing a high sequence homology with drosophila TRP (Montell and Rubin, 1989) which are TRPC (Canonical), TRPV (Vanilloid), TRPM (Melastatin), TRPN (NOMPC "No Mechanoreceptors potential"), and TRPA (Ankyrin) and two group 2 subfamilies TRPP (PKD, Polycystic Kidney Disease) and TRPML (Mucolipin). Thus TRP channels presumably form tetramers, with each subunit containing six transmembrane domains. The N- and C- termini are located on the intracellular side of the membrane, and the channel pore is formed by helices of segments S5 and S6 as well as the S5–S6 linker of the four subunits. Similar to voltage gated channels, many TRP channels are voltage sensitive, albeit only weakly, and the voltage sensor region appears to involve the S4 transmembrane helix (Tombola et al., 2006;Voets et al., 2007). TRP channels have extended cytosolic N- and C- termini that contain a variety of structural features including coiled-coil domains, a TRP domain, and ankyrin repeat domains (Figure 8). These domains, while not present in every TRP channel, have been implicated in channel formation, regulation by cellular factors, etc (Bandell et al., 2006;Jin et al., 2006;McCleverty et al., 2006).

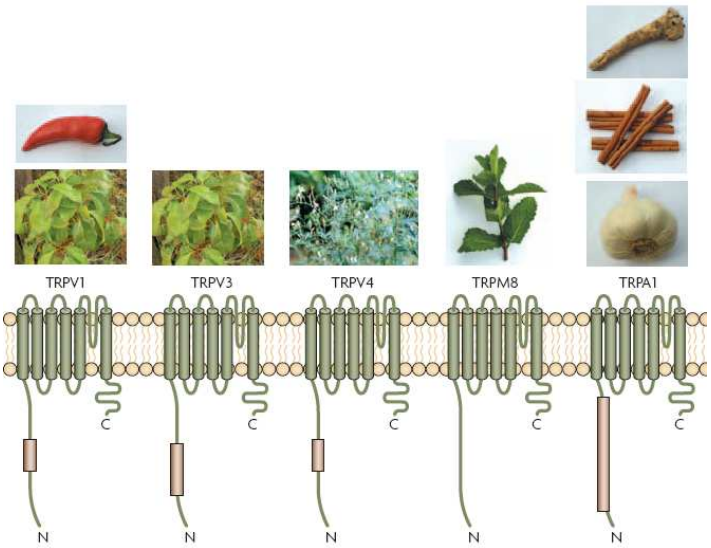

Figure 8. ThermoTRPs are activated by naturally occurring compounds. These channels possess internal N and C-termini, 6 transmembrane domains, a pore between segments 5 and 6. The presence of intracellular ankyrin regions (pink rectangles in the N-termini) varies among channels from absent in TRPM8 to 18 repeats in TRPA1. TRPV1 is stimulated by capsaicin, the burning molecule in red hot chilli peppers and camphor. TRPV3 is activated by camphor and TRPV4 by bisandrographolide (*Andrographis paniculata*). TRPM8 is a channel responding to menthol, the active ingredient in green mint and TRPA1 responds to pungent ingredients such as isothiocyanates (mustard oil, horseradish), cinnamaldehyde (cinnamon), allicin (garlic) and eugenol (cloves). Modified from Szallasi *et al*., 2007.

2.4. The capsaicin receptor TRPV1

David Julius and colleagues achieved a breakthrough in the molecular understanding of thermal and pain sensation by identifying TRPV1 (Caterina et al., 1997). TRPV1, represented in Figure 9, was the first mammalian TRPV identified by expression cloning in a search for channels activated by the inflammatory vanilloid compound capsaicin, which gives spicy foods their characteristic hot sensation. In addition, TRPV1 is activated by heat (≥43°C) (Caterina et al., 1997) and many other chemicals, which include an endocannabinoid, anandamide (Zygmunt et al., 1999); the topical analgesic, camphor (Xu et al., 2005); and the pungent compounds present in black pepper (piperine) (McNamara et al., 2005) and garlic (allicin) (Macpherson et al., 2005). TRPV1-mediated cation influx initiated by the application of noxious chemicals or heat is further enhanced by low pH (Caterina et al., 1997). In fact, pH≤5.9, characteristic of proalgesic tissue acidosis associated with injury, induces a shift in the thermal activation threshold of TRPV1 so that it can be activated at room temperature (Montell, 2005). Shifts in the activation threshold of TRPV1 and potentiation of capsaicin-mediated responses are also brought about by ethanol (Trevisani et al., 2002), nicotine (Liu et al., 2004), and proinflammatory cytokines (Zhang and Oppenheim, 2005). Furthermore, TRPV1 is potentiated by a decrease in PIP2 levels and PKC-mediated phosphorylation of the channel, both following PLC

activation induced by proalgesic agents such as bradykinin and nerve growth factor (Montell, 2005). Therefore, TRPV1 is a multiple signal integrator capable of transducing signals evoked by several noxious stimuli.

The capsaicin-binding domain in TRPV1 was first reported by Jordt and Julius in 2002 (Jordt and Julius, 2002). Using the long recognized fact that only mammalian nociceptors are sensitive to pungent vanilloid compounds (Szallasi, 1994), they constructed chimeric rat-chicken TRPV1 channels. They evidenced a residue (Tyr 511) in the segment of the rat TRPV1 sequence extending from the second to third transmembrane domains, whose mutagenesis resulted in a loss of vanilloid agonism and binding but preservation of TRPV1 activation by protons or heat. In addition, these researchers found that the aromatic ring at Tyr 511 is important for vanilloid interaction and proposed that π-stacking interactions occur with the vanillin moiety of capsaicin. Other residues involved in vanilloid binding implicate Ser 512, Lys 547 and Tyr 550 suggest that a "paddle structure" linking S3 and S4 domains would form a vanilloid binding site (Johnson et al., 2006). Extracellular amino acids located near the pore domain have been implicated in the pH sensitivity of TRPV1 (Jordt et al., 2000), rationalizing pain mediated by tissue acidosis during injury.

Very recently, the reactivity of TRPV1 to allicin, the pungent molecule in garlic, has been characterized. Allicin acts on TRPV1 by covalent modification of cysteine residues (Salazar et al., 2008). In contrast to TRPA1 channels (detailed in 2.5.), modification of a single cysteine located in the N-terminal region of rat TRPV1 (Cys 157) was necessary and sufficient for TRPV1 stimulation. To date, no other covalent agonists of TRPV1 have been reported.

Mice lacking the capsaicin receptor are defective in nociceptive, inflammatory and hypothermic responses to vanilloid compounds, supporting the interpretation that TRPV1 contributes to acute thermal nociception and hyperalgesia after tissue injury (Caterina et al., 2000). In dissociated cultures of dorsal root ganglia, TRPV1 null neurons exhibit a dramatic decrease in the prevalence of heat-evoked cationic currents. In fact, none of these neurons respond to heating below 50°C, in contrast to the wild-type neurons. Behavioral responses to heat were present in temperatures exceeding 50°C, suggesting that TRPV1 accounts for thermosensitive responses below 50°C whereupon other nociceptors are recruited.

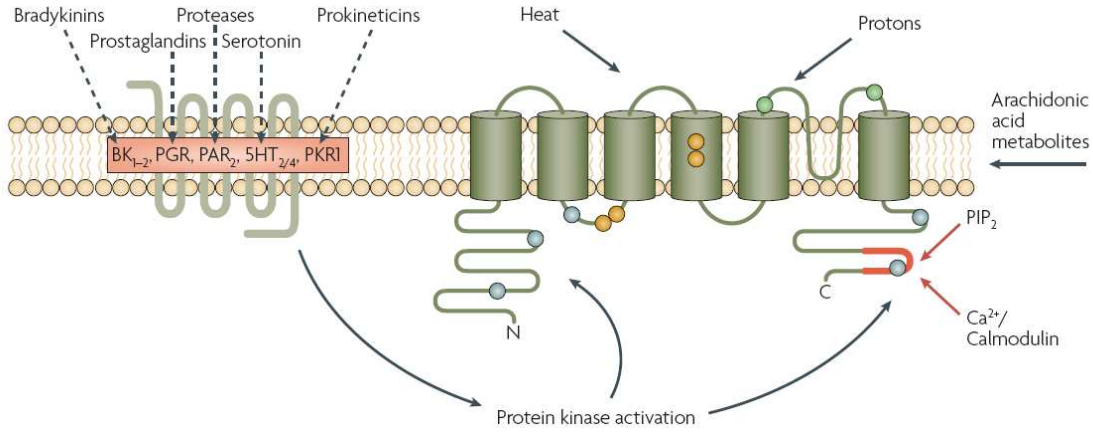

Figure 9. TRPV1 is a polymodal integrator in the peripheral nervous system. Blue arrows represent TRPV1-sensitizing stimuli via protein-kinase activation and red arrows indicate negative regulation by PIP2, Ca^{2+} and calmodulin. Coloured circles represent amino-acid residue for vanilloid binding (orange, Y511, S512, L547, T550), phosphorylation sites (blue, S116, T370, S502, T704, S800) and proton activation sites (green, E600, E648). The C- terminal part of TRPV1 (red line) interacts with PIP2 and calmodulin. From Szallasi *et al*., 2007.

2.5. The cinnamaldehyde receptor TRPA1

TRPA1 is the only TRPA protein present in humans and other mammals (Montell, 2005) and was previously called ANKTM1 because the protein consists of many N terminal ankyrin repeats. This channel is found expressed in a subpopulation of nociceptive neurons where it is co-expressed with TRPV1, but not with TRPM8 (Story et al., 2003a). Also, TRPV1 and TRPM8 are found in distinct neuronal populations (Peier et al., 2002a). TRPA1 is chemically activated by the psychoactive component in marijuana, environmental irritants, and pungent compounds. These include ingredients present in wasabi, horseradish, and mustard oils (isothiocyanates); garlic (allicin); cinnamon oil (cinnamaldehyde); marijuana (tetrahydrocannabinol); and tear gas (acrolein) (Macpherson et al., 2005;Jordt et al., 2004;Bandell et al., 2004a;Bautista et al., 2006). TRPA1 can also be activated by stimulation of the PLC pathway by bradykinin and by the subsequently produced metabolites DAG and PUFAs (Bandell et al., 2004). There is controversy as to whether TRPA1 is a thermally or mechanically gated channel. When exogenously expressed in cultured cells, TRPA1 has been reported as a noxious cold (<17°C) activated channel (Bandell et al., 2004;Story et al., 2003b). However, the cold activation of TRPA1 in both cultured cells and TRPA1-deficient mice is controversial (Bautista et al., 2006;Kwan et al., 2006). TRPA1 channels from mouse and zebrafish have been suggested to be mechanically gated channels, and the multiple ankyrin repeats of TRPA1 may form a gating spring capable of transducing mechanical force and thereby facilitating channel opening (Corey et al., 2004). However, analyses of TRPA1-deficient mice described below mitigate

this possibility (Bautista et al., 2006;Kwan et al., 2006). Despite the controversy concerning TRPA1, three *Drosophila* TRPAs (dTRPA1, Pyrexia and Painless) function in thermosensation. Although TRPA1 may be a cold thermosensor, *Drosophila* TRPA1 and Pyrexia are activated in vitro by warm (≥24–27°C) and noxious (≥40°C) heat, respectively (Viswanath et al., 2003;Rosenzweig et al., 2005). Painless has not been functionally expressed in vitro but is predicted to be gated by noxious heat (≥38°C) and the isothiocyanates present in wasabi (Tracey, Jr. et al., 2003;Al Anzi et al., 2006).

One critical feature to activate mammalian TRPA1 shared by several of its agonists is their chemical reactivity. TRPA1 agonists can be separated into electrophilic and non electrophilic reagents. Recently, two studies (Hinman et al., 2006;Macpherson et al., 2007a) have proposed that cinnamaldehyde and allyl-isothiocyanate, both electrophilic compounds, activate TRPA1 through covalent binding on specific cysteine residues present in the ankyrin repeats of the proteins. These findings could be extended to alkyl chains inferior to 8 carbons possessing α, β unsaturated double bond (Macpherson et al., 2007b), showing that the reactive cysteine residues could attack these compounds through a Michael addition. Interestingly, the mutation of one or several reactive cyteines to serine causes insensitivity of TRPA1 to electrophilic agonists but this is not the case for non electrophilic compounds like the super cooling agent icilin (Macpherson et al., 2007a). The electrophilic compounds elicit large irreversible currents whereas currents produced by icilin are reversible (Macpherson et al., 2007a) suggesting that TRPA1 contains both a traditional binding pocket and intracellular cysteine(s) covalent binding.

2.6. The menthol receptor TRPM8

TRPM8 is a thermally regulated channel activated by moderately cool temperatures (<23–28°C) and by compounds that evoke a sensation of coolness, such as menthol, eucalyptol, and icilin (McKemy et al., 2002;Peier et al., 2002a). Activation by cold and menthol can be separated because several mutations having a profound effect on activation by menthol have only minimal effects on activation by cool temperatures (Bandell et al., 2006). These data indicate that the domains involved in activation by menthol and thermal input are distinct. In further support of this conclusion, modulation of TRPM8 activity by pH has differential effects on activation by icilin and cold versus menthol (Andersson et al., 2004). The mechanism for thermal activation of TRPV1 and TRPM8 by hot and cool temperatures, respectively, appears to be similar (Voets et al., 2004;Brauchi et al., 2004). These channels are voltage dependent, and their respective activation temperatures as well as ligands lead to shifts in their voltage thresholds toward more physiological membrane potentials (Voets et al., 2004;Brauchi et al., 2004).

TRPM8 activity is also regulated by PIP2 (Liu and Qin, 2005), as depletion of PIP2 reduces activity induced by menthol or thermal cool. PIP2-mediated regulation of TRPM8 is reduced by mutations in basic residues in the TRP domain (Rohacs et al., 2005). Although these mutations affect the PIP2-modulated responses to both menthol and cold, they lie close or adjacent to mutations identified in the screen for residues that specifically function in the menthol response (Bandell et al., 2006).

Two independent studies have recently examinated the role of TRPM8 in cold sensation using TRPM8-deficient mice. These mice lack behavioral response to cold-inducing icilin application and display an attenuated response to acetone, an unpleasant cold stimulus (Dhaka et al., 2007). Cultured sensory neurons and intact sensory nerve fibres from TRPM8-deficient mice exhibit profoundly diminished responses to cold (Bautista et al., 2007). These animals also show clear behavioral deficits in their ability to discriminate between cold and warm surfaces, or to respond to evaporative cooling. At the same time, TRPM8 mutant mice are not completely insensitive to cold as they avoid contact with surfaces below 10°C, albeit with reduced efficiency. Therefore, based on these studies, TRPM8 contributes to sensing unpleasant cold stimuli but as TRPM8-deficient mice have normal nociceptive-like responses to subzero centigrade temperatures (Dhaka et al., 2007), this suggests the presence of at least one additional noxious cold receptor.

2.7. TRPV3 and TRPV4

Two additional members of the TRPV subfamily of ion channel proteins that deserve some attention regarding their implication in chemosensation are TRPV3 and TRPV4, both of which share 40–50% homology with TRPV1. TRPV3 can be activated by warm temperatures, with a threshold between 34 and 39°C. Moreover, repetitive stimulation of TRPV3 with heat results in a current response of gradually increasing amplitude (Peier et al., 2002b;Smith et al., 2002;Xu et al., 2002). In addition to heat, TRPV3 can alternatively be activated by camphor (Moqrich et al., 2005), irritant extracts from oregano and cloves (Xu et al., 2006), or synthetic boron compounds containing diphenyl groups, such as 2-aminoethoxydiphenyl borate (Hu et al., 2004). TRPV4 was first described as an ion channel that could be activated by hypoosmotic stimuli (Liedtke et al., 2000). It can alternatively be activated by certain synthetic phorbol esters (Watanabe et al., 2002a). In addition, however, like TRPV3, TRPV4 can be activated by warm temperatures. Depending on the study, the threshold for TRPV4 activation has ranged from 25°C to 34°C (Guler et al., 2002;Watanabe et al., 2002b). Electrophysiologically, TRPV4-mediated currents are distinguishable from TRPV3-mediated currents based upon a relatively pronounced desensitization upon repeated or prolonged thermal challenge (Chung et al., 2004). TRPV3 and TRPV4 are both expressed at very low levels in sensory neurons but are prominently expressed within keratinocytes, the stratified epithelial cells that are the

major constituents of skin epidermis (Moqrich et al., 2005;Guler et al., 2002;Peier et al., 2002b). Consistent with this expression pattern, electrophysiological studies have demonstrated that both sensitizing TRPV3-like and desensitizing TRPV4-like heat-evoked currents can be recorded in cultured keratinocytes. Indeed, each of the respective response types is lost in keratinocytes from the corresponding knockout mice (Moqrich et al., 2005).

The thermal responsiveness of TRPV3 and TRPV4, together with their expression patterns, has led to the hypothesis that, in the skin, some aspects of heat transduction may not be confined to sensory neurons. Rather, activation of thermally gated channels on skin keratinocytes may contribute to temperature sensitivity. In this model, activated keratinocytes would signal, presumably via diffusible chemical substances, to sensory neurons whose terminals are deeply embedded in the epidermis. Keratinocytes can release many different paracrine factors, such as ATP, ACh, opioid peptides, and cytokines, among others, that have the capacity to activate, sensitize, or inhibit the sensory nerve endings (Xu et al., 2006). Although the keratinocyte-signaling hypothesis has not yet been directly tested *in vivo*, physiological evidence has accumulated for the contribution of both TRPV3 and TRPV4 to peripheral thermosensation. Besides its activation by phorbol esters and bisandrographolide, TRPV4 has never been shown to respond to other pungent chemicals and therefore its role in chemesthesis appears limited. Concerning TRPV3, the channel has been shown to respond to pungent stimuli. However, TRPV3's affinity to its agonists shared with other TRP channels such as TRPV1, TRPA1 or TRPM8 and usually requires higher concentrations to induce activation than for other channels. Taken together, these data argue against a physiological role of TRPV3 in chemosensation. Recently, a remarkable role of TRPV3 channels expressed in the brain has been evidenced. Incensole acetate, a Boswellia resin constituent, is a potent TRPV3 agonist that causes anxiolytic-like and antidepressive-like behavioral effects in wild-type mice with concomitant changes in c-Fos activation in the brain (Moussaieff et al., 2008). These behavioral effects were not noted in TRPV3-deficient mice, suggesting that they are mediated via TRPV3 channels.

2.8. Thermo-TRPs are promiscuous

As detailed above and in table 1, these channels are thought to mediate the chemesthetic sensation of a large number of natural (and synthetic) chemicals. The chemical structures of these compounds vary widely. Although certain TRP channels exhibit a specific selectivity for one compound as observed for capsaicin on TRPV1, the cross reactivity is considerable. One striking example is menthol which activates TRPM8, providing a molecular logic for the cooling sensation. Menthol has also been shown to activate TRPV3 and inhibit TRPA1 (Macpherson et al., 2006). However, menthol's inhibitory effect on TRPA1 shows a rapid reversibility masking probably

electrophilic interactions with TRPA1. This compound must act as an allosteric modulator with a global effect on protein structure and gating. The analgesic properties on this compound may rationalize the TRPA1 reactivity.

In addition to menthol, another very promiscuous molecule is camphor. Camphor has the ability to activate TRPV3 and TRPV1, but blocks TRPA1. Pungent molecules such as eugenol (cloves) and thymol (thyme) have the ability to stimulate TRPV1, TRPV3, TRPA1 (Yang et al., 2003;Xu et al., 2006;Lee et al., 2008) with a TRPA1 affinity higher than for the two other TRPs (Bandell et al., 2004b). Cinnamaldehyde, a potent TRPA1 agonist has an inhibitory effect on TRPM8 (Macpherson et al., 2006). Citral, a bioactive component of lemongrass, activates TRP channels found in sensory neurons (TRPV1 and TRPV3, TRPM8 and TRPA1), and produces long-lasting inhibition of TRPV1-3 and TRPM8, while transiently blocking TRPV4 and TRPA1 (Stotz et al., 2008).

Channel	Thermal threshold	Function & phenotype	Chemical agonist	Sensory neuron/ skin expression
TRPV1	>42℃	Noxious heat detector, thermal hyperalgesia under inflammatory conditions	Capsaicin, acidic pH, camphor, ethanol, resiniferatoxin, 2-APB, piperine, eugenol, gingerol	Sensory neuron, TRC (TRPV1t)
TRPA1	<17℃	Noxious cold detector,mustard oil- and bradykinin-induced hyperalgesia	Cinnamaldehyde, mustard oil, eugenol, icilin, allicin, acrolein, methyl salicylate, gingerol, thymol	Sensory neuron
TRPM8	<23℃	Noxious cold detector, cold analgesia	Menthol, icilin, l-carvone, eucalyptol, isopulegol, geraniol	Sensory neuron
TRPV3	>33℃	Warm and noxious heat sensor	2-APB, camphor, menthol, thymol, carvacrol, eugenol	Sensory neuron, keratinocytes
TRPM5	Heat>25℃ increases activity	Transducing element of taste signalling	Cytosolic Ca^{2+}	TRC, gastro-intestinal tract

Table 1. Function of relevant thermo-TRP channels to sensory perception

3. Somatosensation and Taste: do they overlap?

Taste and somatosensory perceptions (thermal, tactile and irritant cues) always accompany the ingestion of a foodstuff. However, are these peripheral systems independent or can they interact? The sensory interactions are thought to occur at cognitive level but persistent hints of a relationship between the two systems are continuously reported.

Daily experience tells us that temperature has a strong influence on how we taste. For example, the perceived sweetness of sugar solutions increases strongly with temperature (Bartoshuk et al., 1982;Green and Frankmann, 1988). In addition, cooling or heating of the tongue by itself is sufficient

to cause sensations of taste in approximately 50% of humans (Cruz and Green, 2000). To interpretate this, a simple explanation would be that thermal taste arises from activation of the receptor mechanisms for normal gustatory coding or that somatosensory elements would enhance the taste function either through physical or chemical interactions.

In 2005, Talavera *et al*. have shown that when expressed heterologously, TRPM5 currents are increased by temperature raises between 15 and 38°C (Talavera et al., 2005). This supports the hypothesis that TRPM5 involvement in taste transduction consists in providing a depolarizing current downstream of taste receptor activation to lead to neurotransmitter release. These researchers showed that TRPM5-dependent taste responses (sweet, umami and bitter) in mouse gustatory nerves are strongly enhanced by heating whereas salty and sour stimuli preferences remain unchanged during temperature increases. However in mice lacking TRPM5, no response to TRPM5-dependent tastants was observed even at 35°C. Whether thermal taste reported by Cruz and Green (Cruz and Green, 2000) is due to direct heat activation of TRPM5 and subsequent excitation of gustatory afferents without the tastant is a possible explanation but is still at large. As TRPM5 shows temperature sensitivity, it was reported in the thermo-TRPs category (table 1).

Histological studies of TRPV1 and TRPM8 expression patterns in the tongue papillae have shown that a dense meshwork of these fibers is specifically present in these organs (Ishida et al., 2002;Dhaka et al., 2008) as shown in Figure 10. In both studies, free nerve endings expressing TRPV1 and TRPM8 were found around but not inside taste buds. However, taste synaptic markers such as SNAP 25, a presynaptic cell marker, were absent from these studies therefore it is not excludible even though poorly probable that somatosensory fibers would form synapses with taste cells. Very intriguingly, the specific arrangement of TRPM8 and TRPV1 fibers into a “bush” around the taste bud is of great interest but the functional reason is unknown.

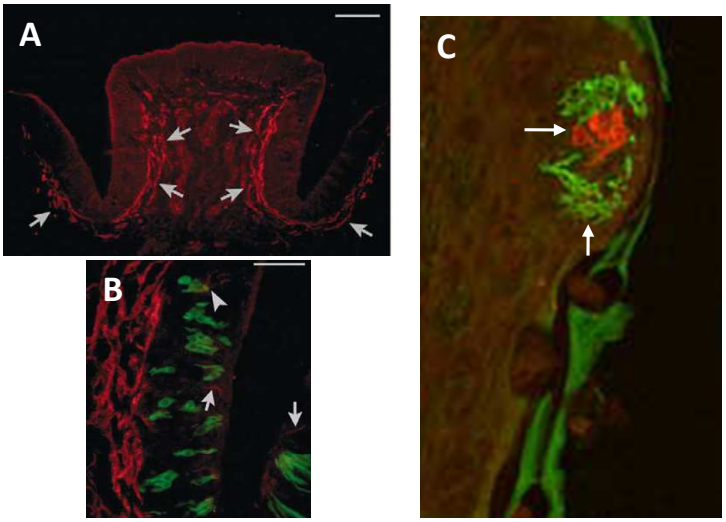

Figure 10. **A.** Histological sections of TRPV1 immunoreactive fibers (anti-TRPV1) in the circumvallate papillae surround the taste cleft and are present in the lamina propria mucosae under epithelia-like bundles (arrows). **B**. High magnification of the colocalization of TRPV1 immunoreactivity (red) and α-gustducin (green) showing perigemmal TRPV1 fibers (arrows) and intragemmal TRPV1 fibers (arrowhead). From Ishida Y. *et al*., 2002 **C**. Confocal images of gustducin (red)-positive taste buds (arrow) and *TRPM8*^{EGFPf} (green) epidermal free nerve endings (arrowhead) in fungiform papillae. From Dhaka A. *et al*., 2008.

In addition, promiscuity between the two systems occurs at the chemical detection level. Indeed, TRCs can also be modulated by trigeminal stimulants such as capsaicin, tannic acid and menthol. Capsaicin modulation of TRCs is thought to be mediated by TRPV1t (Lyall et al., 2004).

However, whether capsaicin really affects TRCs remain puzzling from psychophysical studies observations (Green et al., 2005;Green, 1991). It is well documented that the response latency to capsaicin in the tongue is longer than that of the basic taste substances. Once capsaicin stimulates the tongue, the pungent sensation remains for a while and cannot be easily washed out with water. As most of the TRPV1-immunoreactive fibers are found in the lamina propria beneath the trench wall epithelium of the taste papillae (Ishida, 2002), capsaicin can pass through the epithelium with a latency of a few seconds because of its lipophilic nature. Once capsaicin reaches the TRPV1-expressing fibers in the lamina propria mucosae, the larger pungent sensation cannot be washed out with water due to the lingual epithelium barrier. This explanation is however pure speculation but would rationalize the latencies differences observed between taste stimuli and trigeminal stimulants.

Lowering intra-oral pH levels affects trigeminal neurons in addition to TRCs, producing a painful burning sensation (Carstens et al., 1998;Simons et al., 1999). In a similar manner, the tastant NaCl exhibits a dual effect by stimulating the chorda tympani at low concentrations then when concentration increases, it also affects the trigeminal nerve reponse (Carstens et al., 1998;Wang et al., 1993). The activation of somatosensory afferents do not code for taste quality but are rather signalling the presence of noxious stimuli in the oral cavity.

At the level of the brainstem, the integrative properties of the NTS show taste-somatosensory interactions. The firing activity of taste related NTS neurons can be modulated by trigeminal stimulation, as when lingual stimulation by tastants is preceded by capsaicin treatment (Boucher et al., 2003). Another trigeminal stimulant, nicotine, can directly excites NTS units by gustatory nerves and inhibits their tastant-evoked responses by a nicotinic acetylcholine receptor-mediated excitation of trigeminal afferents that inhibit NTS units centrally (Simons et al., 2006).

Connections between taste and trigeminal systems raise the idea that some complex sensations might be encoded by the interplay of the two systems. Using this approach, we explored for the molecular mechanisms of poorly understood chemosensations such as metallic taste.

Results

1. Multisensory attributes of complex tasting divalent salts and artificial sweeteners

1.1 Introduction

Some dietary molecules impart complex taste sensations described at the sensory level with hedonically positive and negative descriptors (Pfaffmann, 1980). Such sensory profiles are reported for artificial sweeteners and divalent salts of metal cations. Their wide range of sensory descriptors may underlie a recruitment of both gustatory and somatosensory systems. A common feature of these compounds is their metallic taste. Whether this sensation may be considered as a taste modality is a matter of debate in the chemical senses field, specifically because it is poorly understood at the molecular level. To shed light on the sensory pathways involved in the sensory profiles of artificial sweeteners and metal salts, it was assessed which receptors participate in their detection through three complementary approaches. In a first step, chemosensory ion channels from the somatosensory system, TRPV1, TRPA1, TRPM8 and TRPV3 were selected to be investigated in their response to the tastants of interest: sweeteners and metal salts. To do so, the recombinant proteins were expressed in an heterologous expressing system being the Human Embryonic Kydney (HEK) 293 cells. The channels level of opening was determined when stimulated with a ligand through calcium and voltage imaging. However, this technique implies that the channel may behave differently that in its native environment. Ion channels are sensitized and regulated by intracellular factors that are not necessarily present in the in-vitro system. In addition, their proper folding in the recombinant system is not quantifiable. Therefore, it was also necessary to study native receptors in their tissue of origin to confirm the proper behaviour of the channel in the heterologous system. This was achieved for the somatosensory channels by using primary cultures of dissociated Dorsal Root Ganglia (DRG) neurons. Finally, a more global picture of the physiological role of the channel was given by behavioural studies involving targeted deletion of the gene in animals. The knockout mice were an efficient way to study an alteration in the taste behavior of wild type mice to the tested compounds.

The first publication “Artificial sweeteners and salts producing a metallic taste sensation activate TRPV1 receptors” (published in the *American Journal of Physiology Regulatory Integrative Comparative Physiology*) describes the agonistic activity of sweeteners and metal salts on TRPV1 both in HEK cells and DRG neurons observed by calcium imaging techniques. In appendix 3, we reported the characterization of the most potent agonist (saccharin) currents obtained by whole cell

electrophysiology. These results are followed by a second publication, submitted to the J*ournal of Neuroscience,* "Sensory attributes of complex tasting divalent salts are mediated by TRPM5 and TRPV1", in which behavioral evidence using mice null for TRPM5, T1R3 and TRPV1 is provided on metal salts recruiting gustatory and somatosensory transduction to mediate their complex sensations. The third paper, published in *Biochemical and Biophysical Research Communications*, "The capsaicin receptor participates in artificial sweetener aversion" describes the behavioral rationalization of the agonistic activity of these molecules on TRPV1, demonstrating the TRPV1 contribution to the aversive taste of these compounds. In addition, it shows that the mode of action of sweeteners on TRPV1 is unclear as they partially stimulate the capsaicin binding site using a combination of calcium and voltage imaging techniques on point mutants of the channel. The procedures to monitor $[Ca^{2+}]_i$ changes in DRG neurons and the brief-access tests are detailed in appendix 1 and 2, respectively. The three described research articles are provided in the following pages.

1.2 Artificial sweeteners and metallic tasting salts stimulate TRPV1

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Artificial sweeteners and salts producing a metallic taste sensation activate TRPV1 receptors

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Riera CE, Vogel H, Simon SA, le Coutre J. Artificial sweeteners and salts producing a metallic taste sensation activate TRPV1 receptors. *Am J Physiol Regul Integr Comp Physiol* 293: R626–R634, 2007. First published June 13, 2007; doi:10.1152/ajpregu.00286.2007.— Throughout the world many people use artificial sweeteners (AS) for the purpose of reducing caloric intake. The most prominently used of these molecules include saccharin, aspartame (Nutrasweet), acesulfame-K, and cyclamate. Despite the caloric advantage they provide, one key concern in their use is their aversive aftertaste that has been characterized on a sensory level as bitter and/or metallic. Recently, it has been shown that the activation of particular T2R bitter taste receptors is partially involved with the bitter aftertaste sensation of saccharin and acesulfame-K. To more fully understand the biology behind these phenomena we have addressed the question of whether AS could stimulate transient receptor potential vanilloid-1 (TRPV1) receptors, as these receptors are activated by a large range of structurally different chemicals. Moreover, TRPV1 receptors and/or their variants are found in taste receptor cells and in nerve terminals throughout the oral cavity. Hence, TRPV1 activation could be involved in the AS aftertaste or even contribute to the poorly understood metallic taste sensation. Using Ca^{2+} imaging on TRPV1 receptors heterologously expressed in the human embryonic kidney (HEK) 293 cells and on dissociated primary sensory neurons, we find that in both systems, AS activate TRPV1 receptors, and, moreover, they sensitize these channels to acid and heat. We also found that TRPV1 receptors are activated by $CuSO_4$, $ZnSO_4$, and $FeSO_4$, three salts known to produce a metallic taste sensation. In summary, our results identify a novel group of compounds that activate TRPV1 and, consequently, provide a molecular mechanism that may account for off tastes of sweeteners and metallic tasting salts.

multisensory taste; pain; calcium imaging

IN MANY FOODS, ARTIFICIAL sweeteners (AS) represent a major dietary supplement. Their consumption is involved with weight management, prevention of dental decay, and for diabetics the control of blood glucose. Saccharin, aspartame, and acesulfame-K are among the most commonly used AS. Cyclamate, although currently not approved for use in the United States, is used in more than 50 countries worldwide. Unlike sucrose, these compounds are not perceived to be sweet at all concentrations. In fact, for all these compounds, as concentration increases, the taste perception shifts from pleasant (sweet) toward unpleasant (bitter/metallic) (10, 12, 41). This shift has been explained by the activation at low concentrations of sweet tastant-sensing G protein-coupled receptors (GPCRs) T1R2/T1R3 (23) and at higher concentrations, the activation of the bitter tastant sensing GPCRs, T2R43, and T2R44 for saccharin and acesulfame-K (21). Other taste sensations related to the aftertaste elicited by artificial sweeteners have been attributed to their diffusion into taste receptor cells where they can alter intracellular signaling pathways (37, 38, 40, 57). A recent psychophysical study showed that it is sometimes difficult to distinguish between the bitterness caused by quinine and the irritating sensation produced by capsaicin, the principal pungent ingredient in chili peppers that activates TRPV1 receptors (24). This result suggests that some of the bitter taste sensation may involve the activation of capsaicin-sensitive TRPV1 receptors.

TRPV1 receptors or their variants are present in taste receptor cells (TRCs) and in sensory neurons in the mouth (15), where they are activated by acidic and thermal stimuli (5, 30, 51), as well as a wide range of molecules that include vanilloids, alcohols, terpenoids, aldehydes, and lipids. Vanilloids include capsaicin from chili pepper, resiniferatoxin from cactus resin (49), zingerone and gingerol from ginger (7, 28), and eugenol from cloves (55). TRPV1 receptors were also found to be activated by nonvanilloid plant-derived molecules like camphor (54), by the fungal extract scutigeral, and by piperine from black pepper (27, 36, 48). Other TRPV1 ligands include ethanol (1–3%), (52), reducing agents such as dithiothreitol (53), and certain 1,4 dialdehydes (e.g., isovelleral) (47). Endogenous TRPV1 ligands include molecules such as anandamide, *N*-arachidonyl dopamine, and eicosanoids (13, 14, 46, 59). Peptide toxins from tarantula venom also activate TRPV1 receptors (45). As the capsaicin binding site is on the cytoplasmic surface of TRPV1 (17, 18), all of these organic molecules must either be synthesized intracellularly or be able to permeate into the lingual epithelia, where they could diffuse into TRCs (40, 57, 58) or nerve terminals (4, 15) and eventually activate TRPV1 receptors.

TRPV1 receptors have also been shown to be directly activated on the extracellular side by high concentrations of Ca^{2+} and Mg^{2+} (1). At the sensory level, the taste perception of these two minerals is very close and was described as primarily bitter with additional sensations such as salty, metallic, astringent, sour and sweet qualities (25, 43). In addition to AS, metallic taste can be strongly elicited by metal salts containing zinc, iron and copper (22, 25, 56). Consequently, we explored whether TRPV1 receptors would be activated by these metallic salts and thus contribute to some of their gustatory sensations.

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To rationalize the off-tastes of the AS, we tested whether they could act on the somatosensory system by targeting TRPV1 receptors. In summary, we found that TRPV1 receptors are activated by both AS and selected metal salts and therefore can have a role in their irritating and metallic aftertaste perceptions.

MATERIALS AND METHODS

Chemicals. Capsaicin, saccharin, aspartame, acesulfame-K, sodium cyclamate (Fig. 1), capsazepine, sucrose, SB-366791, and ruthenium red were obtained from Sigma-Aldrich (Buchs, Switzerland). Stock solutions in DMSO were prepared: 1 mM capsaicin, 33 mM SB-366791, 33 mM capsazepine, and 1 M saccharin, which were kept at 4°C. When added to cells, the final concentration of DMSO was <0.1% (vol/vol) for capsaicin, SB-366791, and capsazepine and was <0.2% (vol/vol) for saccharin. $CaSO_4$, $MgSO_4$, $CuSO_4$, $ZnSO_4$, and $FeSO_4$ were obtained from Sigma-Aldrich. Bitter tasting molecules caffeine, theophylline, salicin, 6-*n*-propylthiouracil, phenylthiocarbamide, quinine-HCl, and β-glucopyranoside, which were also obtained from Sigma-Aldrich, were dissolved in buffer. All molecules were diluted with buffer to their final concentration.

Cloning and expression of human TRPV1, transient receptor potential ankyrin 1, transient receptor potential melastatin 8. A human TRPV1 expression construct was prepared from a cDNA clone obtained from RZPD (German Resource Center for Genome Research, Berlin, Germany). Following the manufacturer's instructions, the gene was cloned into pcDNA5/FRT (Invitrogen, Carlsbad, CA) and confirmed by sequencing. To obtain the insertion of the TRPV1 expression cassette by Flp recombination, HEK 293 cells containing a recombination site (Flp-In system; Invitrogen) were cotransfected with hTRPV1-pcDNA5/FRT and pOG44 using lipofectamine. Stable clones expressing TRPV1 were selected using hygromycin antibiotic selection, and colonies were expanded to obtain a large stock of TRPV1-expressing cells. TRPV1 protein expression was confirmed by Western blot analysis (positive band at 85 kDa). Human transient receptor potential ankyrin 1 (TRPA1) and transient receptor potential melastatin 8 (TRPM8) cDNA clones were obtained from Origene Technologies (Rockville, MD) and were transiently transfected in HEK 293 cells using lipofectamine 2000 (Invitrogen).

HEK cell culture. HEK 293 cells were grown as monolayers in DMEM (Sigma) supplemented with nonessential amino acids, 10% FBS, 2 mM L-glutamine and maintained at 37°C under 95% O_2-5% CO_2. hTRPV1-expressing cells were cultured with the antibiotic hygromycin. Cells were passaged every 3 or 4 days, and the highest passage number used was 14 for TRPV1-expressing cells and 20 for nontransfected cells. Capsaicin sensitivity in hTRPV1-expressing cells was assessed by imaging intracellular Ca^{2+} concentration (see below) and used as a control for TRPV1 expression.

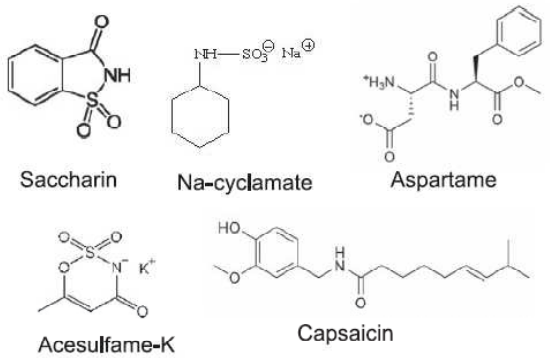

Fig. 1. Chemical structures of the studied artificial sweeteners (AS) diverge from the common vanilloid molecule, capsaicin (Cap). The molecular structure of AS is diverse, comprising dipeptide derivates (aspartame), N-sulfonylamides (acesulfame-K, saccharin), and sulfamate (Na-cyclamate). Capsaicin is a vanilloid (containing the 4-hydroxy-3-methoxybenzyl chemical group). At 25°C, the pK_a values of the corresponding acids are 1.8 for saccharin, 3.2 and 7.9 for the di-acid aspartame, 1.9 for cyclamic acid and 2 for acesulfame.

Measurement of intracellular calcium levels $[Ca^{2+}]_i$ using a fluorescent plate reader. TRPV1-, TRPA1-, TRPM8-expressing cells and nontransfected cells were seeded into 96-well plates (Costar, High Wycombe, UK) coated with poly-D-lysine at a density of ~100,000 cells per well in supplemented DMEM and cultured overnight. Cells were then incubated at room temperature for 20 min with Hank's balanced salt solution (HBSS) supplemented with 2 mM $CaCl_2$ and 20 mM HEPES, containing the cytoplasmic calcium indicator 2 μM Fura-2/AM (Molecular Devices, Sunnyvale, CA) and 0.04% pluronic acid (Molecular Devices). The cells were washed once and resuspended in the same HBSS buffer before being incubated for 25 min at room temperature with either HBSS buffer alone or containing the TRPV1 antagonist capsazepine (10 μM). The 96-well plates were then placed into the plate reader (λ_{ex1} = 340 nm, λ_{ex2} = 380 nm, λ_{em} = 508 nm), and Fura-2 ratios (F340/F380) were recorded to quantify the changes in $[Ca^{2+}]_i$ upon stimulation with AS and metallic salts. Solutions of agonists in HBSS at pH 7.4 were applied at different concentrations after 12 s and were not removed during the recording. The metallic salts acidified the HBSS pH for concentrations superior to 20 mM from pH 7.4 to 5.5.

All experiments were conducted at 37°C except TRPA1 and TRPM8 ligand assays, which were performed at room temperature (22°C), the acid potentiation experiments, which were also performed at 22°C, and the temperature variation experiments (Fig. 4). In the pH variation experiments, the solutions in which pH was adjusted with HCl were applied on the cells immersed in a small volume of buffer at pH 7.4. The final pH of the solutions at 25°C was measured using a pH meter (Methrom, Herisau, Switzerland) in separate vials. In the temperature variation experiments, the agonists' solutions were equilibrated at each temperature and applied on the cells that where incubated in a small volume of HBSS at 22°C.

Responses to molecules were expressed as a percentage of the maximum response evoked by 1 μM capsaicin at 37°C (under these conditions 1 μM was independently assessed to be a saturating concentration). For all experiments, the peak response was taken to be the characteristic value. Data were analyzed using SOFTmax PRO software (Molecular Devices). Dose-response curves and EC_{50} values were calculated using the Hill equation (GraphPad Prism Software, San Diego, CA). The Hill equation was calculated as

$$\%A = \%A_{MIN} + \frac{[\%A_{MAX} + \%A_{MIN}]}{1 + 10^{(\log EC50 - \log C)\cdot n}}$$

where %A is the percentage of activation, C is the concentration of ligand, n is the Hill slope of the curve.

Dorsal root ganglia (DRG) neuron imaging. Dissociated DRG neurons from neonatal (2 or 3 days) rats were obtained frozen in dry ice from Cambrex Bio Science (Walkersville, MD). Cells were thawed and resuspended in neurobasal medium (Invitrogen) supplemented with 2% B27 (Gibco, Gaithersburg, MD), 2 mM glutamine and 100 U/ml penicillin/streptomycin (Sigma). Cells were plated onto coverslips coated with poly-D-lysine (30 μg/ml) and laminin (2 μg/ml) and were cultured with β-nerve growth factor (Sigma) at a concentration of 5 ng/ml. Changes in $[Ca^{2+}]_i$ were measured using ratiometric digital fluorescence imaging (as above). Neurons were loaded at 37°C for 30 min with 5 μM Fura-2-AM and pluronic acid (0.04%) in the supplemented HBSS as above. This was followed by 20-min deesterification of Fura-2-AM in the dark at room temperature. Images of individual neurons were acquired with a cooled, charge-coupled device camera (Cascade II; Photometrics, Tucson,

AZ) mounted to an Axiovert100 inverted microscope. Autofluorescence was negligible and with illumination times of 100–300 ms, F340/F380 remained stable. The projected soma diameters (μm) were measured with a calibrated eyepiece under phase contrast illumination. Traces were recorded from neurons with soma diameters ranging between 18 and 30 μm.

Coverslips with attached neurons were placed in a flow chamber with continuous flow of supplemented HBSS. Chemical stimuli present in HBSS were applied at 30–33°C to the flow chamber for 30 s, and cells were rinsed 2 or 3 min in HBSS between stimuli. For each neuron the average fluorescence ratio F340/F380 was calculated using Metafluor software (Universal Imaging; Molecular Devices). In the SB-366791 antagonism experiments, neurons were first exposed to saccharin (or aspartame) followed by a 2-min wash with HBSS. Subsequently, SB366791 was perfused for 1 min, with saccharin (or aspartame) addition over the last 30 s. After 2-min wash, saccharin (or aspartame) and capsaicin were added sequentially. Neurons were finally washed with buffer, and then KCl (50 mM) was added to verify neuronal activity. Between 225 and 250 neurons were analyzed in 15 separate experiments, and each observation shown in Fig. 5 was made at least in triplicate.

RESULTS

AS activate TRPV1 in a dose-dependent manner but not TRPA1 and TRPM8 receptors. Upon measuring increases in intracellular Ca^{2+}, we found that AS, like capsaicin, activate TRPV1 receptors heterologously expressed in HEK 293 cells (Fig. 2). Such responses are not observed in nontransfected cells (Fig. 2). To ensure that the observed responses were specific to TRPV1 and could not be obtained in other chemosensitive TRP receptors, we recorded from HEK cells with expressed TRPA1 or TRPM8 receptors. As expected, TRPA1- and TRPM8-expressing cells were responsive to their specific ligands, cinnamaldehyde and allyl isothiocyanate for TRPA1 and menthol for TRPM8, respectively (3, 16, 35, 39), but at room temperature they were insensitive to AS (not shown). Moreover, to rule out involvement of bitter taste transduction pathways, we tested 5 mM caffeine, 10 mM theophylline, 10 mM salicin, 1 mM 6-*n*-propylthiouracil, 1 mM phenylthiocarbamide, 0.1 mM quinine-HCl, and 10 mM β-glucopyranoside. None of these bitter tasting molecules elicited a specific response in cells transfected with TRPV1 and only quinine-HCl resulted in equal Ca^{2+} mobilization in both transfected and nontransfected cells (not shown).

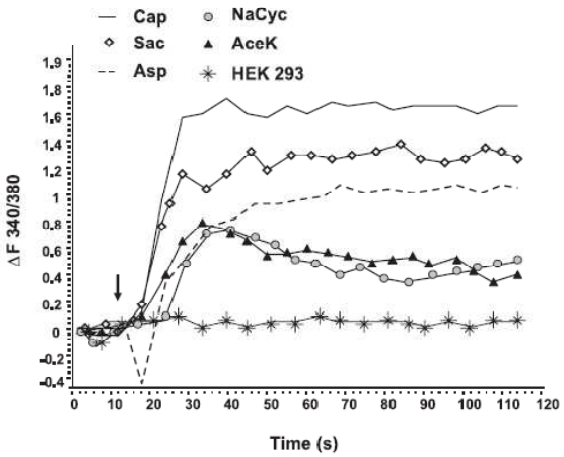

Fig. 2. $[Ca2+]_i$ flux induced by capsaicin and artificial sweeteners stimulation of human embryonic kidney (HEK) cells stably expressing transient receptor potential vanilloid-1 (TRPV1). $[Ca^{2+}]_i$ response induced by 1 μM capsaicin (Cap), 2 mM saccharin (Sac), 8 mM aspartame (Asp), 25 mM sodium cyclamate (NaCyc), and 25 mM acesulfame-K (AceK) were monitored using the FLEX assay in hTRPV1 stably expressing cells. $[Ca^{2+}]_i$ fluxes were measured as changes in fluorescence intensity, before and after the addition of agonists (indicated by arrow). The plotted signal corresponds to the ratio 340/380 nm. None of the tested molecules induced a $[Ca^{2+}]_i$ response in nontransfected cells (HEK 293), or in TRPA1- and TRPM8-expressing cells (not shown) at the used concentrations. One response of HEK 293 cells to 2 mM Sac is shown, the remaining responses were omitted for clarity. Obtained latency values between injection and maximum induced response were (in seconds) Cap (17 ± 1.7), Sac (20.6 ± 3), Asp (32 ± 6), NaCyc (25.3 ± 1.1), and AceK (26.6 ± 3). (n = 3, means ± SE).

Shown in Fig. 2 are typical responses evoked near maximal concentrations of saccharin (2 mM; Sac), aspartame (8 mM; Asp), Na-cyclamate (25 mM; NaCyc) and acesulfame-K (25 mM; AceK). In the TRPV1-expressing cells, the application of 1 μM capsaicin evoked a transient increase in $[Ca^{2+}]_i$ that was characterized by a relatively rapid initial onset and a peak occurring about 16 s after injection. Like capsaicin, the responses to saccharin and aspartame did not markedly desensitize in 30 s, whereas the responses to NaCyc and AceK desensitized. The initial slope of the fluorescence change for the AS is less steep than that found for capsaicin, and their maximal intensities were both smaller than for capsaicin and different from each other. Moreover, all four of the AS tested have a longer onset latency (time between compound injection and maximum induced response) than 1 μM capsaicin.

The initial decrease shown in the response to 8 mM aspartame arose at this high injected concentration by an increase in light scattering caused by small aggregates that reduced the fluorescence. However, the response increased to normal values once the aspartame diffused throughout the chamber. Moreover, at lower concentrations, the transient decrease in light scattering was not observed.

TRPV1-expressing cells respond to all four AS in a dose-dependent manner (Fig. 3*A*). Responses of Sac (0.1–2 mM), Asp (0.5–9 mM), NaCyc (1–25 mM), and AceK (1–50 mM) were measured as the peak increase in fluorescence, expressed relative to the maximum 1 μM capsaicin response. These data were then fitted using the Hill equation. The highest concentration used for each AS was established either because of curve saturation, as observed for Sac, or a TRPV1-independent Ca^{2+} influx (Asp), or by the contribution of the Na^+ or K^+ counter-ions in NaCyc and AceK, respectively. For NaCyc and AceK, the counter-cation influence was assessed using solutions of NaCl and KCl, respectively, having the equivalent Na^+ or K^+ concentrations. Below 25 mM, NaCl did not elicit a Ca^{2+} influx (Fig. 3*A*), whereas 25 mM KCl induced a significant increase in $[Ca^{2+}]_i$. From this result, it is evident that the influence of the K^+ ion in the AceK prevented an accurate calculation of an EC_{50} value.

To characterize further whether TRPV1 is specifically activated by AS, we challenged the AS activation with 10 μM capsazepine (CPZ), a TRPV1-antagonist. We found that AS activation was essentially eliminated in the presence of CPZ (Fig. 3*B*). In the presence of 10 μM CPZ, we found that the response to 1 μM capsaicin was inhibited by about 70%.

We also observed that natural membrane impermanent sweet tastants, such as sucrose at high concentrations (100 mM)

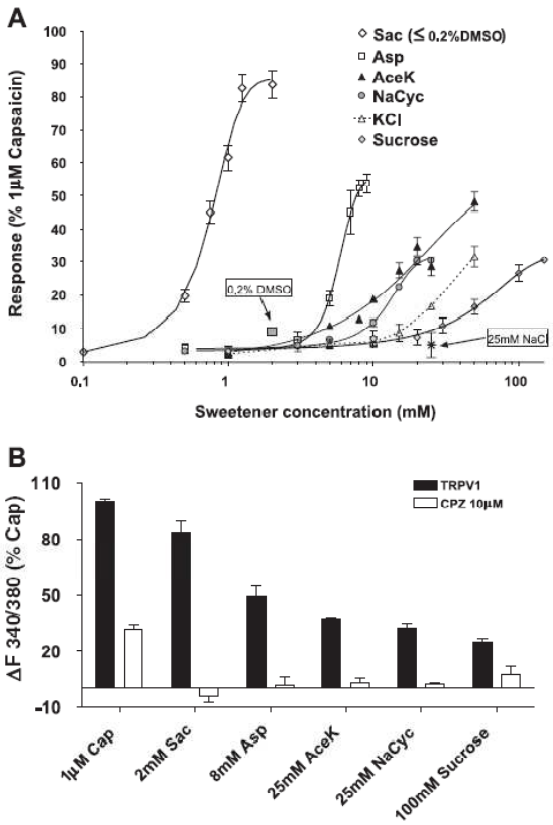

Fig. 3. Artificial sweeteners induce dose-dependent increases in $[Ca^{2+}]_i$ rise in TRPV1 expressing HEK 293 cells. *A*: $[Ca^{2+}]_i$ responses were monitored using Fura-2-AM in hTRPV1 HEK 293 cells before and after the addition of artificial sweeteners and the natural sweet tastant sucrose. Responses of saccharin (Sac; 0.1–2 mM), aspartame (Asp; 0.5–9 mM), Na-cyclamate (NaCyc; 1–25 mM), acesulfame-K (AceK; 1–50 mM), and sucrose (1–150 mM) were measured as a peak increase in fluorescence, normalized responses (means ± SE, n = 3 or 4) were expressed relative to the maximum 1 μM capsaicin response at pH 7.4 and fitted by the Hill equation. Obtained half-maximum activation concentrations were (in mM): EC_{50} (Sac) = 0.8 ± 0.03; EC_{50} (Asp) = 4.6 ± 0.001, EC_{50} (NaCyc) = 15.3 ± 3.2 and EC_{50} (sucrose) = 40 ± 28. Hill coefficients were H (Sac) = 2.04 ± 0.4, H (Asp) = 0.48 ± 0.12, H (NaCyc) = 0.14 ± 0.02 and H (sucrose) = 0.01 ± 0.008. NaCl (1–25 mM) and KCl (1–50 mM) were used to assess the influence of Na^+- and K^+-coupled sweeteners. Note that below 25 mM, NaCl did not induce a rise in $[Ca^{2+}]_i$. Acesulfame-K concentration-dependent increase in $[Ca^{2+}]_i$ was not saturating. At concentrations >25 mM, there is unspecific Ca^{2+} entry due to the K^+ cation, as shown by equimolar KCl response. Saccharin was dissolved in DMSO (final concentration ≤0.2%), which did not elicit a change in Ca^{2+} by itself. *B*: normalized maximum induced responses of the artificial sweeteners and the sweet-tasting molecule sucrose without (solid bars) and with (open bars) pretreatment with 10 μM capsazepine (CPZ). CPZ can selectively block Cap and fully inhibit all sweetener-induced responses. Values are presented as means ± SE; n = 3 or 4.

selectively evoke increases in intracellular calcium in HEK 293 cells with TRPV1 receptors in a dose-dependent manner (Fig. 3*A*). At higher concentrations non-TRPV1 dependent increases were observed (data not shown). These responses may result from changes in osmolality-induced effects on TRPV1 (26, 29, 52). Sucrose response could be inhibited by 10 μM CPZ (Fig. 3*B*).

Artificial sweeteners sensitize the thermal and acid response of TRPV1 receptors. In the absence of capsaicin and in a pH 7.4 buffer, TRPV1 channels are activated at ~42°C (5). In the presence of capsaicin (or at lower pHs), the activation temperature decreases (51). Here, we tested whether AS, like capsaicin, would also increase the thermal sensitivity of TRPV1 (Fig. 4*A*). Saccharin and aspartame were selected to explore their effect on the thermal responses of both transfected (with TRPV1) and untransfected HEK 293 cells, as they are the most potent and structurally different TRPV1 activators (Fig. 3). At

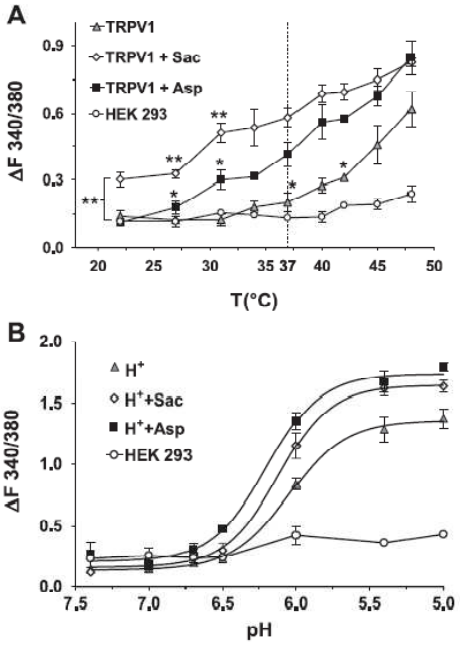

Fig. 4. Saccharin and aspartame shift the threshold and increase the magnitude of heat- and proton-evoked $[Ca^{2+}]_i$ activation in TRPV1-expressing HEK cells. *A*: Ca^{2+} release was induced by a shift of temperature in HEK 293 cells and hTRPV1-expressing cells in the absence or presence of 2 mM Sac or 8 mM Asp. 0.2% DMSO (not shown) had no effect by itself. To quickly shift cells to a desired temperature, buffer heated to a particular temperature, was added to TRPV1 cells incubated at 22°C. Note that both Sac and Asp can lower the threshold of TRPV1 heat activation. Data marked with asterisk and double asterisk indicate significant increase in $[Ca^{2+}]_i$ producing a break in activation ramp ($^*P < 0.05$ and $^{**}P < 0.01$, unpaired t-test). Note that this break shift from 42°C in TRPV1-expressing cells to 31°C when Sac or Asp are added. Vertical line indicates the observed activation at 37°C. Ca^{2+} signaling in nontransfected cells was basal at room temperature and not affected by the temperature changes. Means ± SE (n = 5 or 6). *B*: Buffer with increased acidity (7.4 to 5), adjusted with 1 M HCl was applied either alone or with 2 mM Sac or 8 mM Asp on both TRPV1-expressing cells and HEK 293 cells. One single trace represents the $[Ca^{2+}]_i$ in HEK 293 cells, as the three conditions produced the same fluorescence profile typified by a small increase at pH 6. Concentration-dependent responses were fitted by the Hill equation. Both Sac and Asp increased the efficacy for TRPV1 proton-gated opening by lowering significantly the half-maximal response from pH 6 ± 0.04 to pH (Sac) = 6.13 ± 0.04 and pH (Asp) = 6.2 ± 0.04. Sac and Asp also increase the maximally induced response to 21 ± 3.9 and 27 ± 3.5%, respectively. Hill coefficients were H (H^+) = 2.2 ± 0.63, H (H^+ + Sac) = 2.39 ± 0.43, H (H^+ + Asp) = 2.32 ± 0.37. Values are presented as means ± SE; n = 4.

each particular incubation temperature (22°C to 48°C with intervals of 2–5°C), heated buffer was added to TRPV1 cells to induce a temperature jump. In nontransfected cells with either buffer alone or in the presence of 2 mM Sac or 8 mM Asp, no increase in Ca^{2+} was monitored. In transfected cells, an abrupt increase in Ca^{2+} influx was observed at about 42°C, whereupon it monotonically increased with temperature to 48°C. It is seen that in the presence of saccharin or aspartame, there is no obvious break in the fluorescence-temperature relationship; moreover, at virtually every temperature, including 37°C (vertical dotted line), the fluorescence was greater than it was in the absence of these agonists. These data suggest that, like capsaicin, at physiological temperatures, TRPV1 receptors are sensitized by saccharin and aspartame. As controls, we found no temperature-dependent increase in Ca^{2+} was evoked in nontransfected cells with either buffer alone or in the presence of 2 mM Sac or 8 mM Asp.

Another important activator of TRPV1 are protons. Moreover, in the presence of capsaicin and elevated temperatures, the sensitivity to protons increases (5, 51). To further characterize synergies between AS and other known TRPV1 ligands, we tested the influence of pH on the potency of saccharin and aspartame. Indeed, we found that the addition of 2 mM Sac or 8 mM Asp shifted the half-maximal activation of the pH curve from 6 to 6.13 for 2 mM Sac and from 6 to 6.2 for 8 mM Asp. In addition, AS potentiated the maximum induced pH response and increased the Hill coefficients (Fig. 4*B*).

Sensory neurons are selectively activated by artificial sweeteners. To address whether AS would stimulate native TRPV1-containing cells, we imaged $[Ca^{2+}]_i$ of acutely dissociated DRG neurons in their presence. We choose neurons with small- to medium-diameter somas (<30 μm), as they were likely nociceptors that contain TRPV1 (5). We found that the application of 500 nM capsaicin evoked significant increases in $[Ca^{2+}]_i$ for 89 of the 225 neurons tested (Fig. 5).

As shown in Fig. 5*B*, 2 mM saccharin evoked $[Ca^{2+}]_i$ transients, albeit exclusively in the DRG neurons that were capsaicin sensitive. The capsaicin-insensitive population of neurons only responded to 50 mM KCl (Fig. 5, *A* and *B*). Similar results were found for 8 mM aspartame (Fig. 5*C*). Cells that responded to Sac, Asp, and NaCyc also responded to subsequent application of 500 nM Cap, whereas capsaicin-insensitive cells did not respond to any of these AS (Fig. 5*B*).

The activation of capsaicin-sensitive neurons by capsaicin or AS was blocked by preincubation with 10 μM SB-366791, a highly specific TRPV1 antagonist (9) (Fig. 5, *A–C*). However, after washing SB-366791 away, ligand-evoked responses could be recovered, and moreover, the capsaicin-sensitive neurons were again responsive to subsequent applications of saccharin (or aspartame) and capsaicin (Fig. 5, *B* and *C*). Neurons that did not respond to either sweeteners or capsaicin were found to be responsive to KCl.

The capsaicin-sensitive neurons also responded to AceK (Fig. 5*D*). All neurons responding to KCl were activated by AceK, even those insensitive to capsaicin, which is likely due to the depolarizing activity of K^+. However, capsaicin-sensitive cells responded faster and with a larger amplitude than the other cells, indicating the importance of the acesulfame anion for TRPV1 activation process.

Selected metal salts directly gate TRPV1 expressed in HEK 293 cells. Another category of molecules that exhibit an unpleasant "off-taste" are the salts $CuSO_4$, $ZnSO_4$, and $FeSO_4$ (25). Here, we tested whether like AS, they could activate TRPV1 receptors. All three salts increased $[Ca^{2+}]_i$ levels in TRPV1-expressing cells but did not in nontransfected cells (Fig. 6*A*). The increase in intracellular calcium was not dose-dependent, most likely because these salts will form complexes in solutions like HBSS, and consequently, they do not behave as completely dissociated ions (34). For example, with increasing concentrations, the responses to $CuSO_4$ first augmented,

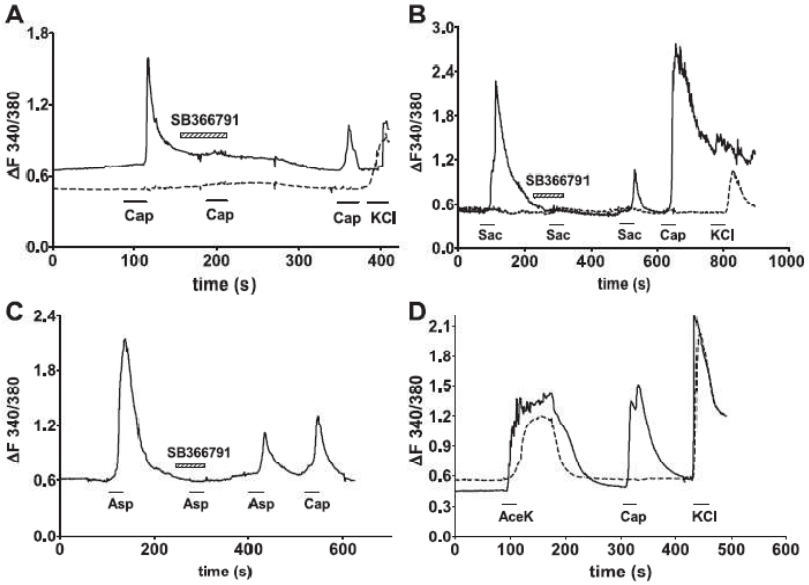

Fig. 5. Artificial sweeteners selectively activate capsaicin-sensitive dorsal root ganglia (DRG) neurons. $[Ca^{2+}]_i$ transients are represented as an increase in fluorescence ratio (F340/F380; basal amplitude = 0.5 ± 0.09) of Fura-2 loaded DRG neurons (225–250 cells from at least three separate cultures were analyzed) evoked by Cap, Sac, Asp, AceK, and NaCyc. Compounds were applied for 30 s, as shown by horizontal bars in the figure, followed a 2-min wash with physiological solution (HBSS). SB366791 applications are shown as indicated by horizontal dashed bars. Successive molecules applications were 500 nM Cap, 10 μM SB366791, 500 nM Cap + 10 μM SB366791, 500 nM Cap and 50 mM KCl (*A*); 2 mM Sac, 10 μM SB366791, 2 mM Sac + 10 μM SB366791, 2 mM Sac, 500 nM Cap and 50 mM KCl (*B*); 8 mM Asp, 10 μM SB366791, 8 mM Asp + 10 μM SB366791, 8 mM Asp, 500 nM Cap, and 50 mM KCl (*C*); 25 mM AceK, 500 nM Cap, and 50 mM KCl (*D*). Typical traces from single neuron represent $[Ca^{2+}]_i$ transients from capsaicin-sensitive (solid line) and capsaicin-insensitive (dashed line) cells.

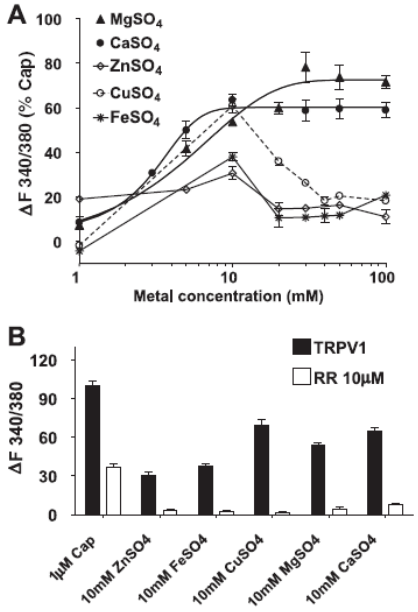

Fig. 6. Multivalent metal salts induce dose-dependent increases in $[Ca^{2+}]_i$ rise in TRPV1 expressing in HEK 293 cells. *A*: $[Ca^{2+}]_i$ responses (% 1 μM Cap) were monitored using Fura-2-AM in hTRPV1 HEK 293 cells before and after the addition of a concentration range (1–100 mM) of $CaSO_4$, $MgSO_4$, $CuSO_4$, $ZnSO_4$, and $FeSO_4$. Note that only $CaSO_4$ and $MgSO_4$ exhibit a dose-dependent effect. The other ions display their maximum activation at 10 mM. None of the metal salts induced a $[Ca^{2+}]_i$ rise in TRPA1 and TRPM8 expressing cells. *B*: Normalized maximum-induced activation of the metals without (solid bars) and with (open bars) pretreatment with 10 μM ruthenium red (RR). RR can selectively block Cap and fully inhibits all metal-induced responses. Values are presented as means ± SE; $n = 3$ or 4.

reached a maximum at 10 mM, and then monotonically decreased. The same pattern was observed for $FeSO_4$ and $ZnSO_4$ even though they induced a smaller Ca^{2+} increase. Using whole cell patch-clamp, Ahern et al. (1) showed in TRPV1 receptors expressed in HEK 293 cells that $CaSO_4$ and $MgSO_4$ salts activate the receptor in a concentration-dependent manner, most likely because these salts are almost completely dissociated. Using measurements of intracellular calcium, we have obtained similar results to Ahern et al. (1) with calcium and magnesium salts (Fig. 6*A*). To further demonstrate that these salts activate TRPV1, we found that they all were completely inhibited by 10 μM ruthenium red, a TRPV1 antagonist (Fig. 6*B*). We also found that with exception of 10 mM $CuSO_4$, 10 μM CPZ also inhibited the responses to the others salts (not shown). Metal salts were also applied on TRPA1- and TRPM8- expressing cells but no response was monitored.

DISCUSSION

The low caloric artificial sweeteners saccharin, aspartame, acesulfame-K, and cyclamate are widely used as a food supplement. However, increasing concentrations of all these compounds shift their sensory attributes from sweet, via activation of T1Rs, to an unpleasant long-lasting taste sensation comprising bitter and metallic components. Although the bitter component can be partially accounted for by the activation of specific T2Rs, the molecular mechanisms of these other taste sensations are poorly understood. To this point, we found that AS and salts that produce a metallic taste are effective agonists for the human TRPV1 receptor. As capsaicin-sensitive TRPV1 receptors are expressed in nociceptive sensory neurons and taste cells, our results not only present a novel class of ligands for TRPV1 channels but also provide a molecular connection that can rationalize the "off tastes" associated with the artificial sweeteners.

Artificial sweeteners activate TRPV1 receptors. We have obtained unequivocal evidence that the four most commonly used AS (saccharin, aspartame, acesulfame-K and sodium cyclamate) activate TRPV1 receptors. In particular, we found all four AS activated TRPV1 receptors in a dose-dependent manner (Fig. 3). In nontransfected HEK 293 cells, AS either did not evoke a response or evoked responses that could be accounted for by an increase in KCl concentration (see below for AceK). Further evidence that AS activate TRPV1 channels is that their responses were largely inhibited by CPZ (Fig. 3*B*). Moreover, like capsaicin, AS were found to sensitize TRPV1 responses to heat (Fig. 4*A*) and acidic stimuli (Fig. 4*B*). Indeed, the sensitization to thermal stimuli suggests that TRPV1 receptors would be active at physiological temperatures in the mouth (34–37°C) (Fig. 4).

In primary sensory neurons, we found that neurons that are responsive to capsaicin (nociceptive) are also responsive to AS (Fig. 6). Moreover, the AS-evoked responses were inhibited by SB366791, a selective TRPV1 antagonist (9).

For heterologously expressed TRPV1 channels, acesulfame-K was able to induce an increase of $[Ca^{2+}]_i$ superior to and independent from the K^+-induced activation (Fig. 3*A*). This behavior correlates well with the Ca^{2+} imaging experiments on DRG neurons in which one subpopulation of neurons was sensitive to capsaicin and Ace-K, whereas the remaining neurons responded only to Ace-K and KCl. One rationalization of these experiments is that increasing KCl will depolarize the neuron and activate voltage-dependent Ca^{2+} channels (VDCC) that are widely expressed in all sensory neurons (20). VDCC typically respond to 50 mM KCl stimulation, which evokes extracellular Ca^{2+} influx.

Do AS diffuse across the plasma membrane to activate TRPV1 receptors? Having established that AS activate TRPV1 receptors, we inquired whether, like capsaicin, the AS bind to an intracellular binding site (17, 18). This is of interest because at pH 7.4, the AS are far from their pK_as and are predominantly in their anionic form (Fig. 1). Therefore, in the absence of any specific transport pathway, AS would be expected to be relatively (to the uncharged form) membrane impermeable because of the large energy barrier involved in transferring a charge from water into the low dielectric environment of a membrane (2).

Recently, Hill and Schaeffer (11) found that trinitrophenol, an anion that does not penetrate HEK 293 cell membranes, activates TRPA1 receptors but not TRPV1 receptors. However, in a series of papers, DeSimone (8) and Naim and colleagues (37, 38, 40) addressed this issue and have demonstrated that at neutral pH, saccharin (presumably in its anionic form) diffuses across liposomes and TRCs, where it accumulates and may affect intracellular pathways. The question of anion transport across membranes remains puzzling (8). However, Thomae et

al. (50), who recently investigated the permeation of weak acids across lipid bilayers, may have provided a rationale for this conundrum. They found that small amphiphilic anions do indeed permeate across bilayers but at a much slower rate than the uncharged form of the acid. They wrote, "The anions, therefore, controlled the total permeation already at 1–2 pH units above their pK_a. These results indicate that in contrast to the expectations of the pH-partition hypothesis, lipid bilayer permeation of an acidic compound can be completely controlled by the anion at physiological pH." In this regard, we note that the pK_as of the AS would be increased by their partitioning into the low dielectric interfacial region of the bilayer (32, 44). Because these results suggest that anions can diffuse into the cytoplasm, they are consistent with an intracellular binding site for the AS. Whether it is the same as that of capsaicin will require further experimentation. How the membrane-impermeable sucrose molecules activate TRPV1 is not at all clear, but one possibility is that at 37°C, where the energy difference between open and closed states is small, the change in osmotic pressure (26) will be sufficient to open the channels. In summary, although TRPV1 channels can be opened by a variety of mechanisms, our data are most consistent with one in which AS activate it via intracellular binding sites.

How the activation of TRPV1 by AS may account for their "off- and aftertastes". From psychophysical studies, it was found that the sensory attributes of AS change and shift from pleasant sweet to an unpleasant bitter and/or metallic taste sensation with increasing concentration. In addition, when AS are compared with sucrose, they exhibit a longer time for the taste sensation to be extinguished, thus giving them a lingering aftertaste. In the following, we discuss the possibilities of how TRPV1 receptors may influence these phenomena.

Chemosensation in the mouth can originate from the activation or inhibition of the corresponding signaling pathways of the TRCs, their associated neurons, and also primary somatosensory neurons expressing TRPV1 receptors in their nerve terminals, which are distributed throughout the oral cavity. Initially, we consider how AS may affect the taste system via transduction pathways in TRCs. The elegant work of Naim and colleagues (40, 57, 58) provides a rationale for the lingering aftertaste of AS that does not require the activation of TRPV1 channels. They showed that AS, like saccharin, can diffuse across the plasma membrane of the TRCs and accumulate, at relatively high concentrations, in the cytoplasm where they may interact with and subsequently delay signal-termination components located downstream of the sweet or bitter responding GPCRs (depending on the concentration). This inhibition in delay in signal termination would therefore extend the taste response, thereby giving rise to the sweet and bitter aftertaste associated with AS. Also, along these non-TRPV1 receptor mechanisms, we note that amphipathic molecules have been shown to open a variety of initially closed ion channels and thus may open channels in TRCs (6, 33). Finally, in *Xenopus laevis* melanophores, it has been shown that saccharin can activate the melatonin receptor, which also is expressed in rat circumvallate papille taste buds (57).

In regard to mechanisms by which AS may activate TRPV1 channels to produce taste sensations, let us now consider the TRPV1 splice variant (TRPV1t) that was identified and characterized by Lyall and colleagues (29–31) in rodent TRCs. These researchers have shown that TRPV1t, like TRPV1, is activated by capsaicin, and temperature and is modulated by ethanol. In addition, upon obtaining whole nerve chorda tympani recordings from rats and wild-type and $trpv1^{-/-}$ mice, they found that TRPV1t activation is involved in the tonic salt response obtained in the presence of inhibitors of ENaCs (e.g., benzamil, amiloride). In this regard, AS activation of TRPV1t could influence the taste sensation in a manner that is dependent on what other taste receptors are present in TRCs containing TRPV1t. To this point, several studies have described the off-tastes of AS as being metallic. Helgren et al. (10) reported that about 25% of a European population characterized an off-taste to saccharin as metallic or bitter. Schiffman et al. (42) found a high variability in the intensity and quality of acesulfame-K that seemed to arise from its bitter and metallic side tastes. Recently, Lim and Lawless (25) characterized metallic taste as the taste of multivalent cations, such as iron, calcium, magnesium, and zinc. This activation by multivalent cations points to a possible role of TRPV1t in metallic taste since Ahern et al. (1) showed that TRPV1 receptors can be activated by high concentrations of calcium and magnesium that bind to sites on the extracellular surface. In this regard, we have found that physiologically relevant concentrations of $CuSO_4$, $ZnSO_4$, and $FeSO_4$ will also activate TRPV1 receptors (Fig. 6*A*) and suggest that the activation of TRPV1t (or other TRP channels in TRCs) by these compounds (as well as Ca^{2+} and Mg^{2+}) could also contribute to metallic taste.

A third candidate AS pathway that would influence taste sensation in a receptor-mediated manner would be TRPV1 channels on the terminals of neurons from the geniculate ganglion. In this regard, using mRNA and immunocytochemistry, Katsura et al. (19) identified TRPV1 channels on the somas geniculate ganglion (GG) neurons. However, it is not known whether these receptors are functional or even on the nerve terminals surrounding TRCs in fungiform papillae. If this were the case, however, then the AS would have to diffuse across the tight junctions between TRCs into the extracellular space between TRCs for them to contact the GG nerve terminals. Interestingly, this may account for the long latency seen with AS.

Our findings that AS activate the somatosensory system through capsaicin-sensitive dissociated primary sensory neurons (Fig. 5) lead to an interesting conclusion: if they can diffuse into the lingual epithelium and activate capsaicin-sensitive trigeminal neurons, then this could contribute to their taste sensation and, moreover, may rationalize the bitter and metallic latencies associated with AS. However, we are unaware of any sensory study describing that AS have a pungent component. Nevertheless, a recent paper from Lim and Green (24) explored at the perceptual level whether bitter and burning/irritating attributes are related. They found, under several conditions, that the bitter taste sensation of quinine and the burning/irritating sensation from capsaicin are extremely similar and even nondistinguishable. We suggest that AS at high concentrations activate T2Rs and TRPV1, which may confuse the sensations. This would provide an explanation for the unclear bitter/metallic aftertaste.

In summary, we found two novel stimulants of TRPV1 receptors: AS and metallic salts. We hypothesize that these interactions may account for of the aversive off-tastes of these compounds.

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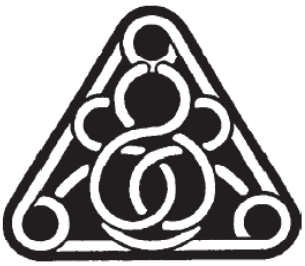

1.3 The taste of metals is mediated by TRPM5 and TRPV1

Sections: Behavioral/Systems/Cognitive; Cellular & Molecular

SENSORY ATTRIBUTES OF COMPLEX TASTING DIVALENT SALTS ARE MEDIATED BY TRPM5 AND TRPV1 CHANNELS

Running title: The taste of metals is mediated by TRPM5 and TRPV1

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ABSTRACT

Complex tasting divalent salts (CTDS) are present in our daily diet contributing to multiple poorly understood taste sensations. CTDS evoking metallic, bitter, salty and astringent sensations include the divalent salts of iron, zinc, copper and magnesium. To identify receptors involved with the complex perception of the above compounds, taste preference tests (two bottle, brief access) were performed in wild type (WT) mice and in mice lacking the T1R3 receptor, the capsaicin receptor TRPV1 or the TRPM5 channel, the latter being necessary for the perception of sweet, bitter and umami tasting stimuli. At low concentrations, $FeSO_4$ and $ZnSO_4$ were perceived as pleasant stimuli by WT mice and this effect was fully reversed in TRPM5 knockout mice. In contrast, $MgSO_4$ and $CuSO_4$ were aversive to the WT mice, but for $MgSO_4$ the aversion was abolished in TRPM5 knockout animals and for $CuSO_4$ decreased in both TRPV1 and TRPM5 deficient animals. Further investigation revealed that the T1R3 subunit of the sweet and umami receptor is also implicated in the hedonically positive perception of $FeSO_4$ and $ZnSO_4$. For several high concentrations of CTDS the omission of TRPV1 reduced aversion. Studies on heterologously expressed channels are consistent with the behavioral studies. Together these results provide a molecular rationalization of the complexity of metallic taste by showing that at low concentrations, compounds like $FeSO_4$ and $ZnSO_4$ stimulate the gustatory system through the hedonically positive T1R3-TRPM5 pathway, and at higher concentrations, their aversion is mediated, in part, by the activation of TRPV1.

INTRODUCTION

Divalent salts such as zinc, iron, copper and magnesium are essential bioavailable elements (O'Dell B.L. and Sunde R.A., 1997) and the molecular mechanisms of their sensory signal transduction remain to be elucidated. All these salts exhibit a complex taste profile. Ferrous sulfate ($FeSO_4$) is the prototypical metallic tasting compound, described as rusty iron, "penny like", fishy and rancid (Civille GV and Lyon BG, 1996;Lawless et al., 2004;Lim and Lawless, 2005b;Lim and Lawless, 2005a;Yang and Lawless, 2005) but is also perceived as astringent/drying, with some sour and bitter attributes (Lawless et al., 2004;Lim and Lawless, 2005b;Lim and Lawless, 2005a). Zinc salts are characterized by astringency with some other qualities such as umami, burning, bitter, sour, salty and metallic (Keast et al., 2003;Yang and Lawless, 2005;Lim and Lawless, 2005a). Copper salts evoke strong bitter, astringent and metallic taste sensations (Lawless et al., 2004;Lawless et al., 2005). Magnesium salts are primarily bitter tasting with additional sensations described as salty, metallic, astringent, sour and sweet (Lawless et al., 2004;Lim and Lawless, 2005b;Yang and Lawless, 2005;Schiffman and Erickson, 1971). Therefore, despite a common metallic denominator, the sensory profiles of the above named divalent salts are complex, vary with the concentration and involve not only taste, but also tactile properties such as astringency and, in the case, of $FeSO_4$, retronasal smell (Lim and Lawless, 2005a;Yang and Lawless, 2005). Whether and how these salts stimulate taste receptors remains unclear, although a recent study has shown that the T1R3 receptor is involved in the detection of Ca^{2+} and Mg^{2+}. In that study, Tordoff *et al.* found that different hedonic values for Ca^{2+} and Mg^{2+} in different strains of mice were related to specific T1R3 haplotypes and that T1R3 knockout mice in a C57BL6 background preferred these two cations whereas they were avoided by their wild type littermates (Tordoff et al., 2008).

In a previous study we found that CTDS activate heterologously expressed hTRPV1, suggesting implication of this channel in the aversive component of their taste (Riera et al., 2007). TRPV1 is a member of the Transient Receptor Potential (TRP) channel family known to be activated by heat, acid, multivalent cations and capsaicin, the primary pungent ingredient in chili pepper (Tominaga et al., 1998;Ahern et al., 2006;Caterina et al., 1997).

The perception of many food borne chemicals is mediated by both the gustatory and somatosensory systems (Simon et al., 2006). The gustatory system detects chemical stimuli dissolved in saliva whereas the somatosensory system detects thermal, mechanical and chemical stimuli many of which, like capsaicin, are irritants. In both systems TRP channels are involved in the transduction pathways. For taste transduction, TRPM5 is a critical element in the downstream signaling of sweet, bitter and umami tastes and TRPM5 deficient mice exhibit complete or markedly diminished

responses to the tastants associated with these sensations (Damak et al., 2006;Zhang et al., 2003). With regard to chemesthesis, polymodal nociceptors in the oral cavity contain several thermally-sensitive TRP channels including TRPV1 (Caterina et al., 1997).

To obtain a better understanding of the pathways involved in the multiplicity of sensations elicited by CTDS, we performed behavioural tests using mice lacking T1R3 receptors, TRPM5 or TRPV1 channels. Our findings indicate that low concentrations of $FeSO_4$ and $ZnSO_4$ are preferred and high concentrations are aversive to C57BL6 mice. We also found that T1R3, TRPV1 and TRPM5 are involved in the transduction of the taste of CTDS.

MATERIALS AND METHODS

Chemicals: Capsaicin, $MgSO_4$, $CuSO_4$, $ZnSO_4 \cdot 1H_2O$ and $FeSO_4 \cdot 1H_2O$ were obtained at chemical grade purity from Sigma Aldrich (Switzerland). Sulfate anions were chosen as a common counter-ion to be coupled to divalent cations of iron, zinc, copper and magnesium because they have little or no impact on the sensory properties of the metals (Lim and Lawless, 2005b;Yang and Lawless, 2005) and they form fewer complex salts than chloride salts (Martell and Hancock, 1989). The range of concentrations and pH values are summarized in table S1.

Expression of human TRPV1 and TRPM5 in HEK293 cells: Human TRPM5 cDNA clone (Origene, USA) in a PCMV-XL6 vector and human TRPV1 cDNA clone (RZPD, Germany) in pcDNA5/FRT (Invitrogen) were transiently transfected in HEK293 cells to monitor voltage changes. For Fura-2 based calcium imaging, TRPV1 was stably expressed in HEK using the Flp-In system (Invitrogen) as previously described (Riera et al., 2007). Cells were grown as monolayers in Dulbecco's minimum essential medium (DMEM, Sigma) supplemented with non-essential amino acids, 10% foetal bovine serum, 2mM L-glutamine and maintained at 37°C under 95%/5% O_2/CO_2. Transfected cells were plated into 96-well plates (Costar, UK) previously coated with poly-D-lysine at a density of 100 000 cells/well and were grown for one day.

Site-directed mutagenesis of TRPV1: Point-mutations Y511A, E600Q and E649A in human TRPV1 homologous to rat Y511A, E600Q and E648A were generated after verifying that these regions were conserved across species. Point-mutants were obtained using the Quick Change Site-Directed Mutagenesis kit (Stratagene) on the hTRPV1 clone. After sequence verification, mutants were transiently expressed in HEK293 cells and the respective sensitivity of Y511A, E600Q and E649A to capsaicin and acidic pH (Jordt and Julius, 2002;Jordt et al., 2000) was verified by calcium and voltage imaging.

Measurement of membrane potential using a fluorescence plate reader: For membrane potential assay, cells at the same density were loaded with a voltage-sensitive dye according to the manufacturer's protocol (Red dye, Molecular Devices). The 96 well plates were then placed into the plate reader to monitor fluorescence intensty (FI) of voltage dye at F530/565 (λ_{ex1}=530 nm, λ_{em}=565 nm) to quantify respectively the changes in $[Ca^{2+}]_i$ and membrane potential upon stimulation with CTDS. Compounds were dissolved in Hank's Balanced Salt Solution (HBSS) supplemented with 2mM $CaCl_2$ and 20mM Hepes buffered at pH 7.4. In TRPM5 assays, calcium was omitted from the HBSS. Experiments were performed at room temperature. For all experiments, calcium fluxes and voltages

changes were measured as increases in fluorescence intensity, before and after the addition of agonists. The fluorescence intensities at the indicated wavelengths were taken to be the characteristic value and were obtained by subtracting the corresponding fluorescence background (value before stimulation). Data were expressed as mean ± S.E.M.

Animals: TRPV1 KO mice (Caterina et al., 2000), were obtained from the Jackson Laboratory (Bar Harbor, ME). TRPM5 KO mice were purchased from Deltagen (San Mateo, CA). The mutation consists of a 174 nt deletion in Exon 14 of TRPM5 corresponding to the second transmembrane domain and some flanking sequence, and introduction of a Neo-LacZ cassette at the site of the deletion. The founder mice were generated in 129/OlaHsd and were backcrossed for six generations with C57BL/6J mice to generate TRPM5 KO mice in 97% C57BL/6J background. The T1R3 KO mice were produced by removing the entire coding sequence of T1R3, which was replaced with a Neo and eGFP cassette. The T1R3 KO mice are in 100% C57BL/6J background.

Two-bottle preference tests: Knockout mice, 10 mice per genotype, and 10 C57BL/6 mice, age and gender matched, males and females, were tested with different compounds. Group 1 (10 TRPV1 KO, 10 TRPM5 KO, 10 WT littermates) was tested with increasing concentrations of $FeSO_4$, $ZnSO_4$, $CuSO_4$ and $MgSO_4$ (see table 1). Group 2 (10 T1R3 KO, 10 WT littermates) was tested with increasing concentrations of $FeSO_4$ and $ZnSO_4$. Mice were caged individually and given access for 48h to two 25-ml bottles, one containing distilled water and the other a tastant solution. After 24h, the bottle positions were switched to control for positional effects (Wong et al., 1996). The volumes of liquid consumed were measured by weighing the bottles. The preference ratio measured as the ratio of tastant volume (V_1) to total liquid consumed (V_t) was recorded. A ratio of 0.5 indicates indifference, a ratio above 0.5 indicates preference and a ratio below 0.5 indicates avoidance. For each tastant, presentation was in ascending concentration. Between tastant trials, the mice were given distilled water for 7 days in two bottles.

Brief-access taste tests: Brief-access taste tests were conducted in a gustometer (Davis MS160-Mouse gustometer; Dilog Instruments, Tallahassee, FL). The training and testing procedures were conducted as described (Damak et al., 2006;Glendinning et al., 2002). The mice were presented alternatively with two bottles, one containing the tastant and the other distilled water for 30 minute testing periods. Each time the mouse licks, it starts a 5 second trial after which the shutter is closed and the other bottle is presented. The number of licks per trial is recorded. Three groups of 15 naïve mice each were used: TRPV1 KO, TRPM5 KO and wild-type C57BL6 age and gender matched controls. Mice that did not get accustomed to the gustometer were excluded from the study. Mice were

tested with four concentrations of $FeSO_4$, $ZnSO_4$, $CuSO_4$ and $MgSO_4$. The lick ratio was calculated as the mean number of licks per trial for the tastant (L_1) divided by the mean number of licks per trial for tastant plus mean number of licks per trial for water (L_t).

Statistical analysis: The preference ratios for the two-bottle preference test and the short access test and the volumes consumed in the two-bottle preference test were analyzed using the general linear model repeated measures of the statistics package SPSS with tastant concentration as within subject factor and genotype as between subject factor. Significance of the between-subject factor indicates difference in the responses between genotypes, whereas significance of the within-subject factor for a given genotype indicates either preference and/or avoidance of certain concentrations of the tastant. A p value of less than 0.05 was considered significant. When a significant difference was found between genotypes, the Tukey test was used to determine which genotypes differ. For biphasic tastant responses, the preferred and aversive concentrations were grouped and analyzed separately using the General Linear Model as described above. For compounds with border line values ($0.05<p<1$) we analyzed each concentration separately with the General Linear Model and used the Bonferroni correction to correct for multiple tests on related data sets.

RESULTS

The cellular responses to Complex Tasting Divalent Salts (CTDS) are mediated by TRPM5 and TRPV1- dependent pathways

To determine the contribution of TRPM5 and TRPV1 to the taste preferences of CTDS, we performed behavioral tests with WT, TRPM5 and TRPV1 KO mice. The CTDS tested include several concentrations of $FeSO_4$, $ZnSO_4$, $CuSO_4$ and $MgSO_4$. These concentrations covered a broad range and were chosen based on concentrations used in human psychophysical studies (Lim and Lawless, 2005a;Lim and Lawless, 2005b;Yang and Lawless, 2005).

Two-Bottle preference test:

$FeSO_4$ & $ZnSO_4$

WT mice exhibited biphasic response profiles to $FeSO_4$ and $ZnSO_4$, that is, these salts were preferred over water at low concentrations and were avoided at high concentrations (Figs 1A, B). Biphasic effects to a tastant can occur if different pathways are activated as a function of tastant concentration (Kawamura et al., 1968;Simon et al., 2006) and are described for many different tastants (Pfaffmann, 1980). For the CTDS we assume that the biphasic response involves at least two hedonically positive and negative pathways. The WT mice preferred these salts over water up to 30 mM for $FeSO_4$ and 10 mM for $ZnSO_4$ with preference ratios (PR) reaching 0.8 for either salt. Over a 48 hour period, this preference corresponded to an intake of 7 mL $FeSO_4$ and 10 mL for $ZnSO_4$ (see supplementary Figure S2). These two salts provoked aversive responses at concentrations higher than 50 mM. The highest concentrations tested, 250 mM $FeSO_4$ and 100 mM $ZnSO_4$, were strongly aversive (PR=0.15 ± 0.05) and (PR=0.2 ± 0.1) respectively. Over this 48 hour period, this corresponded to an intake of 1.1 mL $FeSO_4$ and 1.4 mL for $ZnSO_4$ (see supplementary Figure S1).

For the TRPM5 KO mice, at all concentrations where the two salts could be distinguished from water the PR was less than 0.5 and therefore hedonically negative and globally significantly different from the PR of WT mice ($p<0.0001$). Even at the higher concentrations (> 50 mM) that were aversive to WT mice, the tastants were more aversive to the TRPM5 KO mice than to the WT animals. For these high concentrations, the TRPM5 KO mice consumed less than 1 mL of the tastant over two days. These data indicate the presence of at least two pathways contributing to the transduction of the taste of $FeSO_4$ and $ZnSO_4$, one of which being TRPM5 dependent.

We next inquired whether in TRPM5 knockout mice the residual aversion to $FeSO_4$ and $ZnSO_4$ could be, at least in part, caused by the activation of TRPV1 channels. To answer this question, we compared the PRs from WT and TRPV1 KO mice. For the preferred concentrations of both metals, these KO mice behaved similarly to the WT mice whereas at high concentrations of $FeSO_4$ (> 30 mM $FeSO_4$) the TRPV1 KO mice showed a reduced aversion compared to WT mice ($p<0.01$, Figure 1A). At the highest $FeSO_4$ concentration tested (250 mM), the TRPV1 KO mice ingested on average 1.4 mL versus 1.1 mL for the wild type, whereas for $ZnSO_4$ the TRPV1 KO ingested on average 1.9 mL versus 1.4 mL for the wild type. There was no significant difference ($p>0.05$) between TRPV1 KO and WT mice for the aversive concentrations of $ZnSO_4$.

$CuSO_4$ and $MgSO_4$

Unlike the responses to $FeSO_4$ and $ZnSO_4$, the responses of WT mice to $CuSO_4$ and $MgSO_4$ are monotonically aversive (Figs 1 C, D). For concentrations > 0.01 mM, all mouse strains exhibited an aversive response to $CuSO_4$. The statistical significance of the difference between TRPV1 KO and WT mice is borderline ($p=0.05$), whereas the PRs for the TRPM5 KO mice were indistinguishable from WT animals ($p>0.05$). However, there was a significant decrease in aversion between TRPM5 KO or TRPV1 KO mice versus WT animals for 10 mM $CuSO_4$ ($p < 0.0001$). At the highest concentrations tested, all mice showed a similar pronounced aversion with a PR<0.1 and ingested only small volumes (< 1 mL) of $CuSO_4$ (Figure S2). In summary, the elimination of TRPM5 or TRPV1 did not substantially alter the negative hedonic responses to $CuSO_4$. Because $CuSO_4$ is an emetic (Makale and King, 1992), the remaining aversion in the knockout mice could be caused by post ingestive effects.

The responses of TRPM5 KO mice to $MgSO_4$, for concentrations > 50 mM revealed a markedly decreased aversion to this compound ($p<0.01$). In contrast the responses of the WT and TRPV1 KO's were similar or not statistically different over all concentrations. In summary, the hedonically negative component of the taste of $MgSO_4$ is mediated by a pathway containing TRPM5, but not TRPV1.

Brief-access Test

Since the behavioral responses in the 2-bottle preference tests (TBP) may be partly caused by post-ingestive cues, and some of the compounds tested have emetic properties such as $FeSO_4$, $ZnSO_4$ and $CuSO_4$ (Reissman et al., 1955;Wang and Borison, 1951;Barceloux, 1999;Makale and King, 1992) we carried out brief access tests (BA) to reduce the post-ingestive component of the behavioral test. Brief access tests were performed in naïve animals presented with four to five concentrations that were selected based on the results of the TBP test. To assess the sensory contribution of increased

acidity, we plotted the pH values of the solutions on the graph (also reported in Supplementary Table 1).

$FeSO_4$ and $ZnSO_4$

Consistent with the results of the TBP test, biphasic responses to $FeSO_4$ and $ZnSO_4$ were also observed in the brief access tests (Fig. 2A, B).

$FeSO_4$ was preferred by WT mice at concentrations up to 100 mM and avoided at 250 mM. Knocking out TRPM5 completely eliminated the preference for $FeSO_4$ and increased the aversion for 250 mM $FeSO_4$ ($p<0.001$ compared to WT mice). The responses of WT and TRPV1 KO mice to $FeSO_4$ were not distinguishable ($p= 0.78$).

WT mice preferred $ZnSO_4$ at concentrations up to 50 mM and were indifferent to this compound at 100 mM. TRPM5 KO mice were indifferent to all concentrations of $ZnSO_4$ up to 50 mM and avoided it at 100 mM ($p<0.05$ compared with WT mice). The responses of the TRPV1 KO and WT mice were statistically indistinguishable.

$CuSO_4$ and $MgSO_4$

Consistent with the TBP test, the responses of WT mice to $CuSO_4$ and $MgSO_4$ were found to be aversive for all concentrations greater than 0.1 mM for $CuSO_4$ and 1 mM for $MgSO_4$ (Fig 2C, D). Knocking out TRPM5 reduced the aversion for both salts without completely abolishing it ($p<0.05$ comparing WT and TRPM5 KO). The responses of the TRPV1 KO and WT mice were indistinguishable.

Comparison of 2-bottle preference (TBP) and brief access (BA) tests:

$FeSO_4$ and $ZnSO_4$

The results of the two behavioral tests are in general agreement. That is, both the TBP and the BA tests revealed a biphasic response pattern of WT mice to $FeSO_4$ and $ZnSO_4$ (Figs 1A, B, and 2A, B). The PR maxima occurred at a lower concentration in the TBP than in the BA test (10 versus 50 mM, respectively). Furthermore, the TRPM5 KO mice avoid lower concentrations of these metals in the TBP test but are indifferent to them in the BA test. The most likely explanation for these observations is the added contribution to aversion of the post-ingestive effect, which could be for example a digestive malaise that discourages the mice from drinking the CTDS solution.

The response to $FeSO_4$ of TRPV1 KO mice was significantly different from that of WT mice when measured by TBP test but only a trend was observed in the BA test. This discrepancy is most probably

due to a difference in the experimental method used; in our experience we have found the TBP test more robust in revealing small differences between groups (Damak *et al*. unpublished data).

$CuSO_4$ & $MgSO_4$

The general shape of the concentration-response curves was similar for the TBP test and the BA test for $CuSO_4$ and $MgSO_4$. For $CuSO_4$ the responses of the TRPM5 KO mice were overall significantly different from the WT in the brief access test ($p<0,05$) whereas in the TBP only 10 mM gave a significant difference. This could be explained by a large post-ingestive component in the TBP test that masked relatively smaller differences due to taste. The responses of TRPV1 KO mice were statistically different from the WT in the TBP test ($p=0.05$) but not in the BA ($p>0.05$), although there was a trend for a difference in the BA test. These discrepancies can be explained by differences between the tests used. For $MgSO_4$ both tests are in agreement, highlighting that the aversive response can be fully accounted for by the TRPM5 component. No shift was observed in the PR-concentration profile between the two tests, suggesting that suppression of the post-ingestive component did not alter the preference.

T1R3 is involved in the transduction of the taste responses to $FeSO_4$ and $ZnSO_4$

A subset of TRPM5 expressing cells also expresses T1R3, which is implicated in the transduction of the hedonically positive responses to sweet and umami compounds (Damak et al., 2003;Zhao et al., 2003). Therefore, we investigated whether T1R3 is involved in the transduction of the preferred component of the tastes of $FeSO_4$ and $ZnSO_4$.

T1R3 KO mice were essentially indifferent to concentrations of $FeSO_4$ and $ZnSO_4$ that are preferred by WT mice, and showed a greater aversion to higher concentrations than WT mice (Fig. 3, $p<0.0001$ for $FeSO_4$, $p<0.05$ for $ZnSO_4$). Compared to the TRPM5 KO mice, the T1R3 KO mice showed a trend for less aversion to $FeSO_4$ and $ZnSO_4$ ($p=0.085$ and $p=0.08$, respectively). Therefore, a T1R3 containing pathway accounts for most of the preference imparted by these salts, but a T1R3-independent pathway must also be present.

Effects of CTDS on heterologously expressed hTRPM5 channels

Previously we showed that heterologously expressed hTRPV1 in HEK293 cells was activated by $FeSO_4$, $ZnSO_4$, $CuSO_4$ and $MgSO_4$ (Riera et al., 2007). To determine if CTDS also directly activate TRPM5, we expressed hTRPM5 in HEK293 cells and measured evoked changes in voltage in response to CTDS. TRPV1 transfected HEK293 cells were used as positive control and non transfected HEK293 cells as negative control. Upon stimulation by CTDS, hTRPV1 expressing cells (positive control)

evoked large changes in voltage. Depolarizations were also observed in calcium free HBSS, consistent with previous results showing that HEK293 cells expressing TRPV1 can be depolarized by the influx of divalent cations through the TRP channel (Ahern et al., 2005;Riera et al., 2007).

The response of non-transfected HEK293 cells to CTDS was weak and varied depending on the particular salt tested. At 10mM, $FeSO_4$ evoked a biphasic response - a transient depolarization followed by a small hyperpolarization - whereas $ZnSO_4$ evoked a transient hyperpolarization followed by a long depolarization, $CuSO_4$ evoked a slow depolarizing response and a small hyperpolarization was evoked by $MgSO_4$. The origin of these responses is unknown but could in part reflect responses to changes in volume or complexes of the divalent cations with chloride that could permeate the cell (Gutknecht, 1981).

In TRPM5 transfected cells, the responses to $ZnSO_4$ and $CuSO_4$ were virtually identical to the responses of non-transfected cells (Fig. 4B, C); $FeSO_4$ and $MgSO_4$ evoked marginally larger hyperpolarizing responses than found in non-transfected cells (Fig. 4A, D). No difference in response was observed when 2 mM extracellular calcium was added to the medium for both transfected and non-transfected cells (data not shown). Thapsigargin (5 μM) induced a large and transient increase in fluorescence, which is consistent with an endogenous calcium release activating TRPM5 leading to depolarization of the cell (see supplementary figure S4). The response to thapsigargin shows that heterologously expressed TRPM5 is functional. In summary, the results from these experiments indicate that these salts, should they get to the basolateral side of taste cells where TRPM5 is located (Kaske et al., 2007), unlikely directly activate TRPM5. In this regard, direct activation of the TRPM5 channel is unlikely to account for the behavioral changes seen with these compounds.

Different CTDS stimulate different binding sites in TRPV1

Since TRPV1 governs the behavioral responses to CTDS we explored how this channel may be activated by them. TRPV1 possesses multiple ligand binding sites; among them an intracellular vanilloid pocket (Jordt and Julius, 2002) and an extracellular proton sensitive region (Jordt et al., 2000). Point mutations of these critical residues make TRPV1 channels insensitive to capsaicin (Y511A) or decrease the responses to acidic stimuli (E600Q and E648A) (Fig. 5A). To determine the specificity of the stimulation produced by CTDS we explored which residues among these characterized binding sites were participating in their detection (Fig. 5). As both calcium and magnesium salts stimulate rat TRPV1 channels by direct activation through E600 and by sensitization through E648 (Ahern et al., 2005), we measured how these mutations would affect CTDS responses.

Figure 5 shows typical responses of CTDS in cells transiently expressing the mutant and the wild-type TRPV1 channel proteins. For non-mutated hTRPV1 all four CTDS (green) evoked rapid increases in fluorescence that either reached a plateau or declined slowly ($MgSO_4$). For E649A channels (red) the responses to zinc, copper and magnesium sulfates were significantly decreased compared to the wild type controls. When stimulated with ferrous sulfate, E649A channel showed a maximal fluorescence intensity response very similar as the wild-type channel, but contrary to it rapidly desensitized (Fig. 5A). Compared to the wild-type, E600Q (grey) channels show no responses to $ZnSO_4$, $CuSO_4$ and $MgSO_4$. The capsaicin insensitive mutant Y511A (blue) retained full ability to respond to these divalent salts.

DISCUSSION

The sensory characteristics of complex tasting divalent salts (CTDS) are of general interest due to their use as food supplements and as important nutrients in the diet. In contrast to tastants like sucrose that evoke a single taste quality, CTDS exhibit a multiplicity of taste descriptors, suggesting that several pathways are involved in elaborating their sensation. Moreover, the sensory profiles of two of these compounds change with concentration: they comprise hedonically positive phases at low concentrations and hedonically negative at high concentrations. Here we have shown that $FeSO_4$, $ZnSO_4$ exhibit biphasic responses with an hedonically positive phase mediated by a pathway containing T1R3 and TRPM5 at low concentrations, and a hedonically negative TRPM5-independent and TRPV1-dependent phase at high concentrations. The primarily bitter tasting compounds $MgSO_4$ and $CuSO_4$ exhibit monotonic hedonically negative responses mediated by TRPM5 and TRPV1 pathways. The summarized findings in Figure 6 indicate that tastants with complex descriptors activate several pathways and that particular sensation is critically dependent on the concentration.

The taste of CTDS has multiple components and depends on concentration

To identify some of the molecular pathways of CTDS taste transduction, we selected four divalent salts to be evaluated by WT and KO mice defective in their sensory perceptions, either lacking gustatory (TRPM5 and T1R3) and/or somatosensory (TRPV1) elements. A detailed illustration of the effects on these components is available in Figure S1 for all CTDS.

Our data show that $FeSO_4$ and $ZnSO_4$ present biphasic response profiles in both the TBP and BA tests (Figs. 1 and 2). Their PR-concentration profiles are comprised of a slowly increasing hedonically positive phase and a more rapidly increasing hedonically negative phase. The hedonically positive component arises mostly from the interaction of $FeSO_4$ and $ZnSO_4$ with a T1R3-TRPM5 mediated pathway. In T1R3 or TRPM5 KO animals the loss of these pathways completely suppresses the hedonically positive phase (Figs. 1A, B and 2A, B). As opposed to $FeSO_4$ and $ZnSO_4$, TRPM5 contributes to the hedonically negative response to $CuSO_4$ and $MgSO_4$. Consistent with our findings, previous work reported that the glossopharyngeal nerve responses to $MgSO_4$ are reduced in TRPM5 KO mice compared to WT animals (Damak et al., 2006).

We also found that TRPV1 contributes to the aversive response to $FeSO_4$ and $CuSO_4$ since knocking out TRPV1 increases the PR for these two salts. TRPV1 contribution to the behavioral data is likely to come from its expression in somatosensory nociceptors and/or, from its expression in aversive TRPM5 cells as TRPV1t (Lyall et al., 2004;Lyall et al., 2005b;Lyall et al., 2005a). Bitter, salty, sour and umami compounds are avoided by mice at high concentrations, and this aversion is not

always accounted for by the receptors and pathways known to be involved in the transduction of those tastes. For example, TRPM5 KO mice respond to bitter and umami compounds at high concentrations (Damak et al., 2006), and P2X2/P2X3 double knockout mice avoid citric acid despite a total lack of response to this compound from their taste nerves (Finger et al., 2005). These residual responses could be partly accounted for by TRPV1, as is the case for metals.

Taken together, our data show that there is a TRPM5- and TRPV1-independent pathway of unknown identity for the signal transduction of the four salts tested. The pH of the solutions (table S1 and Fig. 2) may play a role by modifying the response of receptors and channels to the tastants or by activating ASICs and/or TRPV1 in nociceptors (Waldmann et al., 1997;Tominaga et al., 1998). Sour taste could contribute to the response to the highest concentrations of $FeSO_4$ as mice avoid solutions of HCl with pH≤ 3 (Wong et al., 1996) possibly mediated by presynaptic (type III) taste cells (Tomchik et al., 2007;Huang et al., 2008).

Post-ingestive cues likely contribute to the aversion that mice showed to all the salts we tested. Salts of iron, zinc and copper have strong emetic properties and the ingestion of large amounts of these compounds by the mice is likely to cause gastro-intestinal malaise (Wang and Borison, 1951;Reissman et al., 1955;Barceloux, 1999;Makale and King, 1992). The contribution of the post-ingestive effect is observed when we compare the results of the BA test (free of post-ingestive contribution) with those of the TBP test (which includes a post-ingestive contribution). In the BA test, aversion in all three strains of mice tested is less intense and is observed at higher concentrations.

The complex effects of the CTDS observed in mice are a function of concentration and pH (Figs. 1, 2 and table S1) but these components have not been investigated in human psychophysics where often single concentrations are chosen. As CTDS solutions are frequently evaluated at high concentrations, (Yang and Lawless, 2005;Lim and Lawless, 2005b;Keast et al., 2004), where the pH is quite acidic (Table S1) this component may explain some of the descriptors (sour, astringent) associated to the salts. CTDS evoke similar biphasic taste responses as described for NaCl where the hedonically positive phase is taste related and the hedonically negative arises from the activation of nociceptive sensory neurons (Kawamura et al., 1968;Pfaffmann, 1980).

Taste cells and receptors encoding the taste of CTDS at the periphery

Bitter, sweet, and umami tastes are assumed to be encoded at the periphery by discrete subpopulations of taste cells expressing specific receptors (Zhang et al., 2003). Based on this model,

our results together with those reported in the recent publication of Tordoff *et al*. (Tordoff et al., 2008) suggest that many subgroups of both taste receptor cells (TRCs) and receptors are implicated in the transduction of the taste of CTDS. 1) A sub-population of taste cells that express T1R3, respond to magnesium and calcium and are proposed to be wired to the taste areas in the brain that underlay aversion. There are indications that receptors for sweet and umami are heterodimers of T1R3-T1R2 and T1R3-T1R1, respectively, and T1R3 alone responds very weakly to sweeteners (Zhao et al., 2003). Furthermore some taste cells express T1R3 but not T1R1 nor T1R2 (Nelson et al., 2001). Therefore, it is conceivable that the cells responsive to Mg^{2+} and Ca^{2+} express another receptor that dimerizes with T1R3 to form an Mg^{2+} and Ca^{2+} responsive receptor. The partner of T1R3 in such proposed heterodimer is unlikely to be T1R2 or T1R1 since activation of T1R3 heterodimers containing these receptors leads to preference. Tordoff *et al*. suggested that the T1R3 partner might be the calcium sensing receptor CaSR. 2) A sub-population of taste cells that express T1R3, respond to $FeSO_4$ and $ZnSO_4$ and are presumably wired to the areas in the brain underlying preference. The partner of T1R3 in those cells could be either or both T1R2 and T1R1 or another GPCR. 3) A sub-population of taste cells that does not express T1R3 but expresses TRPM5 and is responsible for the preference for Mg^{2+} and Ca^{2+} and part of the preference for Fe^{2+} and Zn^{2+}. This is based on the observations that T1R3 KO mice prefer Mg^{2+} and Ca^{2+} (Tordoff et al., 2008) the preference being masked in WT animals by the aversive contribution of T1R3 expressing cells, and that T1r3KO mice are less aversive to Fe^{2+} and Zn^{2+} than TRPM5 KO animals. Based on the latter observation, we assume that these cells express TRPM5 also because TRPM5 KO mice are indifferent to concentrations of Mg^{2+} preferred by T1R3 KO mice. 4) A sub-population of cells that do not express TRPM5, respond to $FeSO_4$, $ZnSO_4$ and $CuSO_4$ and lead to aversion. In addition, TRPV1 in nerve terminals and possibly TRPV1t in taste cells, but also tactile cues and astringency independent of taste cells play a role in the aversive taste of $FeSO_4$ and $CuSO_4$.

Is metallic taste a unique taste quality?

Our findings suggest that the T1R3 dependent preference for $FeSO_4$ and $ZnSO_4$ could be mediated through activation of T1R1 and/or T1R2 expressing cells. Under this scenario the particular taste of metals is an integration of many modalities, in the same way as umami taste was believed to integrate salty and sweet (Yamaguchi, 1979;Yamaguchi S and Takahashi C, 1984). Psychophysical studies have shown that in addition to their metallic or astringent qualities, $ZnSO_4$ and to a lesser extent $FeSO_4$, evoke savory (*umami*) oral sensations (Keast et al., 2004;Yang and Lawless, 2005;Stevens et al., 2006). Therefore, low concentrations of these salts could produce a 'umami-like' taste that would offset any hedonically negative component and thus explain the preference that we observed for these two salts in mice. The additional taste components beside *umami* that contribute to the taste of CTDS may explain why these salts can be distinguished from other tastants.

It is also possible, alternatively, that the T1R3-dependent taste response to metals underlies a unique taste quality (described in human psychophysical studies as metallic), which would be encoded by a discrete set of taste receptor cells coexpressing T1R3 and (an)other unknown GPCR(s). For the T1R3-dependent aversive taste of Mg^{2+} and Ca^{2+}, this receptor is not T1R1 or T1R2, as discussed above. For $FeSO_4$ and $ZnSO_4$, although it is conceivable that the partner of T1R3 is T1R1 or T1R2, it is also possible that it is another, yet undiscovered receptor. In summary, depending on the concentration salts such as $FeSO_4$ and $ZnSO_4$, may activate several pathways and produce complex taste sensations. In this regard they might be thought of as a mixture rather than a pure component.

Heterologously expressed TRPM5 and TRPV1 channel responses provide information on the molecular mechanisms of CTDS sensory properties.

Several lines of evidence show that divalent salts of Fe^{2+}, Cu^{2+}, Zn^{2+} permeate cells through active and passive transmembrane transport (Powell et al., 1999;Gutknecht, 1981) and can also pass through tight junctions having a selective permeability to divalent cations (Tang and Goodenough, 2003;Ikari et al., 2004). In the taste bud, tight junctions act as a protective barrier of the TRCs and play an important role in salt taste (Elliott and Simon, 1990;Simon, 1992).

We investigated whether these salts would stimulate basolaterally expressed TRPM5 in TRCs. We considered this a possibility since these salts activate heterologously expressed TRPV1 from the extracellular side in TRCs' membranes or tight junctions. However, we found that CTDS did not directly activate heterologously expressed TRPM5 channels in HEK cells (Fig. 4) suggesting that this mechanism is unlikely to occur *in vivo*. These results are consistent with the behavioral data showing a role for T1R3 in the preference for CTDS. Together, these data suggest that TRPM5 is activated by calcium release from stores following activation of PLCβ2 by the action of Fe^{2+} or Zn^{2+} ions on T1R3, not through direct interaction between metal ions and TRPM5.

In addition, consistent with the role of TRPV1 as a sensor of aversive compounds, we found that CTDS are agonists for heterologously expressed TRPV1. Their effects occur via electrostatic interaction with the glutamates E600 and E649 identified as proton binding residues (Jordt et al., 2000) as previously observed in the direct gating by Mg^{2+} and Ca^{2+} (Ahern et al., 2005). Interestingly, we found no TRPV1 contribution to the behavioral response to $MgSO_4$ despite its established role in pain via TRPV1 receptors (Ahern et al., 2005), likely because $MgSO_4$ unlike the other hard divalent salts has a limited diffusion into the epithelium (Powell et al., 1999;Tang and Goodenough, 2003). These results confirm the role of TRPV1 as a sensor of $FeSO_4$, $CuSO_4$ however the agonistic activity of

$MgSO_4$ and $ZnSO_4$ on heterologous TRPV1 *in vitro* does not appear to be physiologically relevant for perception.

Summary

This study provides a molecular rationalization of the sensory properties of divalent salts where TRPM5, T1R3 and TRPV1 are the main molecular targets of these compounds. Other T1R3-, TRPM5- and TRPV1-independent pathways also exist and encode preference for $FeSO_4$ and $ZnSO_4$ and aversion for the four CTDS. Taken together, these components may rationalize the complex metallic taste.

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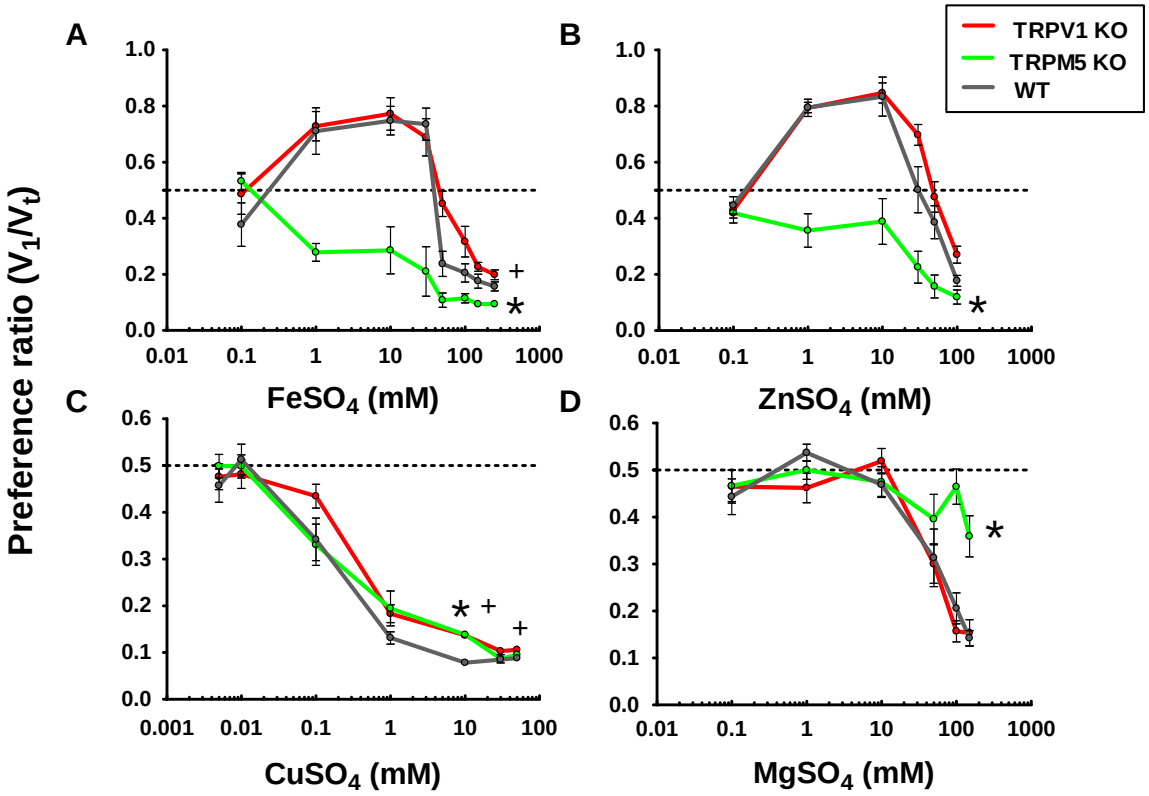

Figure 1. Mean preference ratios from 48-hour two bottle tests (tastant versus water) comparing the responses of TRPV1 KO, TRPM5 KO and wild-type mice to CTDS.

The mice were given two bottles, one containing water and the other a tastant solution. The ratio of tastant drank (V_1) to total liquid consumed (V_t) was measured and compared between groups. Preferences ratio of TRPV1 KO, TRPM5 KO and wild-type mice to (A) $FeSO_4$, (B) $ZnSO_4$, (C) $CuSO_4$ and (D) $MgSO_4$. TRPV1 KO and WT mice show strong preference for $FeSO_4$ and $ZnSO_4$ that is absent in the TRPM5 KO mice. TRPV1 KO mice show a small but significant decrease in aversion to iron and zinc sulfate compared to WT mice. For each group, n=10. Error bars are the SEM. * indicate that the global response of the TRPM5 KO mice are significantly different from those of WT animals, $^+$ indicates that the response of TRPV1 KO mice at the aversive concentrations are significantly different from those of WT animals. The dashed line indicates the indifference line.

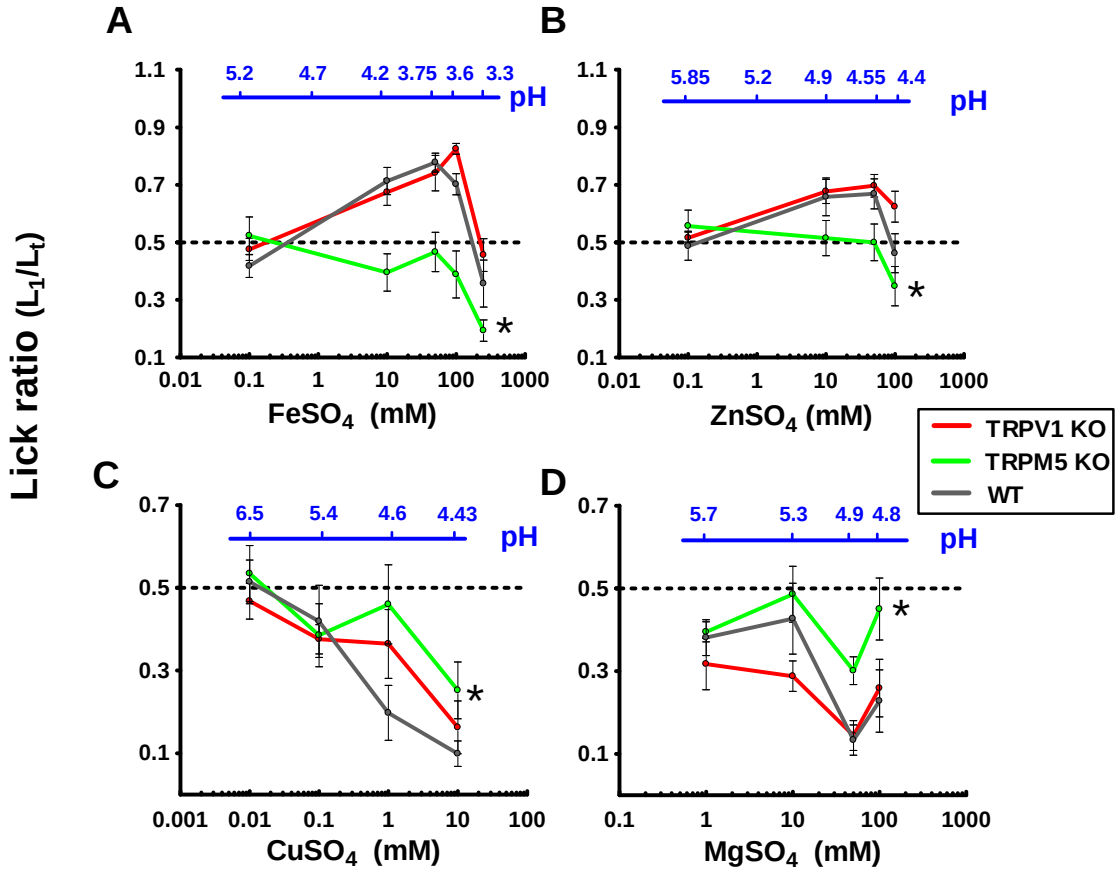

Figure 2. Brief-access lick ratios (tastant versus water) comparing the responses of TRPV1 KO, TRPM5 KO and wild-type mice to CTDS.

The mice had 5s access to alternatively presented water or tastant. The lick ratio of tastant (L_1) to total volume consumed (L_t) is plotted as a function of concentration and the corresponding pH is indicated above the curve. Lick ratios of TRPV1 KO, TRPM5 KO$^-$ and wild-type mice for (A) $FeSO_4$, (B) $ZnSO_4$, (C) $CuSO_4$ and (D) $MgSO_4$. Similar preference curves to those of the 48-hour two bottle tests are obtained, with aversion appearing at higher concentrations in the brief access test. For each group, n=10. Error bars are SEM. * indicate that the global response of the TRPM5 KO mice are significantly different from those of WT animals, $^+$ indicates that the response of TRPV1 KO mice at the aversive concentrations are significantly different from those of WT animals.

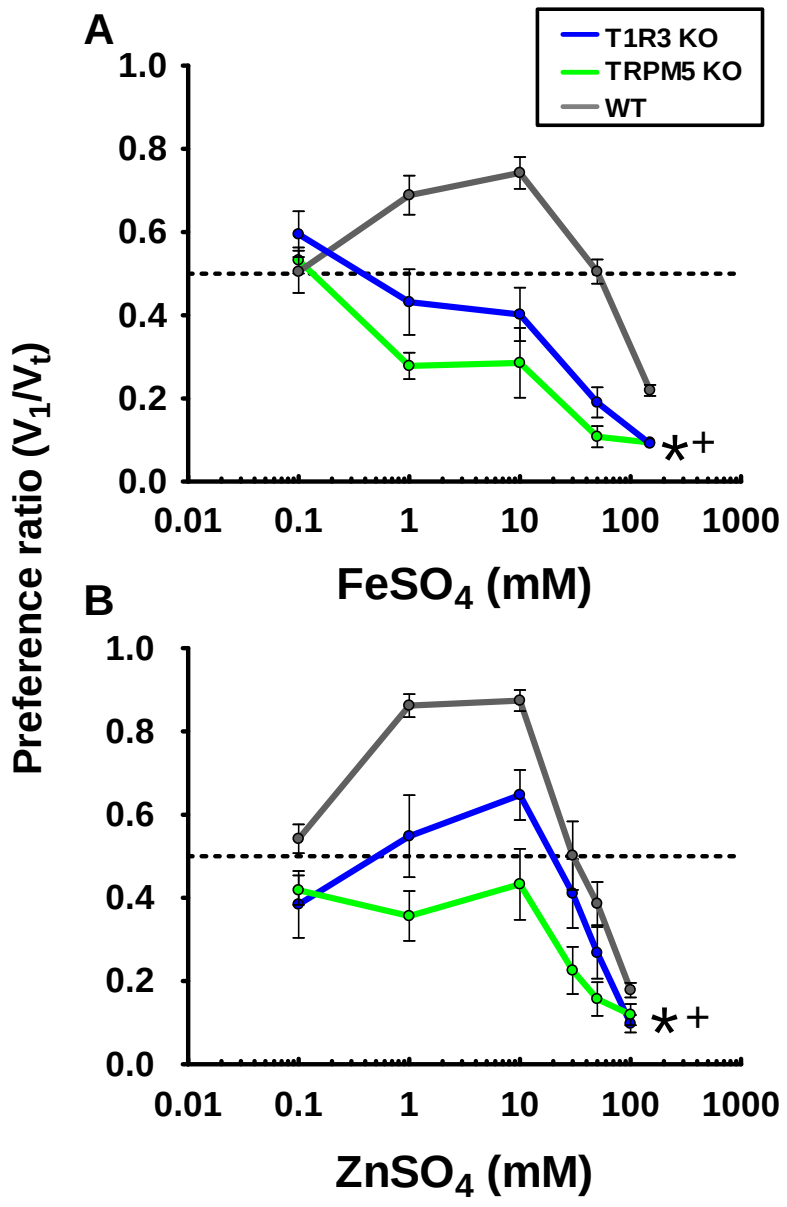

Figure 3. Mean preference ratios from 48-hour two bottle preference tests of responses of T1R3 KO, TRPM5 KO and wild-type mice to Iron sulfate and Zinc sulfate.

The ratio of tastant drank (V_1) to total liquid consumed (V_t) was measured and compared between wild-type, and T1R3 KO mice for (A) $FeSO_4$ and (B) $ZnSO_4$. Plotted together on the graph for comparison is the preference of TRPM5 KO mice for these two salts shown in Fig. 1. T1R3 KO mice are essentially indifferent to concentrations of $FeSO_4$ and $ZnSO_4$ that are preferred by wild type mice. For each group, n=10. Error bars are the SEM. * and $^+$ indicate that the global responses of the TRPM5 KO and the T1R3 KO are significantly different from those of WT mice, respectively.

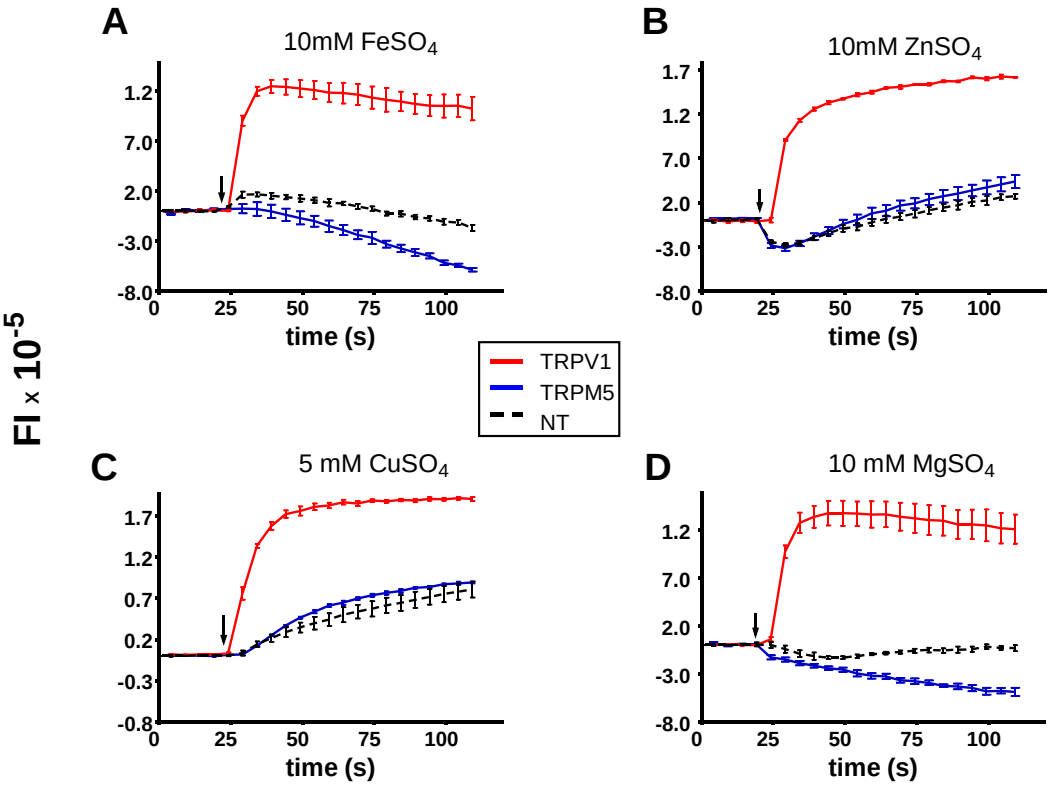

Figure 4. CTDS do not stimulate TRPM5 channels directly

Voltage changes of HEK293 cells loaded with Red dye after stimulation with (A) 10mM $FeSO_4$, (B) 10 mM $ZnSO_4$, (C) 5 mM $CuSO_4$ and (D) 10 mM $MgSO_4$, expressed as fluorescence intensity (FI) versus time. Non-transfected cells (dotted line) and transiently transfected cells with hTRPM5 (blue) and hTRPV1 (red) are shown. All CTDS activate TRPV1 expressing cells, but have little or no effect on TRPM5 expressing or non transfected cells.

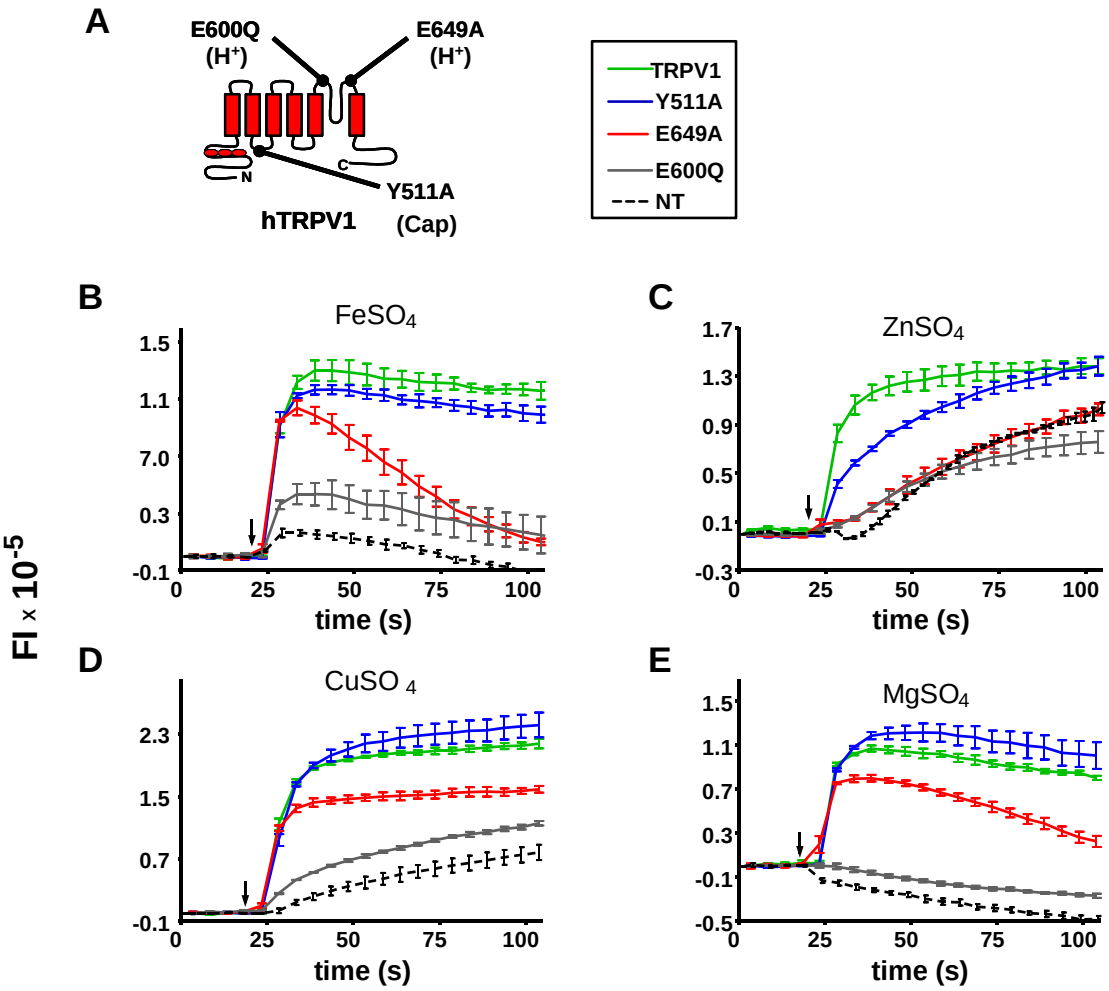

Figure 5. TRPV1 point-mutants in the proton binding site are defective in their response to CTDS. HEK 293 cells expressing the TRPV1 point mutants Y511A (blue), E649A (red), E600Q (black), the wild type TRPV1 (green) and untransfected cells (NT, dashed line) were stimulated with CTDS and voltage changes in the cells were monitored using Red dye. Wild type TRPV1 and the capsaicin-insensitive Y511A were highly responsive to these stimuli, whereas E649A and E600Q, the mutants involved in proton response showed decreased responses to these stimuli. Typical responses to the different CTDS are shown: (A) 10mM $FeSO_4$, (B) 10 mM $ZnSO_4$, (C) 5 mM $CuSO_4$ and (D) 10 mM $MgSO_4$. Means ± S.E.M. (n=3).

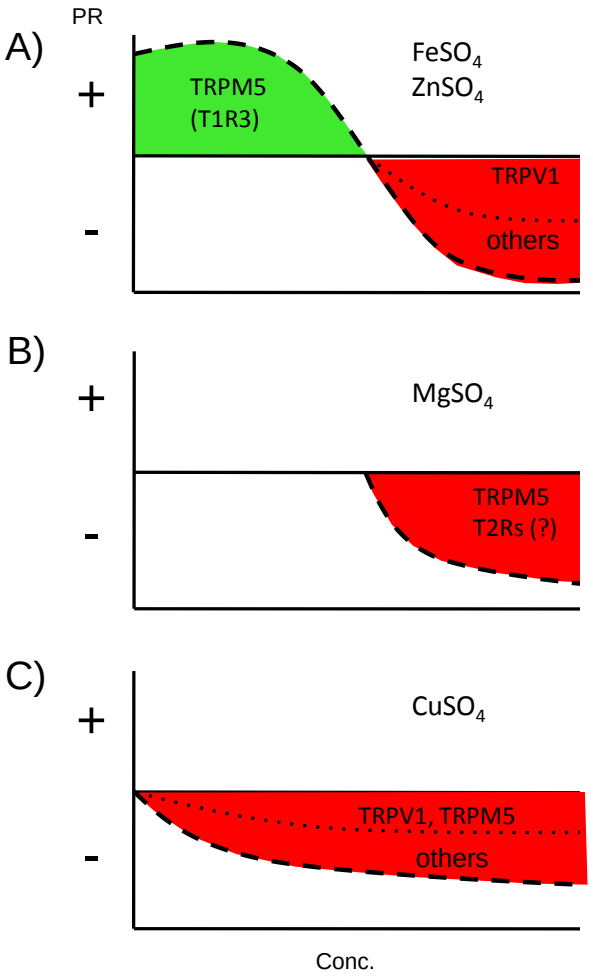

Figure 6. Pathways involved with gustatory and somatosensory perception of CTDS in wild-type mice. Behavioral profiles integrating two bottle preference and brief access tests. Both iron and zinc salts are able to elicit a clear preference behavior up to a concentration range between 50 and 100 mM with contributions from TRPM5 and T1R3 containing signal transduction chains (A, green). At elevated concentrations these salts become aversive, which is driven partly by TRPV1 (red). Conversely, magnesium and copper salts do not induce a preference behavior and while copper salts are aversive across the entire concentration range tested (C) this becomes apparent for magnesium between 10-100 mM.

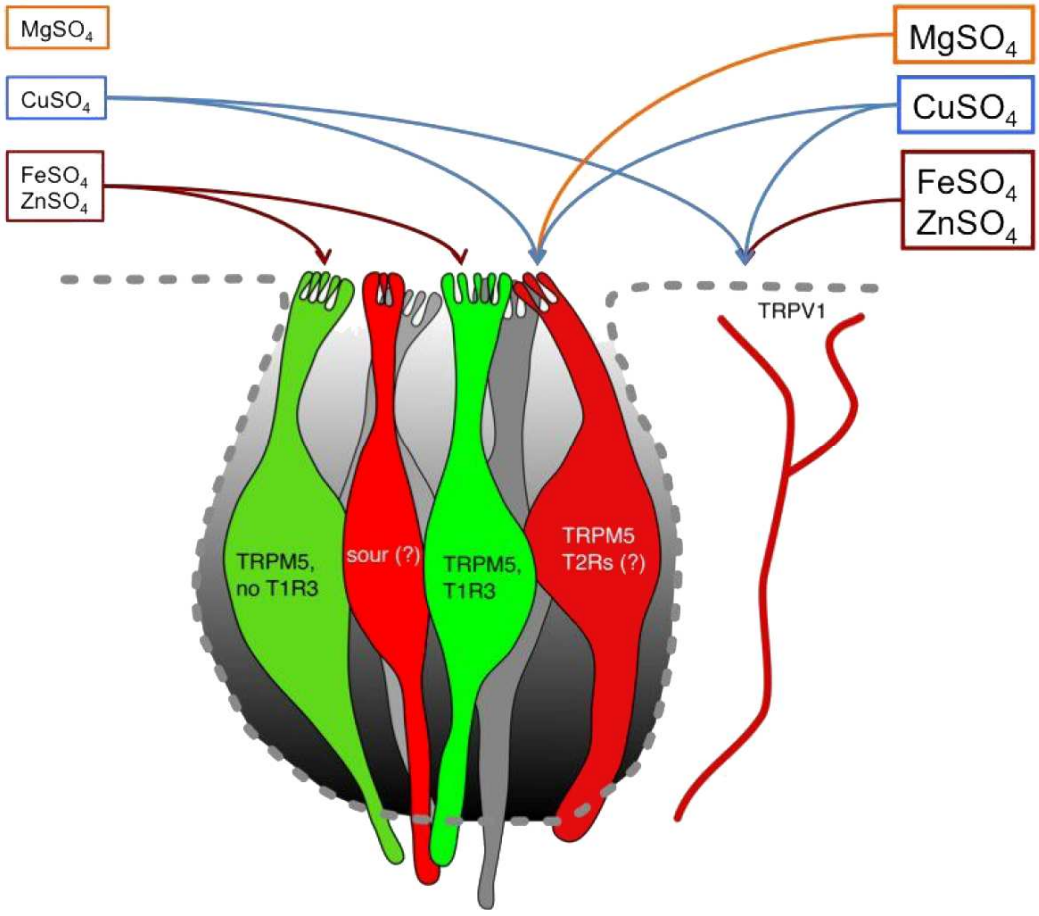

Figure 7. Coding scheme at the periphery integrating gustatory and trigeminal stimuli to explain CTDS hedonic responses. Gustatory cells in the taste bud (grey) segregate in cells imparting preference behavior (green) and avoidance (red). Based on the results of this study hedonically positive cells occur at least in two subpopulations both expressing TRPM5 but only one expressing TRPM5 together with T1R3. Hedonically negative avoidance behavior in part is triggered through activation of another subset of TRPM5 expressing cells, possibly these cells also express T2R bitter taste receptors. Another subset of hedonically negative cells might be expressing sour taste receptors. Another subset of hedonically negative cells might be expressing sour taste receptors and these cells might become activated at 29 high and acidic concentrations of CTDS. Free nerve endings in the taste epithelium of TRPV1-expressing sensory neurons convey hedonically aversive sensations. To explain the switch from preference to avoidance behavior at increasing concentrations the ratio of cells activated changes from a majority of *preference* cells to a majority of *avoidance* cells. CTDS listed at the left in small letters indicate low concentrations and those to the right indicate high concentrations.Free nerve endings in the taste epithelium of TRPV1-expressing sensory neurons convey hedonically aversive sensations.

	2-bottle preference		*Brief-access*		*2-bottle preference*	
	P (TRPM5 KO vs WT)	**P (TRPV1 KO vs WT)**	**P (TRPM5 KO vs WT)**	**P (TRPV1 KO vs WT)**	**P (T1R3 KO vs WT)**	**P (T1R3 KO vs TRPM5KO)**
$FeSO_4$	0.0001	0.009 (50-200 mM)	0.001	0.499	0.0001	0.085
$ZnSO_4$	0.0001	0.229 (30-100 mM)	0.054	0.25	0.012	0.08
$CuSO_4$	0.327 (0.1-50 mM)	0.055 (0.1-50 mM)	0.044	0.759	ND	ND
$MgSO_4$	0.006	0.94	0.022	0.596	ND	ND-

Table 1. Summary of statistical analysis of behavioral data.

The general model for repeated measurements analysis was used to compare the responses of TRPM5 KO, TRPV1 KO, T1R3 KO and WT with tastant concentrations as dependant variables and genotype as a fixed factor. For each compound, the responses for all concentrations were analysed together. The Tukey's test was used to determine which of the means differed when a difference was found. For comparisons of TRPV1 KO versus WT mice, only the results of the aversive concentrations are shown. P values are given for the comparison between two genotypes. ND, not done.

Supplementary information

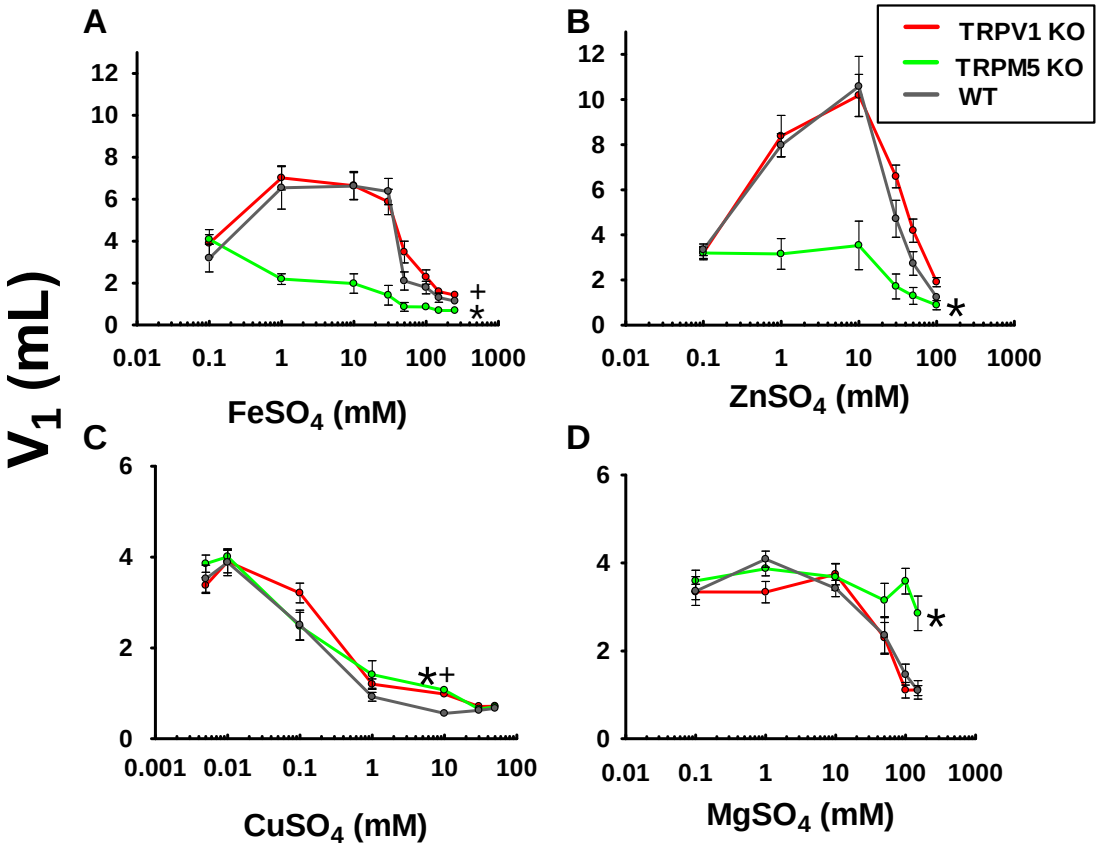

Figure S1. Mean volumes of metallic tasting salts consumed by TRPV1$^{-/-}$, TRPM5$^{-/-}$ and wild-type TRPM5 littermates during 48h two-bottle preference tests.

Drinking volumes (mL) are represented for each genotype as a function of concentration for each genotype. Tastants presented were A) ferrous sulfate, B) zinc sulfate, C) copper sulfate and D) magnesium sulfate. These volumes correspond to the volume of tastant consumed over 48 hours (V_1). For each group, n=10. Error bars are SEM. * and $^+$ indicate that the response of the TRPM5$^{-/-}$ and the TRPV1$^{-/-}$ are significantly different from the WT, respectively.

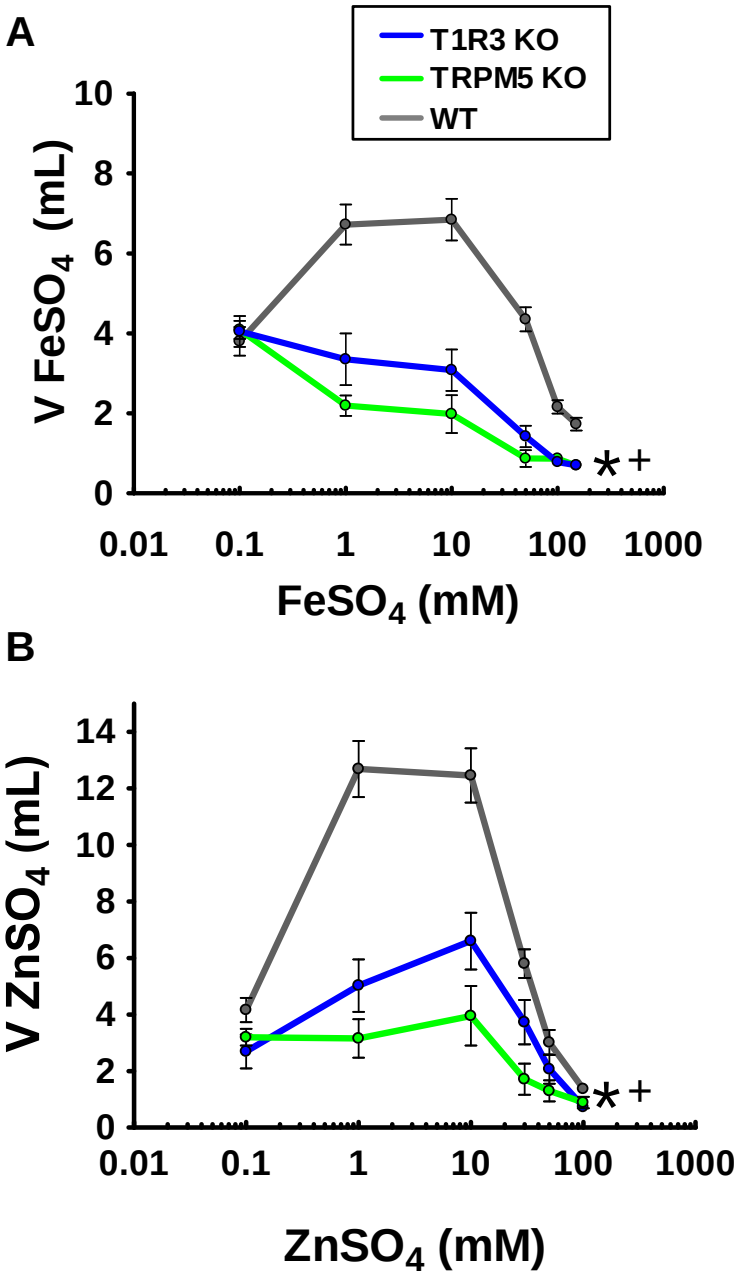

Figure S2. Mean volumes of ferrous sulfate and zinc sulfate consumed by T1R3 KO, TRPM5 KO and wild-type mice during 48 hour two-bottle preference tests.

Drinking volumes (mL) are represented for each genotype as a function of concentration. Tastants presented were A) ferrous sulfate and B) zinc sulfate. These values correspond to the volume of tastant consumed over 48 hours (V_1). For each group, n=10. Error bars are SEM. * and $^+$ indicate that the response of the TRPM5 KO and the T1R3 KO are significantly different from the WT, respectively.

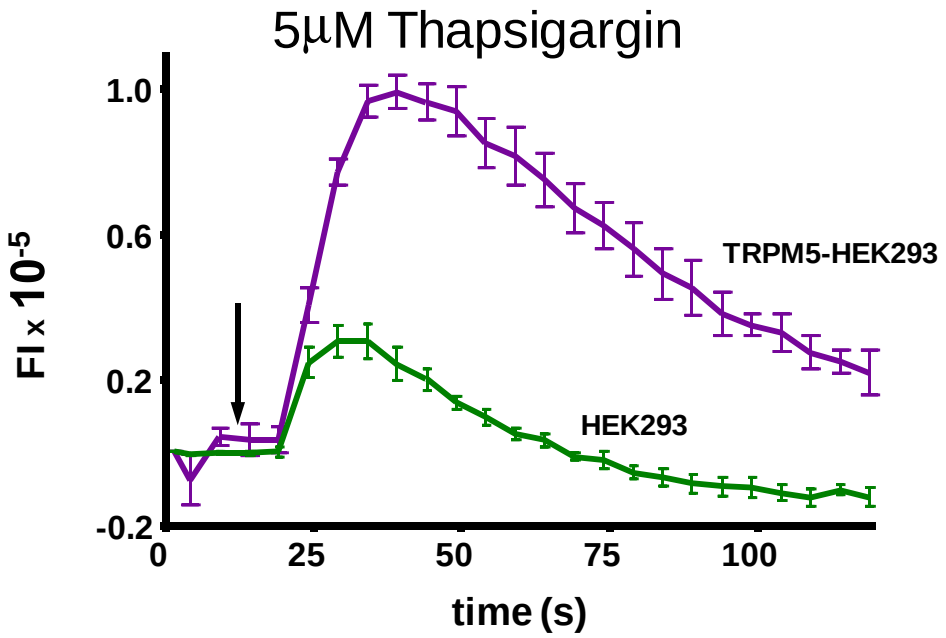

Figure S3. TRPM5 expressing cells respond to stimulation with thapsigargin.

HEK 293 cells were transfected with hTRPM5 (purple) and compared with untransfected cells plated at the same density. Voltage changes of these cells loaded with Red dye expressed as fluorescence intensity (FI) were measured in a calcium free buffer following stimulated with 5µM thapsigargin. TRPM5 sodium fluxes are observed when free endogenous calcium is released from internal stores. Thapsigargin depletes intracellular calcium stores that will indirectly trigger a robust TRPM5 stimulation. This increase is moderately observed in the untransfected cells. values are means ± S.E.M. (n=3).

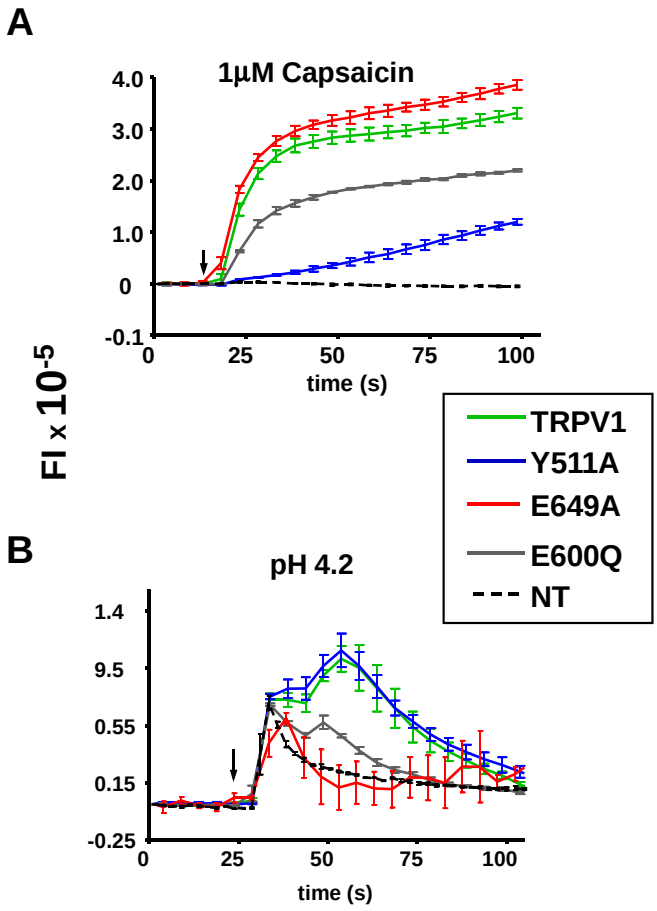

Figure S4. TRPV1 point mutants Y511A, E600Q and E648A responses to capsaicin and acidic pH differ from the wild-type channels.

HEK 293 cells expressing one of the three point mutants, WT TRPV1 and untransfected cells were stimulated with 1µM capsaicin and acidified HBSS (pH 4.2) to monitor voltage changes in the cells using Red dye. Wild type TRPV1 was highly responsive to these stimuli, whereas capsaicin response was absent in the Y511A and proton responses were drastically decreased in E649A and E600Q. Values are means ± S.E.M. (n=3).

	0.1	*1*	*10*	*50*	*100*	*150*	*200*	*250*
$FeSO_4$	5.2	4.7	4.2	3.75	3.6	3.45	3.4	3.3
$CuSO_4$	6.5	5.4	4.6	4.43	-	-	-	-
$ZnSO_4$	5.85	5.2	4.9	4.55	4.4	4.3	4.2	4.1
$MgSO_4$	6	5.7	5.3	4.9	4.8	4.56	-	-

Table S1. pH values of the solutions used in the behavioral tests. pH values highlighted in green correspond to PR >0.5 and in red to PR<0.5 in the two-bottle preference test.

1.4 TRPV1 mediates aversive responses of artificial sweeteners

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The capsaicin receptor participates in artificial sweetener aversion

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ABSTRACT

Artificial sweeteners such as saccharin, aspartame, acesulfame-K, and cyclamate produce at high concentrations an unpleasant after-taste that is generally attributed to bitter and metallic taste sensations. To identify receptors involved with the complex perception of the above compounds, preference tests were performed in wild-type mice and mice lacking the TRPV1 channel or the T1R3 receptor, the latter being necessary for the perception of sweet taste. The sweeteners, including cyclamate, displayed a biphasic response profile, with the T1R3 mediated component implicated in preference. At high concentrations imparting off-taste, omission of TRPV1 reduced aversion. In a heterologous expression system the Y511A point mutation in the vanilloid pocket of TRPV1 did not affect saccharin and aspartame responses but abolished cyclamate and acesulfame-K activities. The results rationalize artificial sweetener tastes and off-tastes by showing that at low concentrations, these molecules stimulate the gustatory system through the hedonically positive T1R3 pathway, and at higher concentrations, their aversion is partly mediated by TRPV1.

The sensory characteristics of artificial sweeteners (AS) are of general interest because of their use in food. In contrast to sugars like sucrose, which have a single taste descriptor, AS have more than one taste descriptor, suggesting several different pathways are involved in their taste transduction. Moreover, for many of these compounds the sensory profiles change with concentration, to the extent that they are comprised of hedonically positive components at low concentrations and hedonically negative ones at high concentrations. Unpleasant taste arises from the aftertaste of AS [1]. The non-sugar sweeteners saccharin (Sac), acesulfame-K (AceK), aspartame (Asp), and cyclamate (NaCyc) display complex sensory properties that have been partially explained. As concentration increases, their taste perception shifts from pleasant (sweet) towards unpleasant (bitter/metallic) [2–4]. This shift has been explained by activation of the heterodimeric G-Protein Coupled Receptors (GPCRs) T1R2/T1R3 at low concentrations of sweet tastants [5] and at higher concentrations of Sac and AceK, the activation of the bitter tastant sensing GPCRs, T2R43, and T2R44, respectively [6]. However, neither of these gustatory mechanisms rationalize the metallic "off taste" of AS. Thus, in an effort to understand the transduction pathways for the aversive tastes of AS we previously found that these compounds activate TRPV1 in capsaicin sensitive DRG neurons and HEK cells heterologously expressing TRPV1 in with a solid pharmacology [7]. These results suggest a putative implication of TRPV1 in the aversive aftertaste.

To obtain a better understanding of the pathways involved in the multiplicity of sensations elicited by sweeteners, we performed behavioral tests using knock-out mice lacking TRPV1 channels or T1R3 receptors. At low concentrations AS are preferred through a T1R3 mediated component and increasing concentrations are aversive to C57BL/6J mice. The suppression of TRPV1 reduced the aversive response to AS. To summarize, non-sugar sweeteners are detected by the gustatory system via a T1R3-dependent pathway and a T2R and TRPV1 mediated component is recruited in the aversive response to these molecules.

Materials and methods

Chemicals. Capsaicin (Cap), sodium saccharin (Sac), aspartame (Asp), acesulfame-K (AceK), and sodium cyclamate (NaCyc) were obtained at chemical grade purity from Sigma–Aldrich (Switzerland) for the behavioral experiments. For calcium and voltage imaging, similar compounds were used except sodium saccharin

Abbreviations: TRP, transient receptor potential; TRPV1, transient receptor potential vanilloid 1; T1R3, taste receptor, type 1, member 3; T1R2, taste receptor, type 1, member 2; AS, artificial sweeteners; Sac, sodium saccharin; Asp, aspartame; NaCyc, sodium cyclamate; AceK, acesulfame-K; Cap, capsaicin; PR, preference ratio; KO, knockout; HBSS, Hank's balanced salt solution

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which was replaced by a sodium free saccharin to minimize the Na^+ content of the solution. Solutions were prepared as previously described [7].

Expression of human TRPV1 receptors in HEK293 cells. Human TRPV1 cDNA (RZPD, Germany) was subcloned into pcDNA5/FRT (Invitrogen) and stably expressed in HEK using the Flp-In system (Invitrogen) as previously described [7]. Cells were grown as monolayers in Dulbecco's minimum essential medium (DMEM, Sigma) supplemented with non-essential amino acids, 10% fetal bovine serum, 2 mM L-glutamine and maintained at 37 °C under 95%/5% O_2/CO_2. Transfected cells were plated into 96-well plates (Costar, UK) previously coated with poly-D-lysine at a density of 100,000 cells/well and were grown for one day.

Site-directed mutagenesis on TRPV1. Point mutations Y511A and E649A in human TRPV1 corresponding to rat Y511A and E648A were generated after verifying that these regions were conserved across species. Point-mutants were obtained using the Quick Change Site-Directed Mutagenesis kit (Stratagene) on the hTRPV1 clone. After sequence verification, mutants were transiently expressed in HEK 293 cells and the respective sensitivity of Y511A and E649A to capsaicin and acidic pH [8,9] was verified by calcium and voltage imaging.

Measurement of intracellular calcium levels $[Ca^{2+}]_i$ and membrane potential using a fluorescent plate reader. For intracellular calcium imaging, cells were loaded with Fura-2/AM as previously described [7]. For membrane potential assay, cells at the same density were loaded with a voltage-sensitive dye according to the manufacturer's protocol (Red dye, Molecular devices). The 96-well plates were then placed into the plate reader to monitor Fura-2 ratios, F340/380 (λ_{ex1} = 340 nm, λ_{ex2} = 380 nm, λ_{em} = 508 nm) or voltage

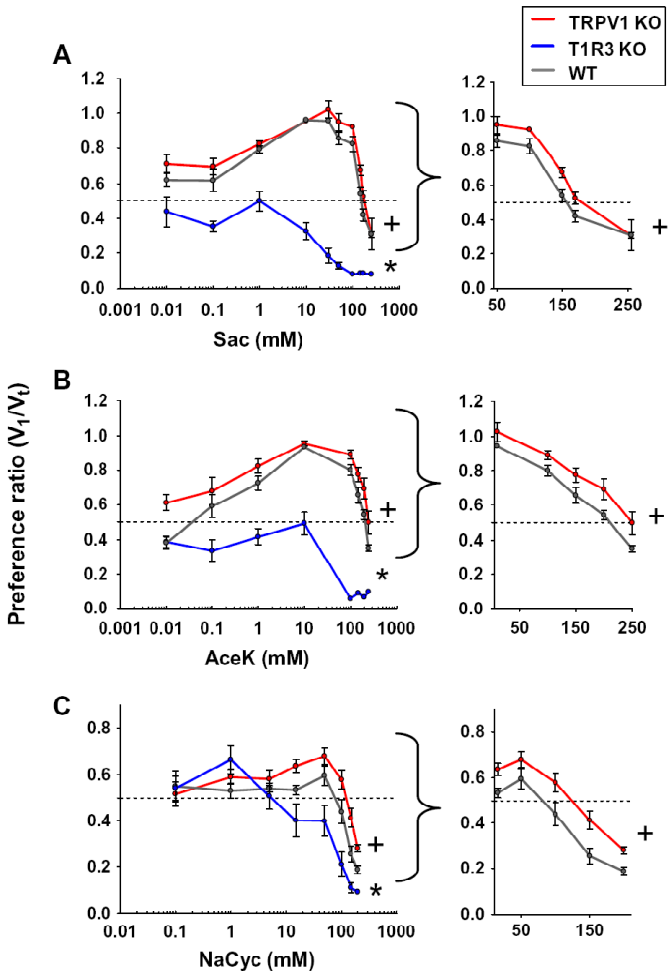

Fig. 1. Mean preference ratios from two bottle tests comparing responses of TRPV1 KO, T1R3 KO, and wild-type mice to artificial sweeteners. The ratio of volume of tastant drank (V_1) to total volume of liquid consumed (V_t) was measured and compared between groups for (A) Sac, (B) AceK, and (C) NaCyc. NaCyc is not preferred over water except for a small preference at 50 mM for the wild-type mice. T1R3 KO mice do not show preference for the three AS present in wild type and TRPV1 KO mice. At high concentrations, an aversive component is observed in all genotypes for the three compounds. TRPV1 KO mice show an increased preference and a decreased aversion versus the wild type animals for the three AS. For each group, n = 10. The error bars are SEM. * and + indicate that the overall responses of the T1R3 KO mice and the TRPV1 KO mice are significantly different from that of the WT, respectively. The responses to post peak concentrations were also analyzed separately and shown in the right panels with a linear x-axis scale.

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dye (λ_{ex1} = 530 nm, λ_{em} = 565 nm) to quantify respectively the changes in $[Ca^{2+}]_i$ and membrane potential upon stimulation with AS. Compounds were dissolved in Hank's balanced salt solution (HBSS) supplemented with 2 mM $CaCl_2$ and 20 mM HEPES buffered at pH 7.4. Experiments were performed at room temperature. For all experiments, calcium fluxes and voltages changes were measured as increases in fluorescence intensity, before and after the addition of agonists. The peak response was taken to be the characteristic value and was obtained by subtracting the peak value from the baseline (value before injection). Data were expressed as means ± SEM.

Animals. TRPV1 KO mice in C57BL/6J background, described in [10] were obtained from the Jackson Laboratory (Bar Harbor, ME). The T1R3 KO mice were produced by removing the entire coding sequence of T1R3 which was replaced with a Neo and eGFP cassette. The T1R3 KO mice are in 100% C57BL/6J background.

Two-bottle preference tests. Three groups of 10 mice, age and gender matched, males and females (10 TRPV1 KO, 10 T1R3 KO, 10 WT littermates) were tested with increasing concentrations of Sac, NaCyc, and AceK. Mice were caged individually and given access for 48 h to two 25-ml bottles, one containing distilled water and the other a tastant solution. After 24 h, the bottle positions were switched to control for positional effects. The volumes of liquid consumed were measured by weighing the bottles. The preference ratio measured as the ratio of tastant volume (V_1) to total liquid consumed (V_t) was recorded. A ratio of 0.5 indicates indifference, a ratio above 0.5 indicates preference and a ratio below 0.5 indicates avoidance. For each tastant, presentation was in ascending concentration. Between tastant trials, the mice were given two bottles of distilled water for 7 days.

Statistical analysis. The preference ratios were analyzed using the general linear model repeated measures of the statistics package SPSS with tastant concentration as a within subject factor and genotype as a between subject factor. Significance of the between subject factor indicates difference in the responses between genotypes, whereas significance of the within subject factor for a given genotype indicates either preference and/or avoidance of certain concentrations of the tastant. When a significant difference was found between genotypes, the Tukey's test was used to determine which genotypes differ. As the tested molecules show a biphasic response, the preferred and aversive concentrations were also analyzed separately using the General Linear Model as described above.

Results and discussion

T1R3 and TRPV1 determine artificial sweetener response profiles

To assess the contribution of TRPV1 channels to the aversive sensations elicited by AS such as Na-saccharin, Na-cyclamate, and Acesulfame-K, 2-bottle preference tests were performed in WT and KO mice lacking either TRPV1 or T1R3 and the preference ratio (PR) for each concentration (Fig. 1) was determined. As described previously, wild-type mice perceive Sac and AceK as hedonically positive [5,11–13] and display a small preference for NaCyc [14] but are believed to be insensitive to its sweetness because heterodimers of mouse T1R3 + T1R2 do not respond to this compound *in vitro* [11].

It is well established that wild-type mice display a biphasic response to these three artificial sweeteners [14]. With increasing concentration of Sac (Fig. 1A) the WT animals preference ratio increases to a peak at 30 mM followed by a decrease and an aversive phase at concentrations above 150 mM. As Sac elicits complex sensations of sweetness, bitterness, and metallic taste, we aimed at determining the concentration where aversive responses were observed in T1R3 KO mice. The data indicate that up to 1 mM, these KO mice are indifferent to Sac then become increasingly aversive to increasing concentrations of this sweetener. In comparing WT with the TRPV1 KO animals only over the concentration range between 50 and 250 mM significant differences are observed ($p < 0.05$). In this range the TRPV1 KO mice establish a higher preference ratio than the wild type. At concentrations below 50 mM Sac no significant difference was observed between TRPV1 and WT mice. Therefore, the TRPV1 contribution to the Sac taste profile mainly occurs in the post peak phase of the response profile, consistent with TRPV1 mediating unpleasant sensations.

For AceK, the WT mice also exhibited biphasic responses (Fig. 1B) having a maximum preference at 10 mM (PR = 0.94 ± 0.03) where the 48-h intake of AceK reached 20 mL (see Supplementary Fig. 1). With increasing concentrations of AceK the T1R3 KO mice showed a small aversion followed by indifference (PR = 0.49 ± 0.19) at 10 mM then strong aversion (PR = 0.05 ± 0.034) at 100 mM. Knocking out T1R3 reduced AceK positive hedonics and increased its aversiveness. The aversive contribution of TRPV1 is observed over the whole range of concentrations tested ($p < 0.005$).

WT mice presented with NaCyc did not prefer this AS to water up to 15 mM then exhibited a small preference response at 50 mM (PR = 0.53 ± 0.07) that is not observed in the T1R3 KOs (Fig. 1C). The overall difference between WT and T1R3 KO mice was significant ($p < 0.005$). The data suggest that high concentrations ($\geqslant$ 15 mM) of NaCyc produce sensations of moderate sweetness in mice but are aversive to T1R3 KO mice (PR < 0.40). As compared with the WT mice the TRPV1 KOs exhibited an increased preference at all concentrations >50 mM ($p < 0.05$).

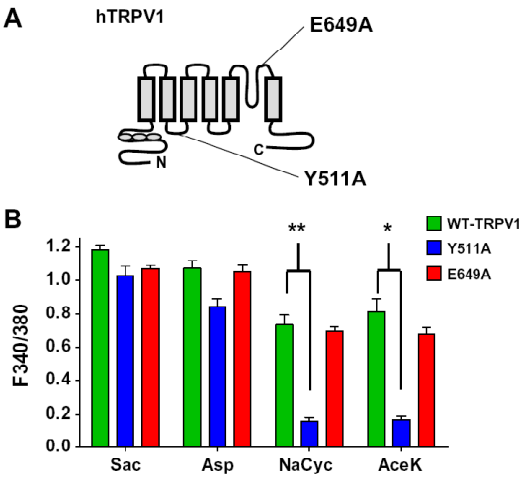

Fig. 2. Artificial sweeteners present divergent modes of action on TRPV1. (A) Schematic representation of a hTRPV1 monomer showing the location of the capsaicin (Y511A) and proton (E649A) insensitive mutations. TRPV1 possesses six transmembrane domains (rectangles), three intracellular ankyrin repeats (ellipses), the N- and C-termini are located on the intracellular side of the membrane, and the channel pore is formed by helices S5 and S6 as well as the S5–S6 linker of the four subunits. Tyr 511 is situated in the S2 region and Glu 649 is in the S5 pore domain. (B) Mean peak responses obtained upon stimulation of cells stably expressing wild type TRPV1 (WT, green), Y511A (blue), E648A (red) with 2 mM Sac, 8 mM Asp, 30 mM NaCyc, and 30 mM AceK obtained by Fura-2 based calcium imaging. Wild type TRPV1 and the proton-insensitive E648A were highly responsive to these stimuli, whereas Y511A shows strong responses to Sac and Asp but decreased responses to NaCyc and AceK. ($^{**}P < 0.0005$ and $^{*}P < 0.001$, unpaired *t*-test). Values are means ± SEM ($n = 3$).

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Taken together these results show that T1R3 mediates the preference for AS, as previously found, and that TRPV1 contributes to the aversive response to high concentrations of AS.

The observed small preference for NaCyc up to 50 mM has previously been described for WT mice but its origin is unknown [14]. It is believed that NaCyc does not taste sweet to rodents because *in vitro* studies showed that heterologously expressed mouse and rat sweet receptors T1R3/T1R2 are not activated by NaCyc [5,11]. However, our results show a clear difference between WT and T1R3 KO mice in their responses to NaCyc indicating that T1R3 plays a role in the preference for NaCyc, and that this preferred component is masked by the aversive component, part of which is mediated by TRPV1. Perhaps T1R3/T1R2 respond weakly to NaCyc, giving a response that is undetectable *in vitro*, but sufficient to elicit a behavioral response *in vivo*. Alternatively, T1R3 may dimerize with another receptor to transduce the taste of NaCyc.

Sac and AceK elicited an aversive response at high concentrations in WT mice, which is partially explained by the contribution of bitter receptors [6] but to a lower extent also through a TRPV1 component (Fig. 1A and B). Suppression of TRPV1 in mice provides a gain of preference for both AceK and NaCyc and reduces the aversive response for the three AS.

Overall, these behavioral results show that the responses to Sac and AceK have a hedonically positive aspect mediated by T1R3 and a hedonically negative one mediated partly by T2Rs, as previously shown, but also by TRPV1 as shown here. The contribution of TRPV1 could be from nociceptors or the variant TRPV1t in taste cells [15–17].

Differential activation of TRPV1 at the molecular level

What is the mechanism of TRPV1 activation used by the artificial sweeteners? It is well established that somato-sensory TRPV1 displays multiple ligand binding sites, among them an intracellular vanilloid binding pocket [8] and an extracellular proton binding domain [9]. Point mutations of critical residues render TRPV1 insensitive to Cap (Y511A) or decrease responses to acidic stimuli (E649A) (Fig. 2A). To determine the specificity of the stimulation produced by different AS we explored, which residues among the characterized binding sites participate in their detection (Figs. 2 and 3). As sweeteners have been shown to permeate taste receptor cells (TRC) [18,19] and stimulate somato-sensory fibers expressing TRPV1 [7], we specifically explored whether their action on TRPV1 was through the intracellular capsaicin binding site. To answer this question, we evaluated the effect of AS on the somato-sensory isoform of TRPV1 as it is the most common form, and is functional in the trigeminal system. TRPV1t is not fully characterized and its molecular nature is unknown [15–17], therefore we cannot speculate on the effect of artificial sweeteners on this isoform.

Using Fura-2 based calcium imaging on cells stably expressing the wild-type (WT) TRPV1, Y511A, and E649A, we monitored channel responses to AS. Fig. 2B shows maximum responses (peak responses) obtained from the cells lines when stimulated with the different AS. Both Y511A and E649A mutants presented similar calcium transients as the wild-type channel to Sac and Asp. Compared to cells expressing WT-TRPV1, the calcium responses to NaCyc and AceK of cells expressing Y511A were al-

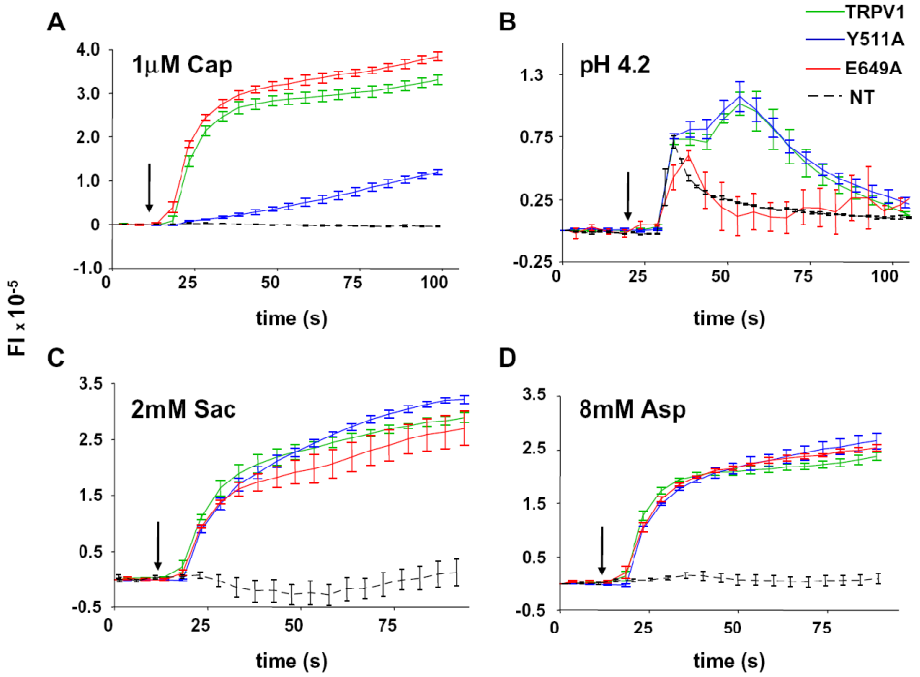

Fig. 3. Sac and Asp do not activate TRPV1 via the capsaicin binding site. HEK 293 cells expressing the two point mutants. WT-TRPV1 and non-transfected cells were stimulated with (A) 1 μM Cap, (B) acidified HBSS (pH 4.2), (C) 2 mM Sac, and (D) 8 mM Asp to monitor voltage changes in the cells using Red dye. WT-TRPV1 was highly responsive to these stimuli, whereas the capsaicin response was ablated in the Y511A variant and the proton responses were drastically decreased in E649A. Sac and Asp responses were unchanged in the TRPV1 mutants compared to the WT. Values are means ± SEM ($n = 3$).

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most fully abolished whereas they were unchanged in cells expressing E649A.

To confirm the observed calcium transients induced by AS application, voltage imaging experiments were conducted using a membrane potential sensitive fluorescent dye. Shown in Fig. 3A and B are the responses of WT-TRPV1 and the two mutants to Cap and protons. As expected, Cap did not produce fluorescence increases in cells expressing Y511A (blue) but induced strong responses in WT-TRPV1 (green) and E649A (red). Acidification of the extracellular medium to pH 4.2 generated similar responses in both TRPV1-WT and Y511A, whereas the response of the E649A mutant was strongly diminished and with identical intensity as observed in non-transfected (NT) cells. The application of Sac and Asp to the capsaicin insensitive mutant Y511A and the proton mutant E649A produced identical fluorescence increase as the wild-type (Fig. 3C and D). Because of the indirect effects of the Na^+ and K^+ counterions associated with NaCyc and AceK on Na^+ and K^+ channels present in HEK cells, assessment of sweetener induced changes in membrane potential were masked by their cation effects (not shown).

Consistent with the role of TRPV1 as a sensor of aversive compounds, all AS tested were agonists for heterologously expressed hTRPV1 but through a different mechanism of stimulation. Our data show that the capsaicin binding residue Tyr 511 is not implicated in Sac and Asp activation whereas the responses of AceK and NaCyc are abolished in the capsaicin insensitive mutant of this residue. No alteration of the response was observed by mutation of the glutamate E649, the proton binding residue [9]. Thus these data point out that AS with divergent chemical structures act on TRPV1 via unknown mechanisms for Sac and Asp and involve the capsaicin binding region for NaCyc and AceK.

In conclusion, our results show that suppression of TRPV1 affects taste preference in mice when presented with non-sugar sweeteners. Specifically, these data point out that TRPV1 is recruited in the aversive response to AS and this stimulation may explain some of the unpleasant taste, notably the metallic off taste at high concentrations. NaCyc and AceK activate TRPV1 at the capsaicin binding site whereas the binding site for Sac and Asp remains undetermined. Taken together, these findings provide a molecular rationalization of the sensory properties of a class of biphasic tasting compounds (artificial sweeteners) where T1R3, T2Rs, and TRPV1 are the molecular targets.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.09.029.

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Supplementary information

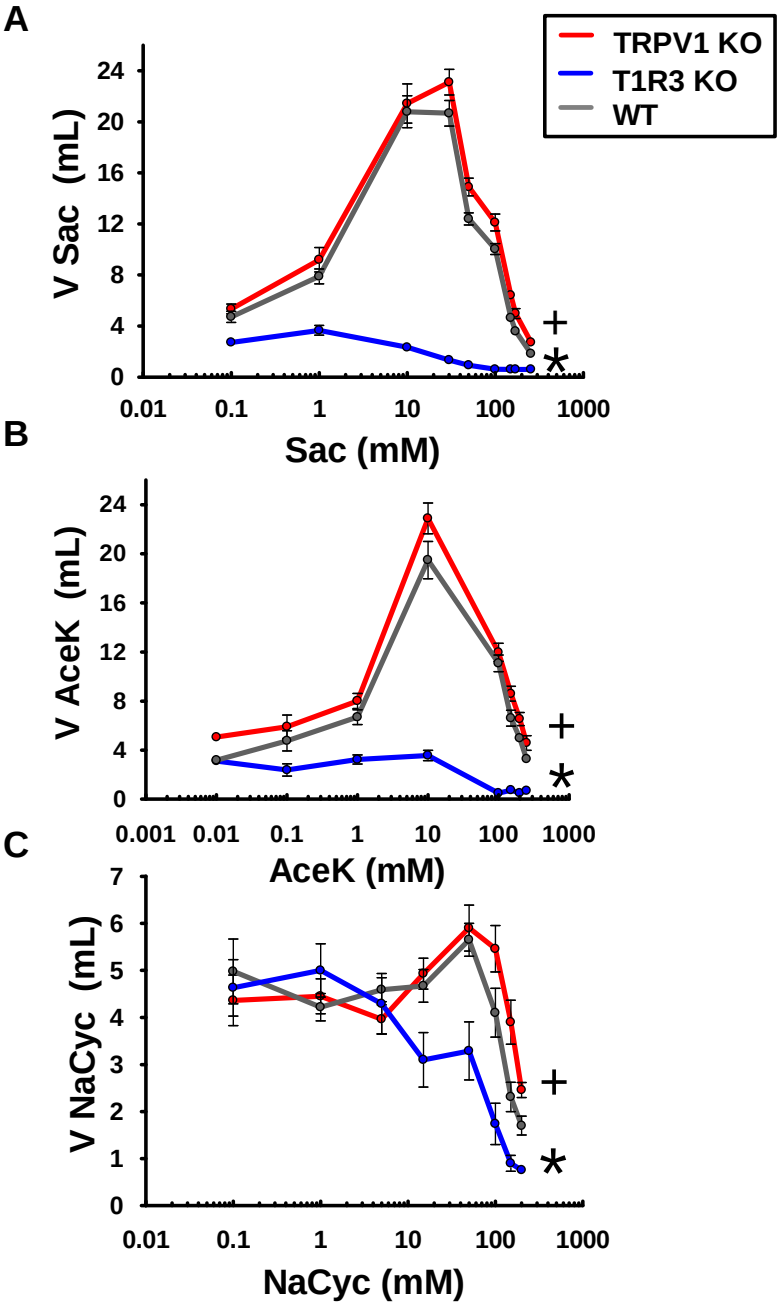

Figure S1. Mean volumes of artificial sweeteners consumed by TRPV1$^{-/-}$, T1R3$^{-/-}$ and wild-type littermates.

Drinking volumes (mL) are represented for each genotype as a function of concentration for each genotype. Tastants presented were A) Sac, B) AceK and C) NaCyc. These volumes correspond to the tastant volume (V_1) used to calculate the preference ratio. For each group, n=10 ± SEM. * and $^+$ indicate that the overall response of the T1R3$^{-/-}$ and the TRPV1$^{-/-}$ are significantly different from the WT, respectively.

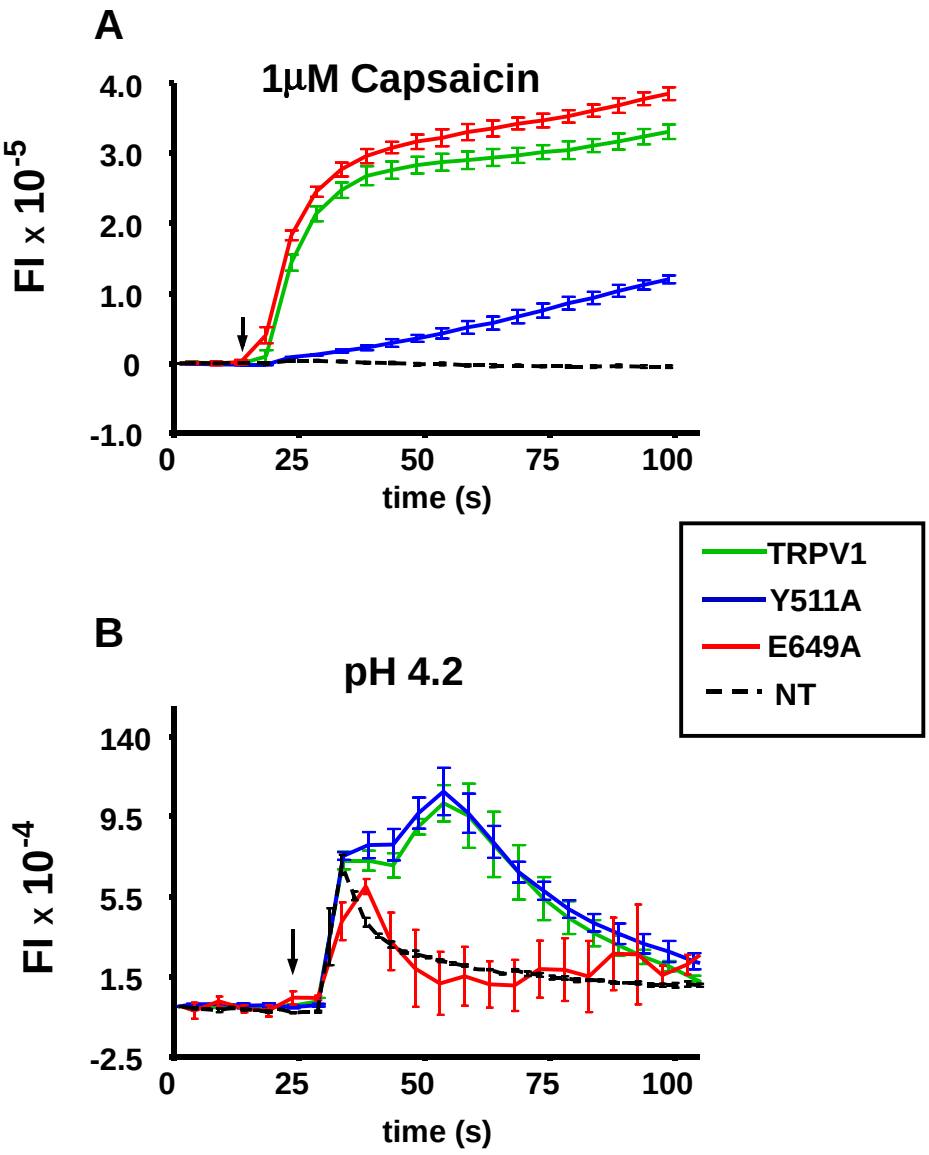

Figure S2. TRPV1 point mutants Y511A and E648A responses to capsaicin and acidic pH differ from the wild-type channels.

HEK 293 cells expressing the three point mutants, the WT TRPV1 and untransfected cells were challenged with 1µM capsaicin and acidified HBSS (pH 4.2) to monitor voltage changes in the cells using Red dye. Wild type TRPV1 was highly responsive to these stimuli, whereas capsaicin response was ablated in the Y511A and proton responses were drastically decreased in E649A. Means ± S.E.M. (n=3).

2 Covalent ligand interactions of TRPA1 and TRPV1 with pungent spices

2.1 Introduction

Having now studied the molecular mechanisms of complex tasting molecules (CTDS and AS) implicating TRP channels, it was aimed at getting more insight into the role of TRP channels in dietary molecules producing specifically chemesthetic but not gustatory sensations such as culinary spices, which are appreciated for their pungent qualities that can enhance the taste of a meal. A spice can be characterized as a dried seed, fruit, root, bark or substance used in nutritionally insignificant quantities as a food additive for the purpose of flavouring. Spices that do not belong to the *capsicum* genus do not contain capsaicin but other pungent molecules. Pungency describes sharp and biting sensory impressions classically produced by mustard oil, garlic or the burning sensation of capsaicin.

Interestingly, as reported in the introduction, the prototypical pungent molecules capsaicin and allyl isothiocyanate (mustard oil) produce their sensations via stimulation of the pain receptors TRPV1 and TRPA1. These compounds, highly toxic, stimulate trigeminal afferents to mediate their noxious effect in a similar manner that bitter toxicants warn our sensory system through T2Rs signaling.

However, while some pungent molecules recognize specifically TRPV1 or TRPA1, others possess the ability to stimulate several receptors on the trigeminal afferents. The promiscuity of TRP channels on sensory afferents raises the question of the coding of these sensations. To address this issue, two spices imparting complex sensations of burning, tingling and numbing were selected and their sensory targets on the DRG neurons were investigated. The results of the fourth article "Covalent and Non-Covalent Ligand Interactions in TRPA1 and TRPV1 Channels with Spicy Molecules from Sichuan and Melegueta peppers", submitted to the *British Journal of Pharmacology*, characterize the pharmacological effects of pungent alkylamides and hydroxyarylalkanones on TRPA1 and TRPV1 through a detailed structure activity analysis. In addition, this paper investigates the molecular mechanisms underlying the binding on both channels by focussing on covalent binding, a relevant mode of action of disulfides garlic derivatives on TRPA1 and TRPV1 (Hinman et al., 2006; Salazar et al., 2008). Finally, the aversive taste of these compounds was investigated in the absence of TRPV1 using taste preference on mice. In this article, it was determined in the alkylamide hydroxy-α-sanshool structure the elements that convey a TRPA1 rather than a TRPV1 stimulation, with the critical involvement of a *cis* double bond in the polyenic chain of the molecule that induces reactivity on TRPA1.

In a second step, the knowledge acquired in this paper was used to generate proprietary compounds with attractive sensory qualities. Briefly, the reactive alkyl backbone of the molecule and other natural fatty acids selected for their *cis*-insaturations were chemically coupled to different amino acids such as glutamate known for its umami taste, glycine, alanine and serine in order to generate a novel compound. Diverse reactivity properties *in vitro* of these compounds were found and it was evidenced as a common feature that they were all moderately stimulating TRPV1, but two potent TRPA1 agonists had a small TRPV1 effect.

These two compounds of interest are likely to be devoid of burning as they lack TRPV1 sensitivity (aversive) but to possess interesting tingling effects and putatively a savory component provided by the amino-acid. The goal is to generate these compounds in a food-based process using the chemical reactivity between the carboxylic acid of the fatty acid and the amine of the aminoacid and perform sensory tests to detail their properties. These results are the subject of a Nestlé patent application and are detailed in another research paper, “Synthesis of new TRPA1 agonists derived from α-hydroxy-sanshool” submitted to the *Journal of Medicinal Chemistry* attached in appendix 7.

2.2 Covalent ligand interactions with TRPA1 and TRPV1

Covalent and Non-Covalent Ligand Interactions in TRPA1 and TRPV1 Channels with Spicy Molecules from Sichuan and Melegueta peppers

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Keywords:

Chemosensory TRP, structure-activity, calcium imaging, covalent binding

Running title: Covalent ligand interactions with TRPA1 and TRPV1

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Number of figures: 7
Number of references: 31
Word numbers in abstract: 286
Word numbers in introduction: 788
Word numbers in discussion: 2053

List of non-standard abbreviations: TRP, transient receptor potential; TRPV1, transient receptor potential vanilloid 1; TRPA1, transient receptor potential ankyrin 1; TRPM8, transient receptor potential melastatin 8; TRPV3, transient receptor potential vanilloid 3; Cap, capsaicin; Cinna, Cinnamaldehyde; GSH, glutathione; α-SOH, hydroxy-α-sanshool, 2-APB, 2-aminoethyl diphenyl borate; MTSEA, 2-aminoethyl methanethiosulfonate hydrobromide; ESI-MS, electrospray ionization mass spectrometry; DRG, dorsal root ganglia; HEK, human embryonic kidney.

ABSTRACT

The oil extract of Sichuan (*Zanthoxylum* spp.) and Melegueta (*Aframomum* spp.) peppers evoke burning and tingling sensations that are mediated respectively by different alkylamides and hydroxyarylalkanones. The alkylamide hydroxy-α-sanshool (α-SOH) has been shown to stimulate both cinnamaldehyde-sensitive TRPA1 and capsaicin-sensitive TRPV1 channels whereas two pungent vanilloids in Melegueta pepper, 6-shogaol and 6-paradol were found to stimulate TRPV1 receptors. Here we determined the relative importance of the double bonds present in the polyenic chain of α-SOH and four synthetic analogues that vary in their alkyl chain structure in activating TRPA1, TRPV1 and TRPM8 channels. In addition, 6-shogaol and 6-paradol, which differ by a single double bond were characterized. Finally, we investigated the effect of linalool, a terpene found in Sichuan pepper oil extract, on activation of TRP channels. For all compounds we also explored whether they activate the channels via covalent modification by using a site directed mutagenesis strategy and glutathione trapping as a model to probe TRPA1 activation. We show that the *cis* C6 double bond in the polyenic chain of α-SOH is critical for TRPA1 activation, whereas no structural specificity is required for TRPV1 stimulation. The two vanilloids in Melegueta pepper activate TRPA1 at 100 fold higher concentration than TRPV1 whereas linalool stimulates only TRPA1. None of these compounds activate TRPM8. Of the investigated compounds the alkylamides and 6-shogaol act on TRPA1 via covalent mechanisms whereas none of the stimuli activated TRPV1 through these interactions. TRPV1 mutant mice retain sensitivity to 6-shogaol but are indifferent to α-SOH, showing a partial role for TRPA1. The results present novel insight into the co-stimulation of TRPA1 and TRPV1 where the two channels, even though sensitive to the same compounds, react via different modes and code for different sensations.

Introduction

Identification of several channels of the Transient Receptor Potential (TRP) superfamily in sensory fibers of the oral cavity has led to advances in the understanding of oral sensations associated with spices. For example, TRPV1 is stimulated by capsaicin the primary pungent ingredient in chilli pepper (Caterina et al., 1997), TRPV3 by pungent components of oregano, savory, thyme and cloves (Xu et al., 2006), and TRPA1 by mustard, garlic and cinnamon (Bandell et al., 2004a;Jordt et al., 2004;Macpherson et al., 2005;Bautista et al., 2005).

Some spices impart complex sensations including pungency. Pungency describes sharp and biting sensory impressions classically produced by mustard oil, garlic or the burning sensation of capsaicin. Among these spices, Sichuan pepper (genus *Zanthoxylum*) imparts unique sensory qualities, characterized as tingling, numbing and burning. These pungent sensations have been attributed to several alkylamides present in the pericarp of the dried fruit (Sugai et al., 2005b). Recent work on alkylamide based pungency demonstrated that these compounds were agonists of both TRPV1 (Sugai et al., 2005a) and TRPA1 (Koo et al., 2007). Very recently, two-pore potassium channels sensing volatile anesthetics (Patel et al., 1999;Talley and Bayliss, 2002) were found to be stimulated by applications of α-SOH, rationalizing some of the complex numbing and long lasting perceptions associated with the spice (Bautista et al., 2008) whereas the pungent “sharp” and “biting” sensations may be attributed to TRPV1 and TRPA1 stimulation.

Interestingly, oil extracts from Sichuan pepper are extremely rich in terpene compounds, with linalool being the most abundant (75% in weight). Linalool has been reported as a weak agonist of the menthol receptor TRPM8 (Behrendt et al., 2004) but little is known about its activity on other TRPs or its gustatory profile.

Recently, two studies (Hinman et al., 2006;Macpherson et al., 2007a) have proposed that cinnamaldehyde and allyl-isothiocyanate activate TRPA1 through covalent binding on specific cysteine residues present in the ankyrin repeats of the channels. These findings could be extended to alkyl chains less than 8 carbons long possessing α, β unsaturated double bonds (Macpherson et al., 2007b). Interestingly, the mutation of one or several reactive cysteines to serine leads to insensitivity of TRPA1 to electrophilic agonists, but not to non-electrophilic compounds (Macpherson et al., 2007a). Electrophilic compounds elicit irreversible currents whereas currents produced by non-electrophilic compounds are reversible (Macpherson et al., 2007a) suggesting that TRPA1 contains both a traditional binding pocket and cysteine residues involved in covalent activation.

The stimulation of TRPV1 receptors by capsaicin or other vanilloids such as resiniferatoxin (Szallasi and Blumberg, 1999), zingerone and gingerol (Dedov et al., 2002;Liu and Simon, 1996) is believed to occur via a non covalent binding pocket in the transmembrane domain through π-stacking interactions between the aromatic moiety of Tyr 511 and the vanilloid ring moiety of capsaicin (Jordt and Julius, 2002). Plants of the *Allium* genus (onion and garlic) also stimulate TRPV1 in addition to TRPA1 (Macpherson et al., 2005) and recently they have been shown to act covalently on one intracellular cysteine residue in the N-terminal region of TRPV1 (Salazar et al., 2008). These findings raise the question if covalent modification of cysteine residues in the cytoplasmic terminus of the channels is the common mechanism for pungent TRPA1 and TRPV1 activation. α-SOH contains an α, β unsaturated double bond, which could account for its activity on TRPA1 and TRPV1. However, this double bond does not seem to be sufficient for channel activation because the *trans* isomer hydroxy-β-sanshool is unable to stimulate TRPA1 and TRPV1-expressing DRGs (Koo et al., 2007) even though the two compounds differ only by the *cis*/*trans* configuration of their C6 double bond.

Essential oil of Melegueta pepper (*Aframomum melegueta* K. Schum) contains the hydroxyarylalkanones 6-shogaol and 6-paradol in approximately equal concentrations (Tackie et al., 1975). As they contain a vanilloid moiety as in capsaicin (see Fig. 1) these compounds activate TRPV1 and have been reported to be pungent (Lee and Surh, 1998;Witte et al., 2002). Since 6-shogaol and 6-paradol differ only by an α, β unsaturated double bond we also explored their effect on TRPA1 and TRPV1.

As many pungent compounds stimulate either TRPA1 and/or TRPV1 we evaluated the effects of the main constituents of Sichuan and Melegueta pepper and four synthetic analogues of α-SOH on both dissociated rat DRG cells and on HEK293 cells expressing the human TRPA1 and TRPV1 receptors. We established that molecules present in these spices stimulate specifically TRPA1 and TRPV1 containing neurons with the exception of linalool stimulating only TRPA1. None of the compounds tested stimulated TRPM8 expressing neurons. We also characterized the importance of side-chain double bonds in the alkylamides configuration to modulate TRPA1 but not TRPV1 where the *cis* C6 bond of α-SOH accounts for full TRPA1 specificity. In addition, we tested the effects of these molecules on cysteine mutants of TRPA1 and TRPV1 to address whether their mode of action on both TRPs would be similar. We found that covalent binding is critical for the stimulation of TRPA1 whereas it is not required for TRPV1. These results provide new insight in the understanding of TRPA1 and TRPV1 coding and their pharmacological responses to pungent compounds.

Materials and Methods

Chemicals: Capsaicin, cinnamaldehyde, linalool, 2-APB, menthol, *cis*-6-nonenal and MTSEA were obtained from Sigma Aldrich (Buchs, Switzerland). 6-shogaol was purchased from Extrasynthese (Lyon, France) and 6-paradol was synthesized from 6-shogaol (see Supplementary information S3). α-SOH was purified from the extract of green Sichuan pepper, *Zanthoxylum* spp. (see Supplementary information S1). Four analogues of α-SOH (**I, II, III, IV** see Fig. 1) were synthesized as outlined in S2 and published elsewhere in more detail.

Technical sensory trials: Solutions of food-grade linalool (Sigma-Aldrich) diluted in Vittel® were evaluated by three volunteers. Solutions of 10 µM, 100 µM, 500 µM and 1 mM were kept in mouth for 30s to evaluate the pungency with rinsing the mouth between each trial. Pungency of analogues (**I, II, III, IV**) of α-SOH was not assessed as these molecules are non-food grade synthetic reagents.

Glutathione (GSH) adduct reaction: Compounds at 10 mM in water were incubated for several hours with an equimolar concentration of GSH to form adducts. Products of reactions were diluted 10 times in a solution of 50% MeOH and measured by electrospray ionization mass spectrometry (ESI-MS).

Cloning and expression of human TRPV1 and TRPA1 receptors in HEK293 cells: Cloning and expression of these receptors was performed following previously published protocols (Riera et al., 2007). Briefly, cloned human TRPV1 cDNA was obtained from RZPD (Germany) and hTRPA1 cDNA from OriGene (Rockville, MD). Genes were subcloned into pcDNA5/FRT (Invitrogen, Carlsbad, CA) to generate stable cell lines using the Flp-In system (Invitrogen) after sequencing verification.

Site-directed mutagenesis on TRPA1 and TRPV1: Point-mutants were generated using the Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) on the hTRPA1 and the hTRPV1 clone. A triple TRPA1 cysteine mutant (C621S-C641S-C665S) and the cysteine point mutant of TRPV1 C158A were generated. After sequence verification, mutants were transiently expressed in HEK293 cells using Lipofectamine 2000 (Invitrogen) and the respective response to various agonists was obtained using voltage imaging (see below).

Measurement of intracellular calcium levels $[Ca2+]_i$ and membrane potential variation in HEK 293 cells using a fluorescent plate reader: Cell-lines stably expressing TRP channels were seeded into 96 well plates previously coated with poly-D-lysine. Cells were incubated in Hank's Balanced Salt

Solution (HBSS) supplemented with 2mM $CaCl_2$ and 20mM HEPES (pH 7.4), containing the cytoplasmic calcium indicator Fura-2/AM at 2 μM (Molecular Devices, Sunnyvale, CA). For membrane potential assays, cells were loaded with a voltage-sensitive dye according to protocol (Red dye, Molecular Devices) and fluorescence changes were measured upon application of compounds (λ_{ex1}=530 nm, λ_{em}=565 nm). Experiments were conducted at room temperature. $[Ca2+]_i$ fluxes from an homogenous cell population (approximately 100'000 cells) were measured as changes in fluorescence intensity when stimulated with agonists using a FLEXstation (Molecular Devices) (Riera et al., 2007). Cells expressing TRP channels were then challenged with the different compounds shown in Fig. 1.

Dorsal root ganglia (DRG) culture and calcium imaging: Dissociated DRG neurons from neonatal (2-3 days) rats were obtained frozen from Cambrex Bio Science (Walkersville, MD). Cells were cultured as previously described (Riera et al., 2007) and supplemented with nerve growth factor (β-NGF, Sigma) at a concentration of 100 ng/ml. Changes in $[Ca^{2+}]_i$ were measured using ratiometric digital fluorescence imaging using Fura-2/AM. Images of individual neurons were acquired with a cooled, charge-coupled device (CDD) camera (Cascade II, Photometrics, USA) mounted to an AxioObserver D1 inverted microscope. Autofluorescence was negligible and with illumination times of 100-300 ms, F340/F380 remained stable. Coverslips with attached neurons were placed in a chamber with continuous flow of supplemented HBSS. Chemical stimuli present in HBSS were applied at 30-33°C to the flow chamber for 5s and cells were rinsed in supplemented HBSS between stimuli. $[Ca2+]_i$ transients are represented as an increase in the fluorescence ratio of Fura-2 loaded DRG neurons (450 cells from 2 separate cultures were analyzed). To prevent channel desensitization, we tested one or two compounds per coverslip and then applied cinnamaldehyde (TRPA1), capsaicin (TRPV1) and menthol (TRPM8) to determine TRPA1, TRPV1 and TRPM8 expressing DRGs. For each neuron the average fluorescence ratio F340/F380 was calculated using Metafluor software (Universal Imaging Corp.).

Brief access taste tests: 2 groups of mice consisting of 10 C57BL/6J wild type (WT) mice and 10 TRPV1 KO mice (Jackson Laboratory; Bar Harbor, ME), age and gender matched, males and females, 100% C57BL/6J background were tested with different compounds. Brief-access taste tests were conducted in a gustometer (Davis MS160-Mouse gustometer; Dilog Instruments, Tallahassee, FL). The training and testing procedures were conducted as described (Damak et al., 2006;Glendinning et al., 2002). The mice were presented alternatively with two bottles, one containing the tastant and the other distilled water for 30 minutes testing periods. Each time the mouse licks it starts a 5 second trial after which the shutter is closed and the other bottle is presented. The number of licks

per trial is recorded. Mice that did not get accustomed to the gustometer were excluded from the study. The lick ratio was calculated as the mean number of licks per trial for the tastant (L_1) divided by the mean number of licks per trial for tastant plus mean number of licks per trial for water (L_t). A ratio of 0.5 indicates indifference, a ratio above 0.5 indicates preference and a ratio below 0.5 indicates avoidance.

Data analysis: Responses of molecules in HEK 293 cells were expressed as a percentage of maximum responses evoked by 150µM cinnamaldehyde for TRPA1 and 1µM capsaicin for TRPV1 (these concentrations were assessed independently to be saturating under these conditions). For all experiments, calcium fluxes were measured as changes in fluorescence intensity, before and after the addition of agonists. The peak response was taken to be the characteristic value and was obtained by substracting the peak value from the baseline (value before injection). Doses response curves were fitted using the Hill equation (GraphPad Prism Software, San Diego, CA) to obtain EC_{50} values and Hill coefficients. Data obtained from this study were expressed as mean ± S.E.M.

Results

Natural Sichuan and Melegueta pepper components and synthetic alkylamides derived from hydroxy-α-sanshool (α-SOH)

Extracts of Sichuan and Melegueta peppers contain molecules that impart perceptions of burning, numbing and tingling (Tackie et al., 1975;Sugai et al., 2005a;Sugai et al., 2005b). Sichuan pepper oil extract primarily contains linalool, an acyclic monoterpene, as well as various alkylamides, the most abundant being α-SOH (Fig. 1). To evaluate the role of double bonds and their configuration in the polyenic chain of α-SOH, we synthesized four analogues of this compound and measured their activity on DRG neurons, hTRPV1 and hTRPA1 channels. The analogues of α-SOH comprise a fully saturated α-SOH (**I**), a mono α, β unsaturated analog (**II**), a diunsaturated analog of α-SOH possessing both the α, β double bond and the *cis* C6 double bond (**III**) and mono-*cis* C5 unsaturated compound (**IV**). Melegueta pepper contains hydroxyarylalkanones: 6-paradol and 6-shogaol, which differ from each other by the unsaturation in the α, β position (Fig. 1). We determined which channels are activated by these compounds, their concentration dependence and if the activation occurs via covalent or non-covalent interactions.

Responses of DRG neurons to Sichuan and Melegueta compounds

To address if any of the compounds shown in fig. 1 would stimulate sensory fibers expressing TRPA1 and/or TRPV1 and/or TRPM8 channels, we measured compound-induced changes in $[Ca2+]_i$

from dissociated DRG cells. TRPV1 channels are found in the presence or absence of TRPA1 channels, whereas TRPA1 is expressed in a subset of TRPV1-DRG cells (Story et al., 2003a). For the latter reason purely cinnamaldehyde (TRPA1)-sensing DRGs were not identified in any of the 450 neurons tested. To identify TRPM8-expressing neurons, we selected neurons stimulated by menthol (McKemy et al., 2002).

In fig. 2, changes in $[Ca2+]_i$ are shown in response to specific stimuli for three representative neurons. Cap–sensitive (TRPV1) neurons are in red, cinnamaldehyde -sensitive (TRPA1) are in black and menthol-sensitive (TRPM8) are shown in green. Fig. 2A shows representative responses to α-SOH. One representative neuron responded to α-SOH, cinnamaldehyde and capsaicin suggesting it expresses TRPA1 and TRPV1; another responded to α-SOH and capsaicin but not to cinnamaldehyde suggesting it contained TRPV1 but not TRPA1, and a third responded to menthol and not to α-SOH or capsaicin consistent with expression of TRPM8. Analogues of α-SOH (**I-IV**) were found to stimulate capsaicin and cinnamaldehyde-sensitive DRGs, but they had no effect on Menthol-responsive DRGs (Fig. 2 B & C). Of the 450 individual neurons imaged 135 responded to capsaicin, 85 responded to cinnamaldehyde and capsaicin and 89 responded only to menthol**.** The remaining neurons did not respond to any of these compounds.

Both 6-shogaol and 6-paradol stimulated cinnamaldehyde- and capsaicin-sensitive DRGs, but had no effect on menthol-responding DRGs (Fig 2D & E). Neurons activated by linalool respond both to cinnamaldehyde and capsaicin but no changes in $[Ca2+]_i$ were evoked on purely capsaicin-sensitive DRGs, suggesting the involvement of TRPA1 (Fig 2F). Linalool did not induce an increase in $[Ca2+]_i$ in menthol-sensitive DRGs.

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Heterologously expressed TRPA1 and TRPV1 channels respond to natural and synthetic stimuli derived from Sichuan and Melegueta peppers.

For all the compounds tested, only DRG cells putatively expressing TRPA1 and TRPV1 responded, and therefore menthol activated TRPM8 channels were excluded for further investigation. Although the responses evoked in DRG neurons are suggestive of the channels they activate, the assignment is not definitive because many compounds activate numerous receptors and common antagonists in use for TRPA1 and TRPM8 are non-specific. Thus to address this issue more directly we used a heterologous system to test if these molecules would activate the cloned cinnamaldehyde-activated channel (hTRPA1) and the capsaicin-receptor (hTRPV1).

Typically, changes in $[Ca2+]_i$ were monitored over time at different agonist concentrations to test the stimulus effect on TRPA1 and TRPV1 in HEK293 cells. Although all eight compounds were tested, for illustration only two are shown (Fig. 3). Typical cell responses are shown for increasing concentrations of compound **IV** and 6-paradol on TRPA1 (Fig. 3A) and TRPV1 (Fig. 3B). On exposure to both compounds, robust increases in $[Ca2+]_i$ were observed in TRPA1- and TRPV1-expressing cells. Neither of these compounds produced an effect in non-transfected cells (data not shown).

Each of the eight stimuli displays a characteristic dose-response relationship for TRPA1 and TRPV1 (Fig. 4). Figures 4A and B show the responses for TRPA1 relative to the maximal concentration of cinnamaldehyde. Table 1 gives the EC_{50} and the Hill coefficients of these compounds. Upon ranking the EC_{50}'s for TRPA1 (cinnamaldehyde > 6-shogaol > α-SOH = 6-paradol > **IV** > linalool> **III** > **II** > **I**), it is important to note that the unsaturated analogue (**I**) did not evoke an appreciable effect and the monounsaturated α, β sanshool analogue was a weak agonist, whereas the two vanilloids were the most potent after cinnamaldehyde. Linalool and 6-shogaol evoked about 90%, and 6-paradol 70% of the maximum cinnamaldehyde response. In contrast, α-SOH and analogues **III** and **IV** produced about 60% of the maximum response. As seen in the dose-responses curves, compound **III** displayed the same potency as compound **IV** suggesting the replacement of the *cis* C6 by a *cis* C5 olefin is a suitable model to study the structural arrangement of the natural molecule. Compound **III** and **IV** were about as potent as α-SOH suggesting it is the *cis* C6 double bond, which largely accounts for α-SOH specificity.

Figures 4C and D show the dose-response obtained on hTRPV1 relative to the maximal capsaicin response. Upon ranking the EC_{50} values (capsaicin > 6-shogaol > 6-paradol > α-SOH > **III = IV** > **I** > **II** >> linalool) we observed small differences in the EC_{50} of compounds **I, II, III, IV** meaning that TRPV1 responses are not very sensitive to alkyl chain unsaturation (at least to the extent of the compounds tested). We observed that the presence of the C8-C10 conjugated system increased α-SOH potency from compounds **III** and **IV** 5 fold, more likely through hydrophobic gain. Similarly, a 4 fold increase in potency from 6-paradol to 6-shogaol was noted indicating the significance of the α, β unsaturation in the alkyl chain. High concentrations (>1mM) of linalool induced weak changes in $[Ca2+]_i$ about 30% of the maximum capsaicin response (not shown). All tested compounds were less potent than capsaicin, with 6-shogaol and 6-paradol producing maximum increases in calcium reaching 90% of the capsaicin response. All sanshool responses saturated at about 70% of the capsaicin response.

Covalent binding of tested compounds to TRPA1 and TRPV1 channels

TRPA1

Cinnamaldehyde and other α, β unsaturated aldehydes react via a Michael addition on several cysteine residues situated in the ankyrin binding repeats of TRPA1 (Hinman et al., 2006;Macpherson et al., 2007a;Macpherson et al., 2007b). To determine if the above tested TRPA1 ligands would have the same ability, we constructed a reactive triple TRPA1 cysteine mutant (TRPA1-3C) and monitored, using a membrane voltage sensitive assay, the responses at maximal concentrations (Fig. 5).

As shown in the panels, in comparison between the mutant and the wild type the response to cinnamaldehyde was, as expected (Macpherson et al., 2007a), drastically decreased, whereas the response to 2-APB, a non-electrophile TRPA1 agonist (Hinman et al., 2006) was identical in both wild-type and mutant, as it was the case for linalool thus arguing for a non-electrophilic binding mechanism. In contrast, responses to α-SOH and analogues **II, III, IV** were almost fully abolished in the TRPA1 triple cysteine mutant. Compound **I** was not tested as it was unable to produce calcium increases in TRPA1 (Fig 4A). The inhibition of α-SOH and the 3 analogues argues for a covalent binding via the cysteines. We also observed that the response of 6-paradol was unchanged by the mutant, although under the same conditions 6-shogaol was decreased by 35%. This can be rationalized, in part, by the presence of the α, β unsaturation making 6-shogaol a Michael acceptor.

To further demonstrate that the tested compounds could act covalently on TRPA1, we used GSH as a model for adduct formation (Macpherson et al., 2007a). We found that cinnamaldehyde and 6-shogaol reacted covalently with the cysteine on GSH whereas 2-APB, linalool and 6-paradol did not (see supplementary S4). We also found that α-SOH and the analogs **II, III, IV** reacted covalently with GSH. **II** and **III** adducts can be explained by the α, β unsaturation, whereas **IV**-GSH adducts might be due to the *cis* C5 unsaturation. To test whether a cis unsaturated bond in the carbon backbone of the α-SOH system would be sufficient for covalent bonding, we used *cis*-6-nonenal, an aldehyde possessing the same *cis* unsaturation feature as α-SOH and found the latter did not form adducts with GSH. Surprisingly, the fully saturated compound **I** formed adducts with GSH like its analogs. Based on these findings, we hypothesize that the amide moiety increases the electrophilicity of the carbonyl, turning it into an accessible electron acceptor for the thiol of the reactive cysteines.

TRPV1

For rat TRPV1, a single reactive cysteine residue C157A has recently been characterized as a reactive residue for the stimulation by pungent sulfide compounds from garlic and onion (Salazar et

al., 2008). After verifying that this region is conserved across human, rat and mice, we generated the point mutant C158A in hTRPV1 channels. We then assessed if TRPV1 reactivity for the molecules tested was mediated by this cysteine. The results, shown in fig. 6, show that none of the compounds tested, with the exception of the cysteine-modifying agent MTSEA, gave a response suggesting these ligands act via different mechanisms on TRPV1 and TRPA1.

TRPV1 KO mice show diminished aversion to α-SOH and 6-shogaol

To quantify the taste aversion to α-SOH in the absence of TRPV1, we performed brief-access tests to both wild-type and TRPV1 KO mice when presented with 1 mM α-SOH over water. Our results indicate that α-SOH is largely aversive to wild-type animals (PR=0.24±0.15) however the suppression of TRPV1 fully reverses this aversion to indifference ($p<0.005$). When tested with 1 mM analog **I,** selected for its absence of agonistic activity on heterologously expressed TRPA1 (Fig 4A), WT mice showed decreased avoidance than observed for α-SOH ($p<0.05$). Interestingly, even though avoided by wild-type mice, it was not preferred over water for TRPV1 KO animals. Comparison between the lick ratio of α-SOH and analog **I** indicate that these compounds were not perceived as significantly different in the TRPV1 KO mice ($p>0.05$).

When presented with 6-shogaol, WT mice display a strong aversion (PR=0.19±0.08) that is largely decreased in TRPV1 KO ($p<0.001$). However, TRPV1 KO present still a residual aversive response to the compound as their PR is slightly negative (PR=0.36±0.1).

In both experiments, TRPV1 largely accounts for the negative perceptions associated with the compounds, however these results highlight the presence of an additional component to fully explain the aversive responses.

DISCUSSION

Our goal was to determine whether and how compounds present in *Zanthoxylum spp*. and *Aframomum Melegueta* stimulate sensory neurons via activation of TRPA1, TRPV1 and TRPM8 channels. First, we found that these molecules target TRPA1 and/or TRPV1 but not TRPM8. Second, by using unique synthetic analogues of α-SOH that mediate TRPA1 and TRPV1 activation, we evaluated the relative importance of unsaturations (number and position) in the molecule for activation of these TRP channels. Third, we found that the vanilloids, 6-shogaol and 6-paradol, stimulate both TRPV1 and TRPA1 but are 100 times more potent in activating TRPV1. We then assessed if these compounds stimulated TRPA1 and TRPV1 via covalent modification of cysteine residues located at the channel N-terminus. Our data indicate that a *cis* double bond at position 6 in α-SOH is important for TRPA1, but not TRPV1 specificity. In addition, covalent interactions occur with

TRPA1 stimuli possessing either an α, β double bond or an electrophilic carbon, however TRPV1 stimulation by pungent stimuli is independent of covalent modification. Finally, through a taste preference test, we show that the aversive effect of α-SOH and 6-shogaol is primarily accounted by TRPV1. Taken together these data show that even though a compound may activate both TRP channels they do so via different mechanisms.

Vanilloids and sanshools stimulate specifically TRPA1- and TRPV1- expressing DRGs

Several chemosensory TRP channels are expressed in neurons of the sensory ganglia: TRPA1 is found in a subpopulation of nociceptive neurons where it is co-expressed with TRPV1, but not with TRPM8 (Story et al., 2003a). Also, TRPV1 and TRPM8 are found in distinct neuronal populations (Peier et al., 2002a). In agreement with these expression patterns, we identified a subpopulation of cinnamaldehyde-sensitive DRG neurons (19% of neurons) that also responded to capsaicin (TRPV1), but not to menthol (TRPM8). We found that 30% of neurons responded to capsaicin and 20% responded only to menthol. The remaining 50% of DRGs were not stimulated by any of the agonists. The lack of response in neurons might be interpreted either as an absence of effect or as an inhibitory effect produced by the compounds (see physiological section).

Stimulation with sanshools and hydroxyarylalkanones induced calcium influx in populations of neurons responding to capsaicin and cinnamaldehyde, but not to menthol (Fig. 2). None of the compounds tested, including linalool, stimulated menthol-sensitive neurons, even at their maximal concentrations (Fig. 2F). Linalool's absence of effect on TRPM8-expressing neurons is consistent with it being only a weak agonist in heterologously expressed TRPM8 channels (Behrendt et al., 2004). Our results showing that α-SOH had no effect on DRG neurons not responding to capsaicin diverge from another recent study where it was found that low-threshold mechanoreceptors neurons were stimulated by long applications of this compound in addition to capsaicin responding neurons (Bautista et al., 2008). These differences may be interpretated in term of temporal activation as we applied very short stimuli latency to avoid channels desensitization.

Vanilloids 6-shogaol and 6-paradol stimulate TRPA1 and TRPV1 channels

Activation of TRPV1 by 6-shogaol and common gingerols (Iwasaki et al., 2006) is consistent with their burning sensory profile (Govindarajan, 1982). Gingerols are highly similar to the shogaols and paradols, 6-gingerol differing from 6-paradol only by a single hydroxyl group at C6 of the alkyl chain. Increasing the hydrophilicity of these compounds in the transition of 6-shogaol to 6-gingerol (see Fig. S5) coincided with about a 10 fold decrease in potency on TRPV1 (Dedov et al., 2002).

Given its structural similarity to 6-shogaol, 6-paradol stimulation of TRPV1 was not surprising. However, as a consequence of the missing α, β double bond 6-paradol is about four times less potent than 6-shogaol. The presence of this bond might change steric interactions by altering configuration and stiffness of the molecule in the TRPV1 binding pocket. The concentration-response profile for 6-shogaol and 6-paradol clearly indicates the less potent nature of these compounds than capsaicin and maximum activations inferior to capsaicin (Table 1). The dramatic changes in the Hill coefficients from capsaicin to 6-paradol is not understood, but probably do not mean that 'N' molecules are needed to activate the channel.

In addition to their effect on TRPV1, our results suggest that 6-shogaol and 6-paradol are agonists at TRPA1-expressing DRG neurons. This effect was confirmed by challenging them in heterologously expressed hTRPA1 cells (Fig 4A). We found that the two vanilloids stimulated both TRPA1 and TRPV1 in a dose-dependent manner, with a 100 fold stronger potency on TRPV1 (Fig. 4 B & D), likely because of the better fit of the vanilloid moiety in the TRPV1 binding pocket.

In addition, 6 shogaol and 6-paradol were less potent than cinnamaldehyde on TRPA1 with weaker maximum responses. Bandell et al. characterized 8-gingerol's activity on TRPA1 (Bandell et al., 2004a). The gingerols, shogaols and paradols differ from the non TRPA1 agonist capsaicin mainly by the amide moiety in the alkyl chain, suggesting that the phenol core is not sufficient to confer TRPA1 specificity.

Role of the *cis* C6-double bond in the structure-activity-relationship of α-SOH on TRPA1 and TRPV1

To determine the structure-activity relationship defining α-SOH recognition properties on TRPA1 and TRPV1, we investigated the role of the double bonds in the polyenic chain using the synthetic analogues **I-IV**.

Our detailed characterization of the effects of α-SOH and its analogues on TRPA1 and TRPV1 establish that these compounds are receptor agonists with a diminished efficacy compared to the control compounds cinnamaldehyde and capsaicin. The incapacity of these alkylamides to produce total activation of the channels may arise from their temporal effects such as receptor desensitization or shorter open times.

For α-SOH our data show that the *cis* C6 bond is critical for TRPA1, but not for TRPV1 activity (Figs 4A and 4C). In this regard, the fully saturated (**I**) and α, β unsaturated (**II**) α-SOH analogues produce small TRPA1 responses while the *cis* C6 di-unsaturated (**III**) and *cis* C5 mono (**IV**) analogues stimulated TRPA1 to practically the same extent than α-SOH (Fig. 4A), thereby highlighting the role of the *cis* double bond in the tail. Even though we did not test the *cis* C6 mono-unsaturated analogue, our data show that the *cis* C5 compound stimulate TRPA1 and TRPV1 with similar potency as compound **III** suggesting the placement of this unsaturation at either C5 or C6 provides similar effects on the channels. The other mono-unsaturated and fully saturated compounds display the ability to stimulate TRPV1 potently, even though small differences in efficacy were observed (Fig. 4C). These relatively small changes in efficacy are consistent with small decreases in hydrophobicity or molecular flexibility of the tested compounds as α-SOH being the most unsaturated, is also the most potent. Taken together, the observed structure-activity relationship shows that α-SOH is recognized differently by TRPA1 and TRPV1 channels.

α, β unsaturation of alkylamides does not provide TRPA1 specificity and is moderately required in shogaols to activate TRPA1.

Thiol-reactive pungent chemicals from mustard, garlic and cinnamon have been shown to activate TRPA1 by covalent modification of N-terminal cysteine residues (Macpherson et al., 2007a;Hinman et al., 2006). In contrast to its *cis* isomer, the 6 *trans* hydroxy-*β*-sanshool contains an α, β conjugated bond but does not stimulate TRPA1, (Koo et al., 2007). The poor effect on TRPA1 of the α, β unsaturated analogue (**II**) was unexpected (Figs. 4A & 5E) because compounds with α, β unsaturation have always been shown to be TRPA1 agonists, as they react as Michael acceptors for intracellular reactive cysteines (Macpherson et al., 2007b). This effect does not seem to be due to hampered membrane permeation as another monounsaturated molecule with the same chain length (**IV**) and hydrophobicity stimulates TRPA1 through the N-terminal cysteines (Figs. 4A & 5F).

We have made the important observation that covalent bonding at the electrophilic carbonyl (see Fig. S4) occurs with all tested TRPA1 reactive alkylamides via the intracellular cysteines (Fig. 5D, E, F & G). Indeed, independent of its unsaturations, GSH forms covalent adducts with any of the alkylamide tested (see S4). However, TRPA1 activity cannot be rationalized just in terms of covalent binding to a reagent as the configuration of the *cis* C6 unsaturation in the alkylamides clearly determines their effect on TRPA1 (see Fig. 4A).

Stimulation of TRPA1 by 6-shogaol and 6-paradol shows an interesting example of compounds acting via covalent and non-covalent interactions. To this point, the α, β unsaturation of 6-shogaol

increases TRPA1 efficacy and the compound partially targets the three reactive cysteines of TRPA1 (Fig. 5H &I).

TRPV1 reactivity to pungent chemicals does not require covalent binding at the intracellular cysteine C158.

Pungent extracts from onion and garlic that stimulate both TRPA1 and TRPV1 channels act on TRPV1 by covalent modification of one cysteine residue of rat TRPV1, C157A (Salazar et al., 2008). By analogy, we looked for similar effects of the sanshools and the hydroxyarylalkanones. However, among the molecules that covalently bind to TRPA1, none activated TRPV1 through its reactive cysteine (Fig. 6).

Possible Physiological implications

Among the alkylamides in Sichuan pepper, the α-sanshools (*cis* C6) are characterized as tingling and burning and β-sanshools (*trans* C6) as bitter and numbing. Interestingly, hydroxy-β-sanshool evoked a moderate TRPV1 activation (Sugai et al., 2005a) and did not activate TRPA1 (Koo et al., 2007) suggesting that the *cis* configuration of this specific C6 double bond is required to produce the tingling of α-SOH. Beside a *cis* C6 stereospecificity of TRPA1, it is unlikely that TRPA1 mediates the tingling sensation as none of its agonists produce this sensation. The numbing sensation has been recently attributed to the modulation of two-pore potassium channels (Bautista et al., 2008). Based on these assumptions, we speculate the sensory properties of the synthetic analogues **I**-**IV** to elicit burning whereas only compounds **III** and **IV** might be perceived as tingling. The observed behavioural responses of the TRPV1 KO mice show that TRPV1 accounts fully for the aversive response of analogue **I** and α-SOH with no apparent support to the pharmacology observed in vitro on TRPA1 and TRPV1 and thus to a TRPA1 aversive effect but rather explaining the differences in potency at the TRPV1 level.

Sichuan oil is rich in linalool, which stimulates TRPA1 in the micromolar range but does not stimulate TRPV1 (Fig 4B & D). Our technical sensory trial revealed that the compound is neither burning nor tingling but elicited a weak but unpleasant taste sensation and a strong floral odor. Clearly, the absence of pungency of this compound raises the question as to why linalool which activates TRPA1 is not pungent. It is well documented that the pungent chemicals cinnamaldehyde, allyl-isothiocyanate and garlic derivatives potently target TRPA1 (Bandell et al., 2004a;Jordt et al., 2004;Macpherson et al., 2005;Bautista et al., 2005). Behavioral data on TRPA1 KO mice presented with mustard oil suggest that TRPA1 is partially involved in the aversion to allyl-isothiocyanate (Kwan et al., 2006) while TRPV1 contribution to capsaicin pungency is well documented (Caterina et al.,

2000). However, in our behavioural results (Fig. 7B), it is observed a residual aversive response to the taste of 6-shogaol when TRPV1 is suppressed which could be accounted to a TRPA1 stimulation. Contrary to α-SOH, TRPV1 KO mice retain ability to detect 6-shogaol which is correlated to TRPA1 higher affinity to 6-shogaol. These findings suggest that from our tested compounds, TRPV1 mediates most of the aversion whereas TRPA1 may convey minor aversive response for its high affinity ligands such as 6-shogaol.

In addition to its pungent organoleptic properties, α-SOH evokes a characteristic numbing paresthesia (Bryant and Mezine, 1999). The authors reported that α-SOH applied to the tongue excited tactile sensitive fibers of the lingual branch of the rat trigeminal nerve. The origin of the numbness is now thought to result from inhibitory effects of α-SOH on two-pore potassium channels in tactile fibers (Bautista et al., 2008). In this regard α-SOH may act like local anaesthetics such as lidocaine which diffuse into nociceptive neurons and block their excitability (Cahalan, 1978). In our brief-access tests, these effects were masked by the temporal latency of the numbness which prevents the mice from identifying the origin of the stimulus but also meaning that numbness probably doesn't affect taste preference. It is striking that in our DRG experiments we did not observe a calcium response in the other 50% of the DRG population of neurons than the TRPA1, TRPM8 and TRPV1 expressing cells - however, this would be consistent with a neuronal inhibitory effect of α-SOH.

Summary

We found that the detection of pungent tasting molecules from Sichuan and Melegueta peppers is mediated at least in part, by TRPA1 and TRPV1, and their implication may rationalize the pungent properties of both the alkylamides and hydroxyarylalkanones. Finally, while TRPV1 stimulation by these molecules operates through non-covalent binding, TRPA1 responses present complex interactions of stereoselectivity and cooperation between covalent and non-covalent gating.

FIGURE LEGENDS

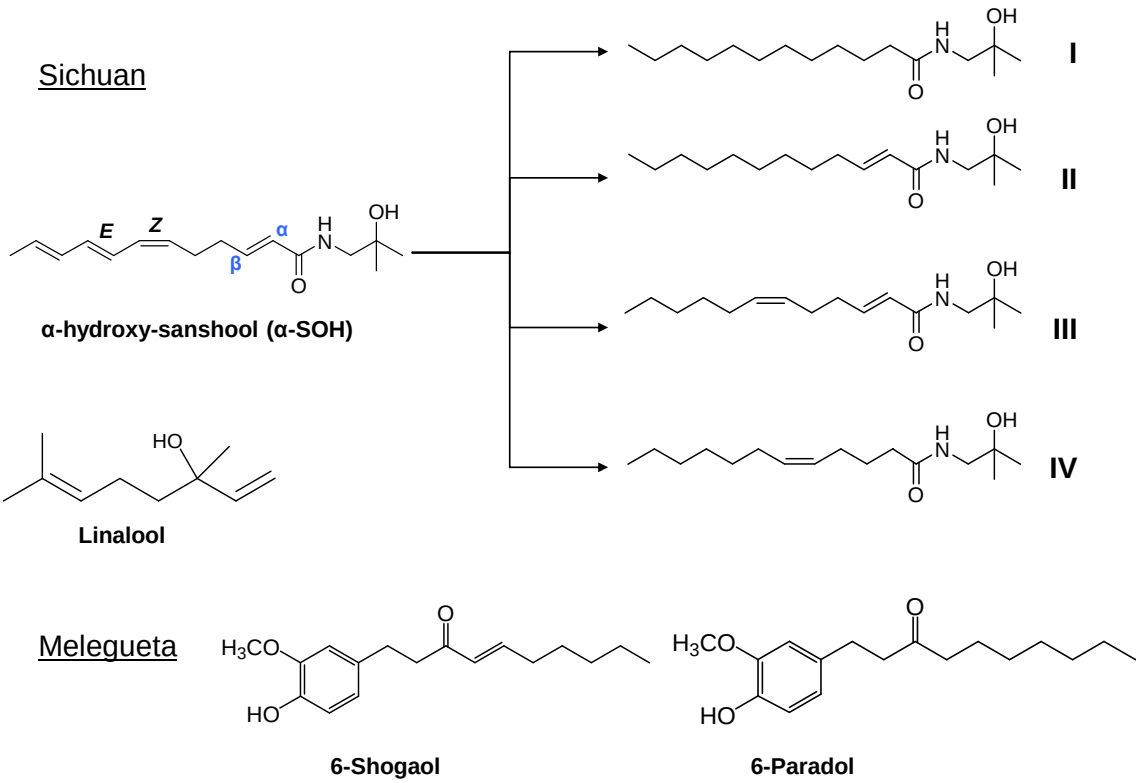

Figure 1. Chemical structures of compounds tested to determine if they activate TRPV1, TRPA1 and TRPM8 channels.

The natural compounds are contained in Sichuan (α-hydroxy-sanshool and linalool) and Melegueta (6-paradol and 6-shogaol) peppers, whereas the synthetics **(I–IV**) are analogues of α-SOH**.** The four synthetic analogues **I, II, III** and **IV** were tested to obtain their structure–activity relations. Linalool, is a monoterpene that markedly differs from the sanshools. The vanilloids 6-paradol and 6-shogaol only differ from each other in the α, β unsaturation.

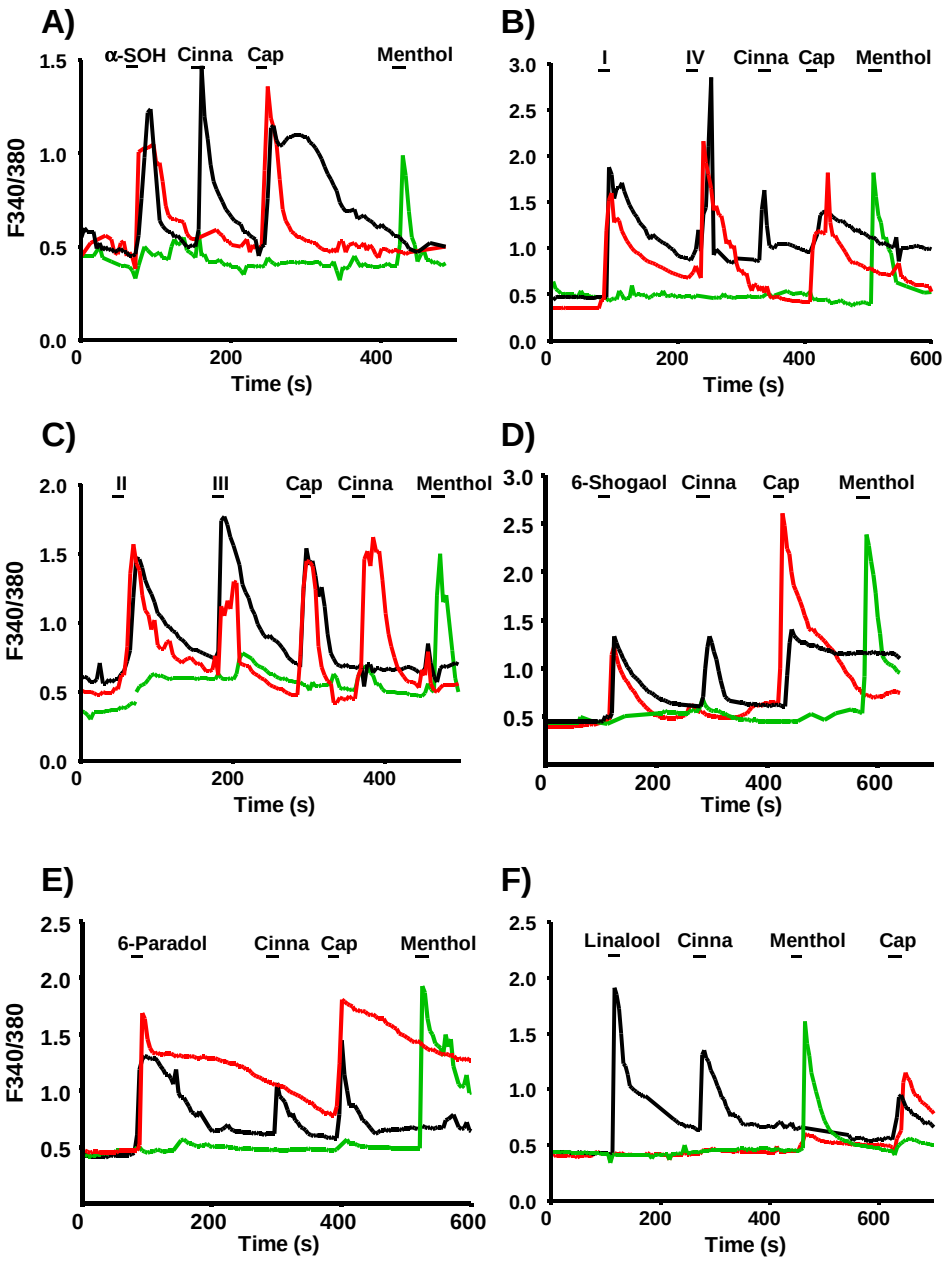

Figure 2. Sanshools, 6-shogaol, 6-paradol and linalool activate Capsaicin and Cinnamaldehyde sensitive DRG neurons.

Each panel shows representative responses of three neurons to changes in $[Ca2+]_i$. Cap–sensitive (TRPV1) neurons are in red, Cinna-sensitive (TRPA1) are in black and Menthol-sensitive (TRPM8) are shown in green. Compounds were applied successively for 5s, as shown by horizontal bars. Between stimulations neurons were perfused with supplemented HBSS. Maximal concentrations were used in these experiments. (**A**): 500µM α-SOH, 100 µM Cinna, 1µM Cap, 500 µM Menthol; (**B**) 500µM **I**, 500µM **IV**, 100 µM Cinna, 1µM Cap, 500 µM Menthol; (**C**) 500µM **II**, 500µM **III**, 1µM Cap, 100 µM Cinna, 500 µM Menthol; (**D**) 500µM 6-shogaol, 100 µM Cinna, 1µM Cap, 500 µM Menthol ; (**E**) 500µM 6-paradol, 100 µM Cinna, 1µM Cap, 500 µM Menthol; (**F**) 500µM linalool, 100 µM Cinna, 500 µM Menthol,1µM Cap.

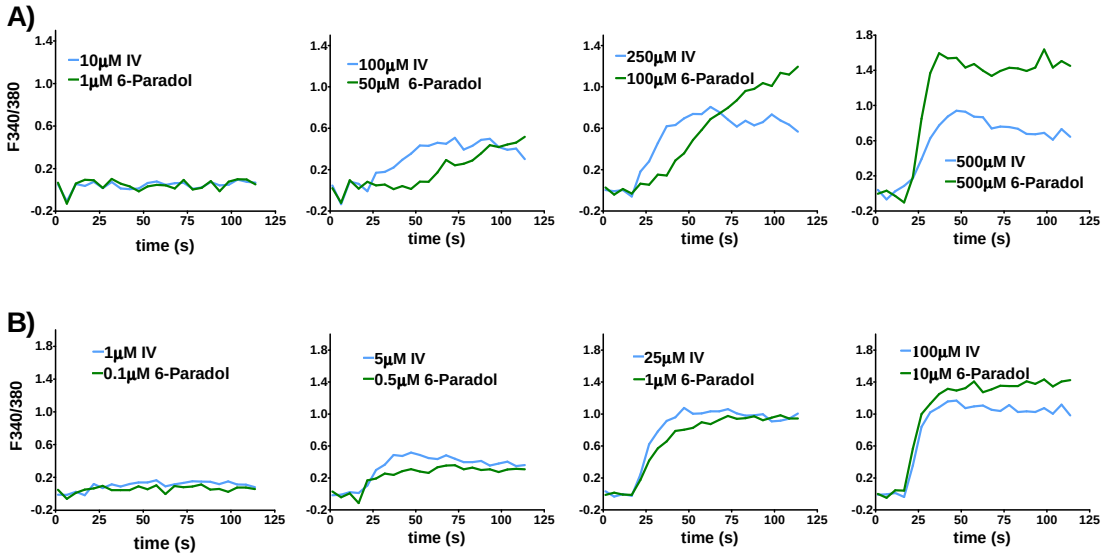

Figure 3. [Ca2+]$_i$ transients in TRPV1 and TRPA1-expressing HEK293 cells.

Typical traces obtained for TRPA1 and TRPV1 containing cells are shown for compound **IV** and 6-paradol. The changes in concentrations were obtained in separate wells. (**A**) TRPA1-expressing HEK293 cells respond to increased concentrations of **IV** (blue) and 6-paradol (green). (**B**) TRPV1-expressing HEK293 cells respond to increased concentrations of **IV** (blue) and 6-paradol (green). At the end of each of these test compounds, maximal concentrations of cinnamaldehyde (TRPA1) or capsaicin (TRPV1) were tested (not shown).

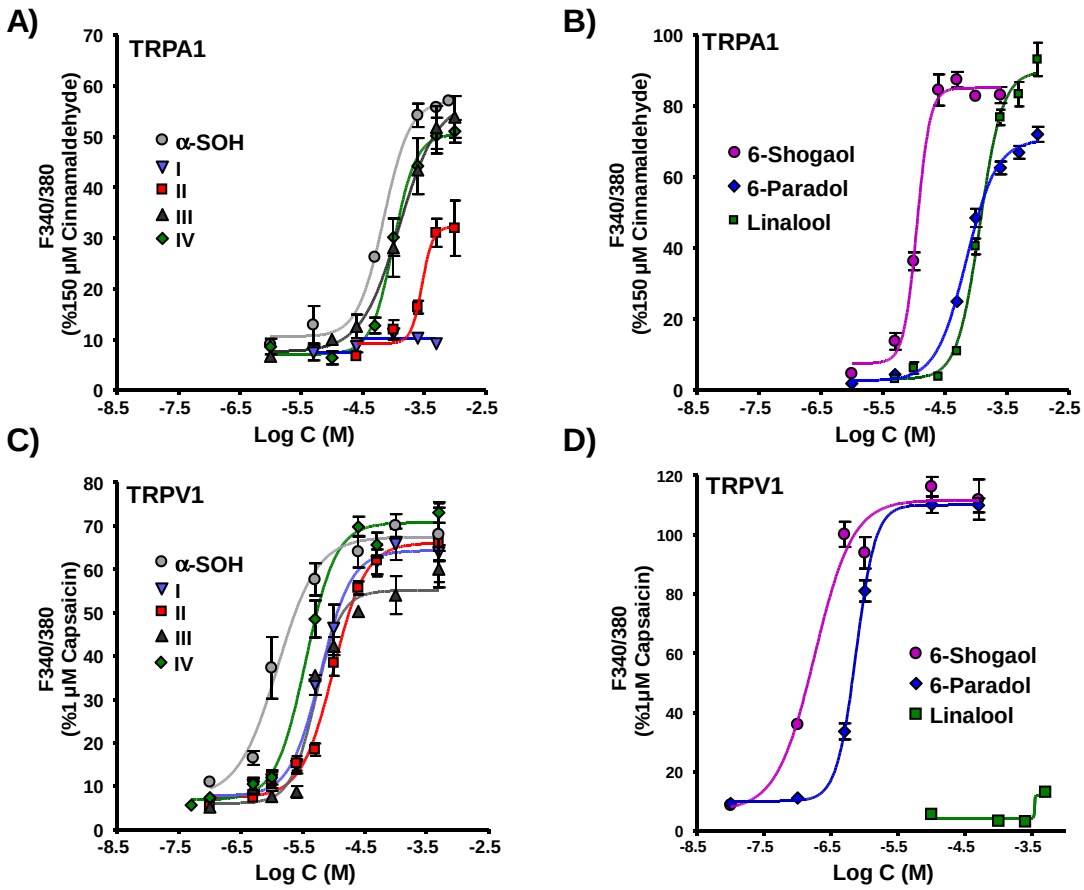

Figure 4. Dose-response profiles in HEK293 cells expressing TRPV1 or TRPA1.

Responses were normalized to maximal concentrations of cinnamaldehyde and capsaicin for TRPA1 and TRPV1, respectively. Data were fit to the Hill equation. **A** and **B** TRPA1. **B** and **C** TRPV1. (**A**) Dose-responses for α-SOH and its analogues **I-IV. I** and **II** evoke small changes. (**B**) Dose-responses of 6-shogaol, 6-paradol and linalool. (**C**) Dose-responses for α-SOH and its analogs on TRPV1. (**D**) Dose-responses of linalool, 6-shogaol and 6-paradol. Note that linalool has no effect on TRPV1 until 1mM.

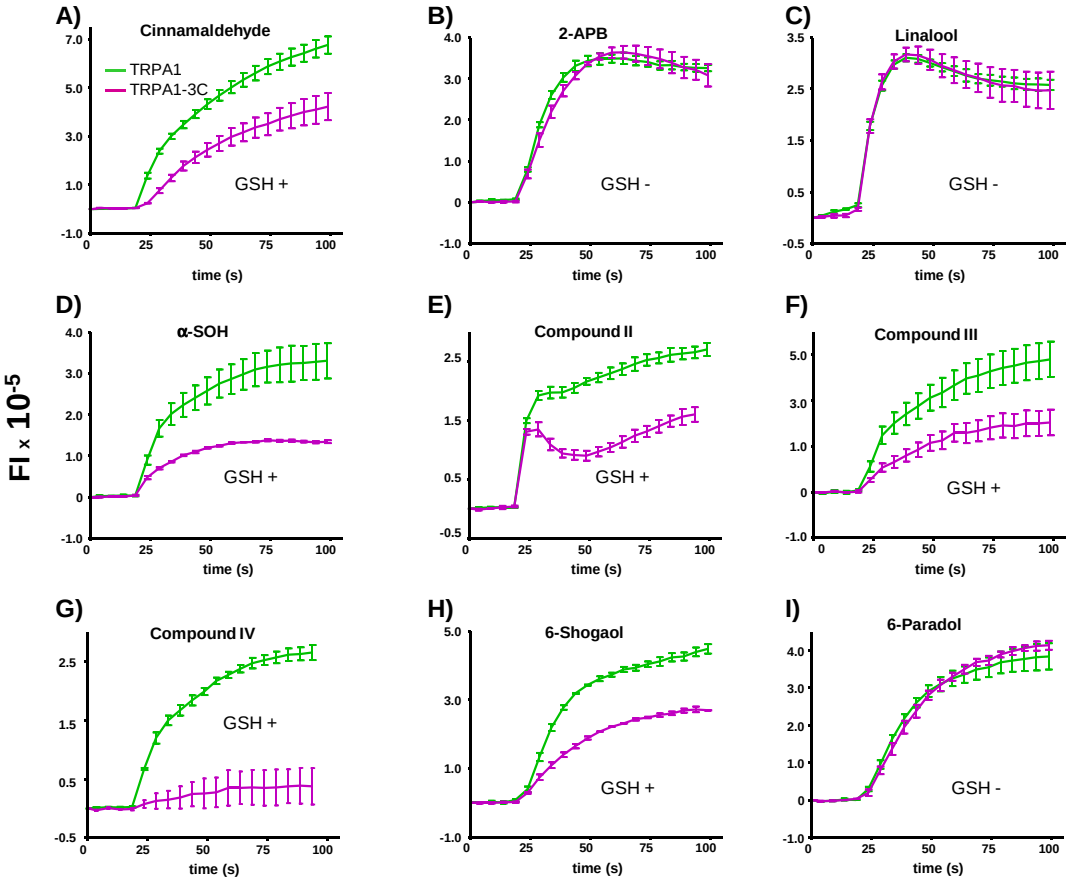

Figure 5. Selected compounds target the N-terminal reactive cysteines in TRPA1.

Voltage changes of HEK293 cells loaded with Red dye expressed as fluorescence intensity (FI) when stimulated with maximal concentrations of the tested compounds. Cells were transiently transfected with wild-type TRPA1 (green) and TRPA1-3C (purple) and stimulated with (**A**) 150 µM Cinna, (**B**) 100 µM 2-APB, (**C**) 500 µM linalool , (**D**) 500 µM α-SOH , (**E**) 500 µM **II**, (**F**) 500 µM **III**, (**G**) 500 µM **IV**, (**H**) 100 µM 6-shogaol, (**I**) 500 µM 6-paradol. Means ± S.E.M. (n=3).

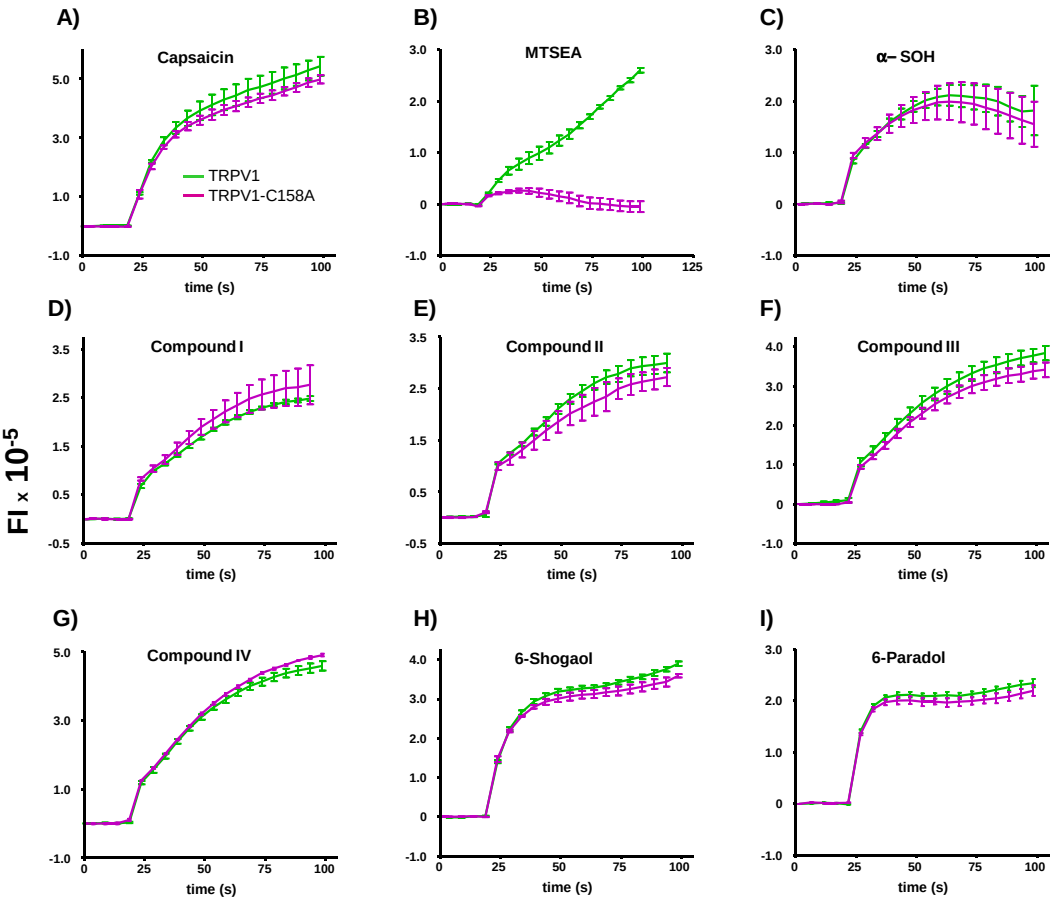

Figure 6. Selected compounds activate TRPV1 via non-covalent gating.

Voltage changes of HEK293 cells loaded with Red dye expressed as a fluorescence intensity (FI) when stimulated with saturating concentrations of compounds. Cells were transiently transfected with wild-type TRPV1 (green) and TRPV1-C158A (purple) and stimulated with (**A**) 1 µM Cap, (**B**) 2mM MTSEA, (**C**) 500 µM α-SOH, (**D**) 500 µM **I**, (**E**) 500 µM **II**, (**F**) 500 µM **III**, (**G**) 500 µM **IV**, (**H**) 100 µM 6-shogaol, (**I**) 100 µM 6-paradol. Means ± S.E.M. (n=3).

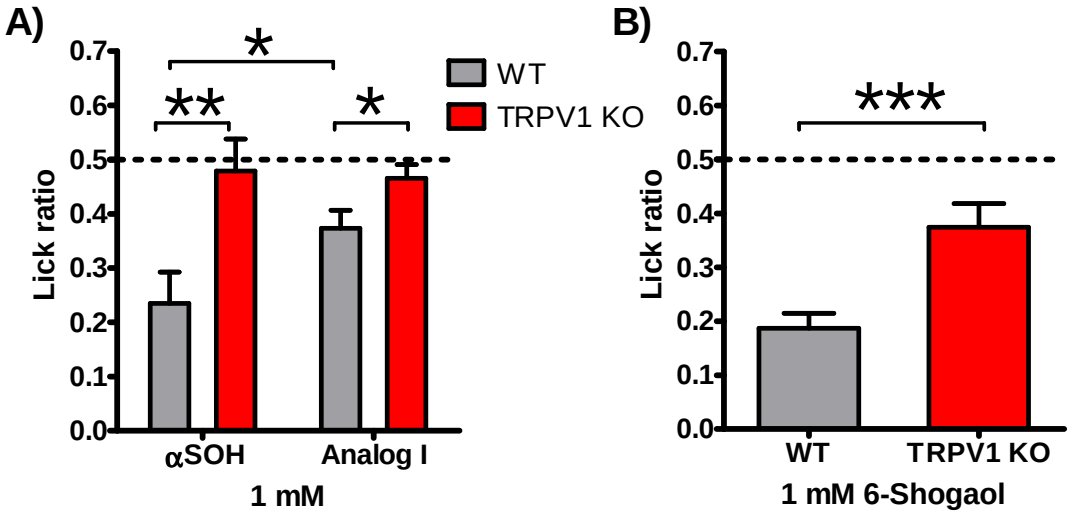

Figure 7. Brief-access taste preference comparing the responses of TRPV1 KO and WT mice.

Lick ratios of TRPV1 KO and WT mice for (A) 1mM α-SOH and 1mM **I**, (B) 1mM 6-shogaol. For each group, n=10 ± SEM. *p<0.05, **p<0.005, ***p<0.001, unpaired student *t*-test.

	TRPA1		TRPV1	
	EC_{50} (µM)	**Hill Coeff.**	**EC_{50} (µM)**	**Hill Coeff.**
Cinnamaldehyde	6.75	1.8	-	-
Capsaicin	-	-	0.037	0.5
α-SOH	69.4	2.0	1.1	0.8
I	-	-	7.0	1.4
II	-	-	10.2	1.8
III	125.2	1.4	5.0	2.5
IV	100.1	2.3	3.5	1.8
6-Shogaol	11.2	4.1	0.2	1.5
6-Paradol	70.7	1.8	0.7	3
Linalool	116.9	2.4	-	-

Table 1: EC50 values and Hill coefficients of selected TRPV1 and TRPA1 ligands.

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Supplemental Data

Organic chemistry materials.

All commercially available reagents were used as received, from their respective suppliers. The Green Sichuan extract was purchased from Hela (Singapour) and was obtained by CO_2 extraction from a chinese specie (*Zanthoxylum* spp.). Analytical thin layer chromatography (TLC) was carried out on Silica 60 F254 (Merck) and plates. The TLC plates were visualized by shortwave UV light, ceric ammonium molybdate stain. Flash chromatography was performed using a Biotage SP1 HPFC system and FLASH cartridge (25+M and 40+M KP-SIL). *^{1}H NMR (360.13 MHz) and ^{13}C NMR (90.56 MHz) spectra* were recorder on a Bruker DPX-360 spectrometer equipped with a broadband multinuclear z-gradient probehead. The chemical shifts (in ppm) were expressed with respect to an internal reference (TMS or TSP). Multiplicities are reported as follows: s= singlet, d= doublet, t= triplet, q= quatruplet, m= multiplet, bs= broad singlet. HPLC chromatogram of purified green Sichuan extract was carried out with a column N-Nucleosil 100 (Macherey-Nagel, 5 um, C-18, 250 x 4 mm), with UV detection at 254 nm and a flow rate of 1 mL/min. Solvents used are A = 0.1% (g/vol) TFA in H_2O, and B = Acetonitrile.

S1- Purification of Green Sichuan Extract

800 mg of Green Sichuan extract were purified by chromatography column on silica gel using a gradient of ethyl acetate in petroleum ether. Column *FLASH* 40+M was used on a Biotage HPFC Purification system with a flowrate of 40 ml/min. The hydroxy-sanshools were detected at 275 nm and collected in 21 ml fractions. Fractions were concentrated under reduced pressure to give finally 200 mg of hydroxy-sanshools as a mixture of 3 isomers which are not separable by this technique of purification (25% yield in weight). The ratio after purification was established by HPLC and stayed unchanged (*m*/α/β 3.7/75.4/20.8); R_f = 0.35 (Ethyl acetate); *α-Hydroxy-sanshool* ^{1}H NMR (360 MHz, $CDCl_3$, TMS as reference) δ 6.88 (dt, *J* = 15.3, 6.7 Hz, 1H), 6.36-5.92 (m, 5H), 5.84 (dt, *J* = 15.3, 1.3 Hz, 1H), 5.74 (dt, *J* = 13.8, 6.5 Hz, 1H), 5.37 (dt, *J* = 10.8, 6.7 Hz, 1H), 3.32 (d, *J* = 6.1 Hz, 2H), 2.54 (s, 1H), 2.36-2.27 (m, 4H), 1.78 (d, *J* = 6.7 Hz, 3H), 1.23 (s, 6H); ^{13}C NMR (90 MHz, $CDCl_3$, TMS as reference) δ 166.95, 144.47, 133.51, 131.77, 130.2, 129.64, 129.53, 125.44, 123.70, 71.06, 50.41, 32.07, 27.34, 26.46, 18.34; *β-Hydroxy-sanshool* ^{13}C NMR (90 MHz, $CDCl_3$, TMS as reference) δ 166.93, 144.35, 132.03, 131.63, 131.57, 131.44, 130.07, 129.39, 123.71, 71.06, 50.42, 31.90, 31.37, 27.34, 18.29.

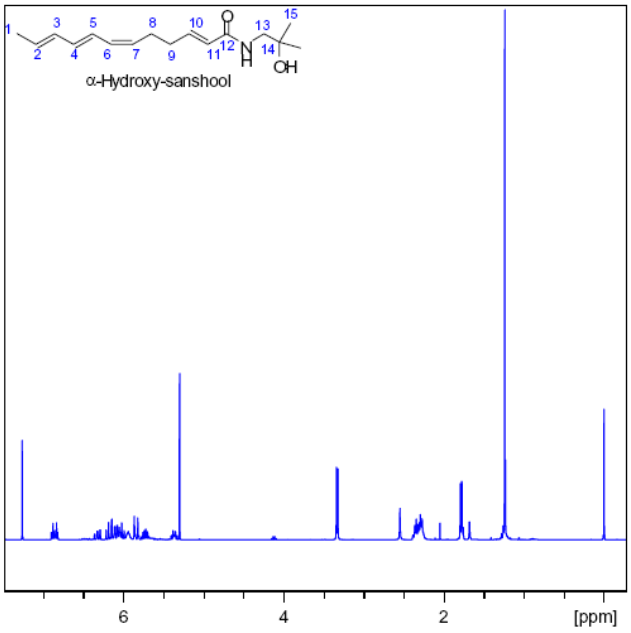


Fig. S1: ^{1}H NMR (360 MHz, $CDCl_3$, TMS as reference) of purified Sichuan extract

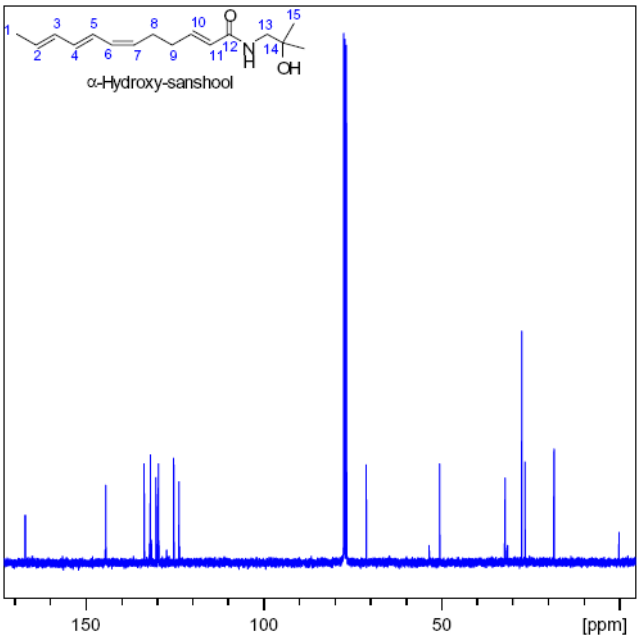
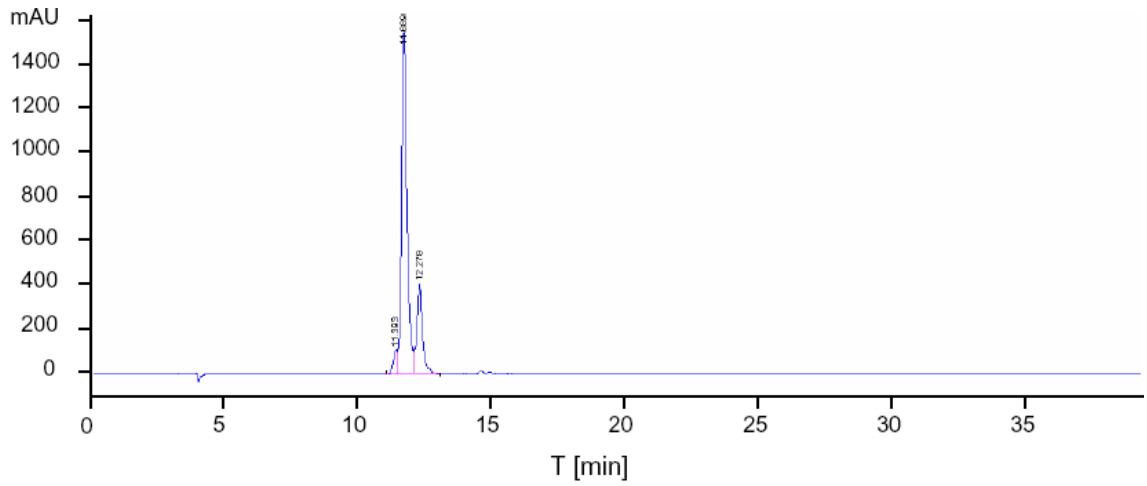


Fig. S2: ^{13}C NMR (90 MHz, $CDCl_3$, TMS as reference) of purified Sichuan extract



Peak No	Ret Time [min]	Width [min]	Area [mAu*s]	% Area
Unidentified OH-Sanshool *m*	11.393	0.1528	1094.8	3.743
α-OH-Sanshool	11.689	0.2074	22077.3	75.477
β-OH-Sanshool	12.279	0.2114	6078.5	20.781

Fig. S3: HPLC Chromatogram of purified Sichuan extract

S2- Chemical synthesis of I, II, III, IV.

Sanshool analogues **I-IV** were prepared from the corresponding carboxylic acids and 1-amino-2-methylpropan-2-ol by classical coupling conditions (di-*iso*propylethylamine, benzyltriazol-1-yloxy-tripyrrolidinophosphonium hexafluorophosphate in DMF).

***N*-(2-Hydroxy-2-methylpropyl)-dodecanamide I.** white solid; ^{1}H NMR (360 MHz, $CDCl_3$, TMS as internal reference) δ 6.48 (s, 1H), 3.28 (d, *J* = 5.7 Hz, 2H), 3.17 (s, 1H), 2.28 (t, *J* = 7.5 Hz, 2H), 1.67-1.61 (m, 2H), 1.34-1.23 (m, 16H), 1.23 (s, 6H), 0.87 (t, *J* = 6.6 Hz, 3H); ^{13}C NMR (90 MHz, $CDCl_3$, TMS as internal reference) δ 177.83, 73.71, 53.41, 39.28, 34.71, 32.41, 32.29, 32.14, 32.10, 30.05, 28.74, 25.49, 16.92.

(2*E*)-*N*-(2-Hydroxy-2-methylpropyl)-dodec-2-enamide II. pale orange oil; ^{1}H NMR (360 MHz, $CDCl_3$, TMS as internal reference) δ 6.79 (dt, *J* = 15.3, 7.0 Hz, 1H, H-10), 6.03 (s, 1H), 5.75 (dt, *J* = 15.2, 1.5 Hz, 1H), 3.26 (d, *J* = 6.0 Hz, 2H), 2.06 (qd, *J* = 7.7, 1.4 Hz, 2H), 1.40-1.34 (m, 2H), 1.24-1.17 (m, 12H), 1.16 (s, 6H), 0.81 (t, *J* = 6.7 Hz, 3H); ^{13}C NMR (90 MHz, $CDCl_3$, TMS as internal reference) δ

167.35, 145.79, 123.23, 71.14, 50.57, 32.21, 32.00, 29.93, 29.55, 29.42, 29.33, 28.36, 27.42, 22.79, 14.23.

(2*E*-6*Z*)-*N*-(2-Hydroxy-2-methylpropyl)-dodeca-2,6-dienamide III. (ratio (6*Z*-2*E*)/(6*E*-2*E*) 87.5/12.4); colourless oil; ^{1}H NMR (360 MHz, $CDCl_3$, TMS as internal reference) δ6.87 (dt, *J* = 15.3, 6.7 Hz, 1H), 5.95 (s, 1H), 5.83 (d, *J* = 15.4 Hz, 1H), 5.44-5.30 (m, 2H), 3.33 (d, *J* = 5.9 Hz, 2H), 2.59 (s, 1H), 2.27-2.15 (m, 4H), 2.04-1.97 (m, 2H), 1.36-1.21 (m, 6H), 1.23 (s, 6H), 0.88 (t, *J* = 6.5 Hz, 3H); ^{13}C NMR (90 MHz, $CDCl_3$, TMS as internal reference) δ 166.99, 144.81, 131.21, 127.89, 123.34, 71.04, 50.41, 32.19, 31.47, 29.27, 27.31, 27.22, 25.91, 22.54, 14.05.

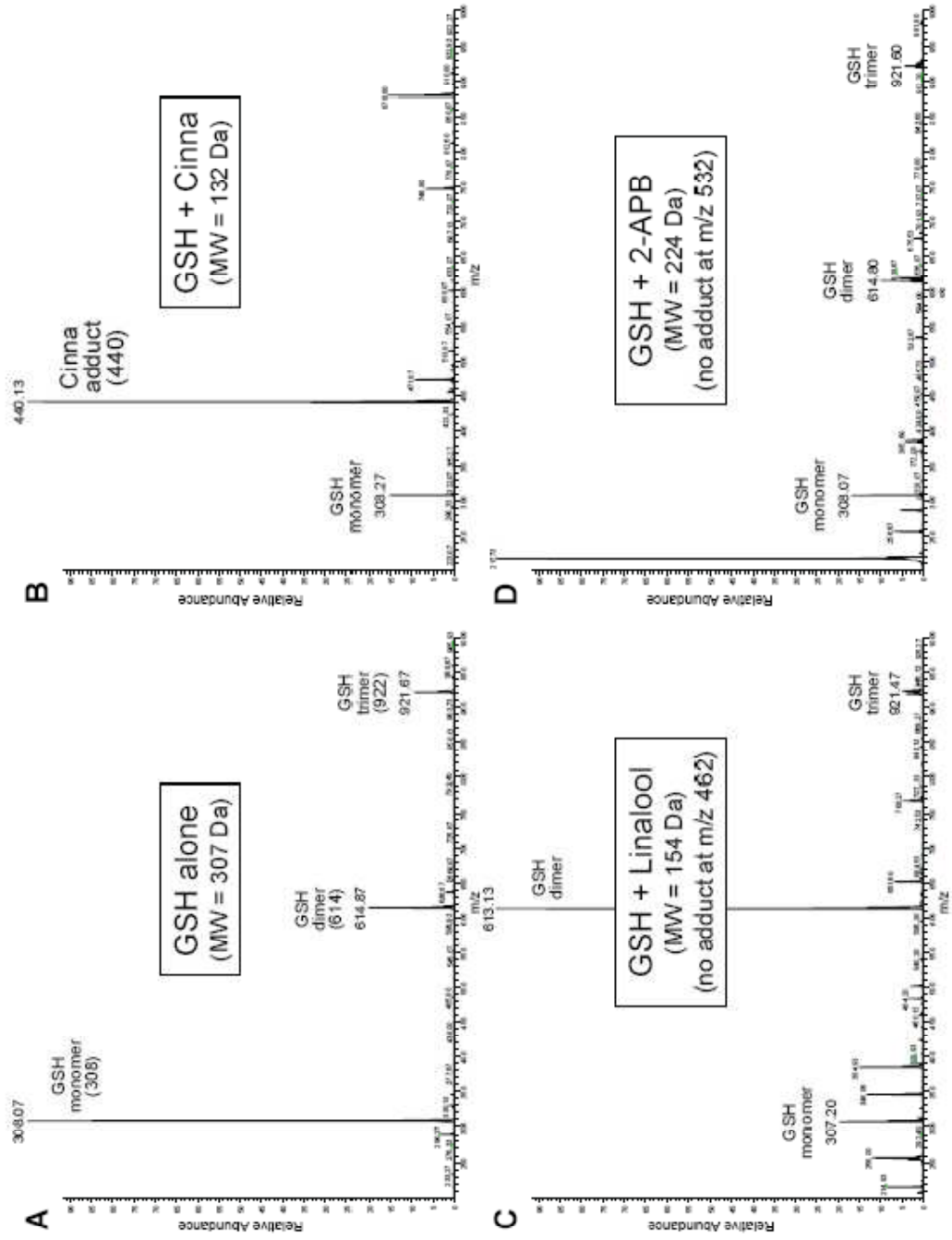
(5*Z*)-*N*-(2-Hydroxy-2-methylpropyl)-dodec-5-enamide IV. pale orange oil; ^{1}H NMR (360 MHz, $CDCl_3$, TMS as internal reference) δ5.87 (s, 1H), 5.37-5.22 (m, 2H), 3.19 (d, *J* = 6.0 Hz, 2H), 2.16 (t, *J* = 7.4 Hz, 2H), 2.01 (q, *J* = 6.9 Hz, 2H), 1.93 (q, *J* = 6.4 Hz, 2H), 1.64 (q, *J* = 7.5 Hz, 2H), 1.27-1.15 (m, 8H), 1.15 (s, 6H), 0.81 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR (90 MHz, $CDCl_3$, TMS as internal reference) δ 176.95, 133.96, 131.21, 73.73, 53.13, 39.94, 34.57, 32.48, 31.80, 30.10, 30.09, 29.48, 28.56, 25.45, 16.91.

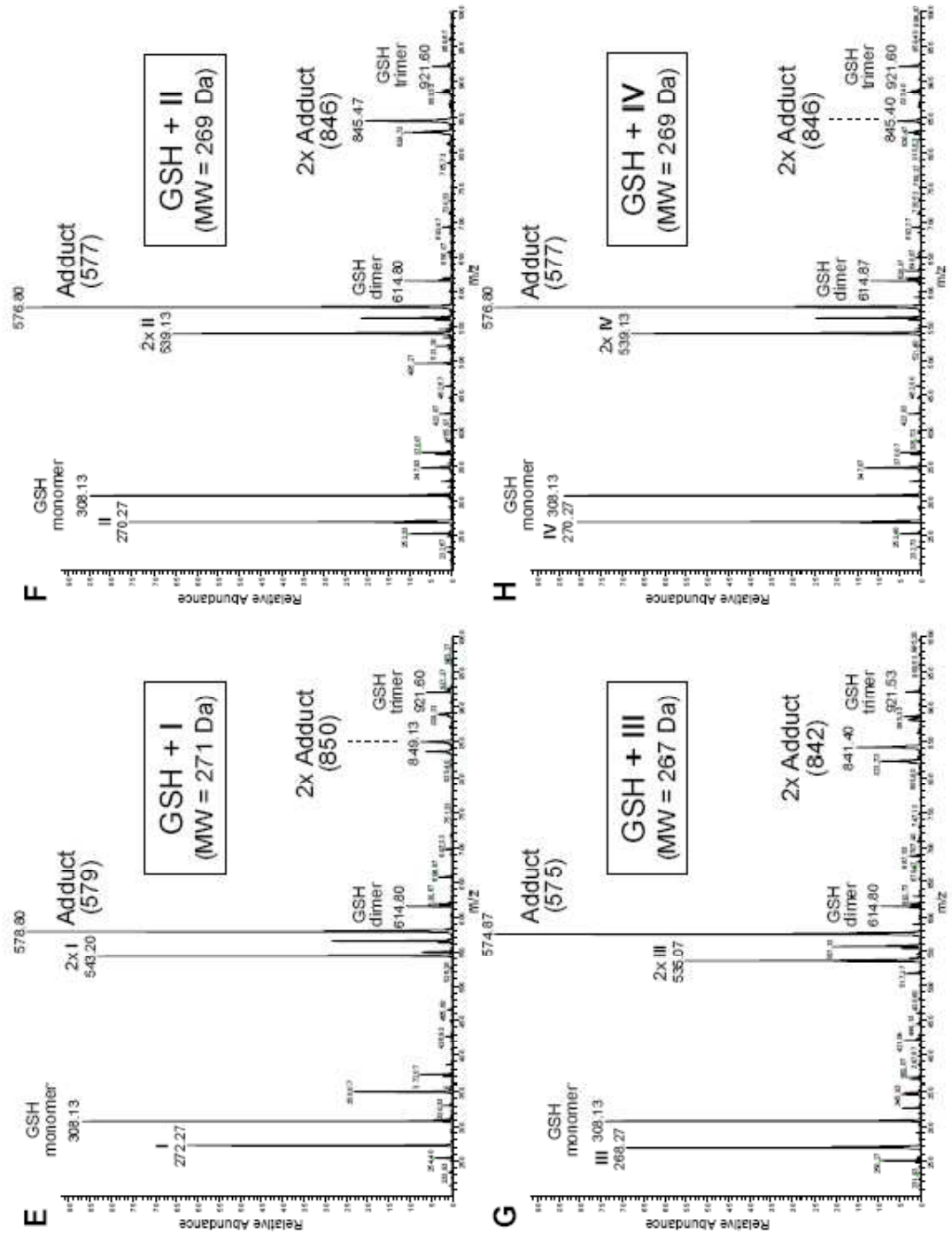
S3- Synthesis of 6-Paradol.

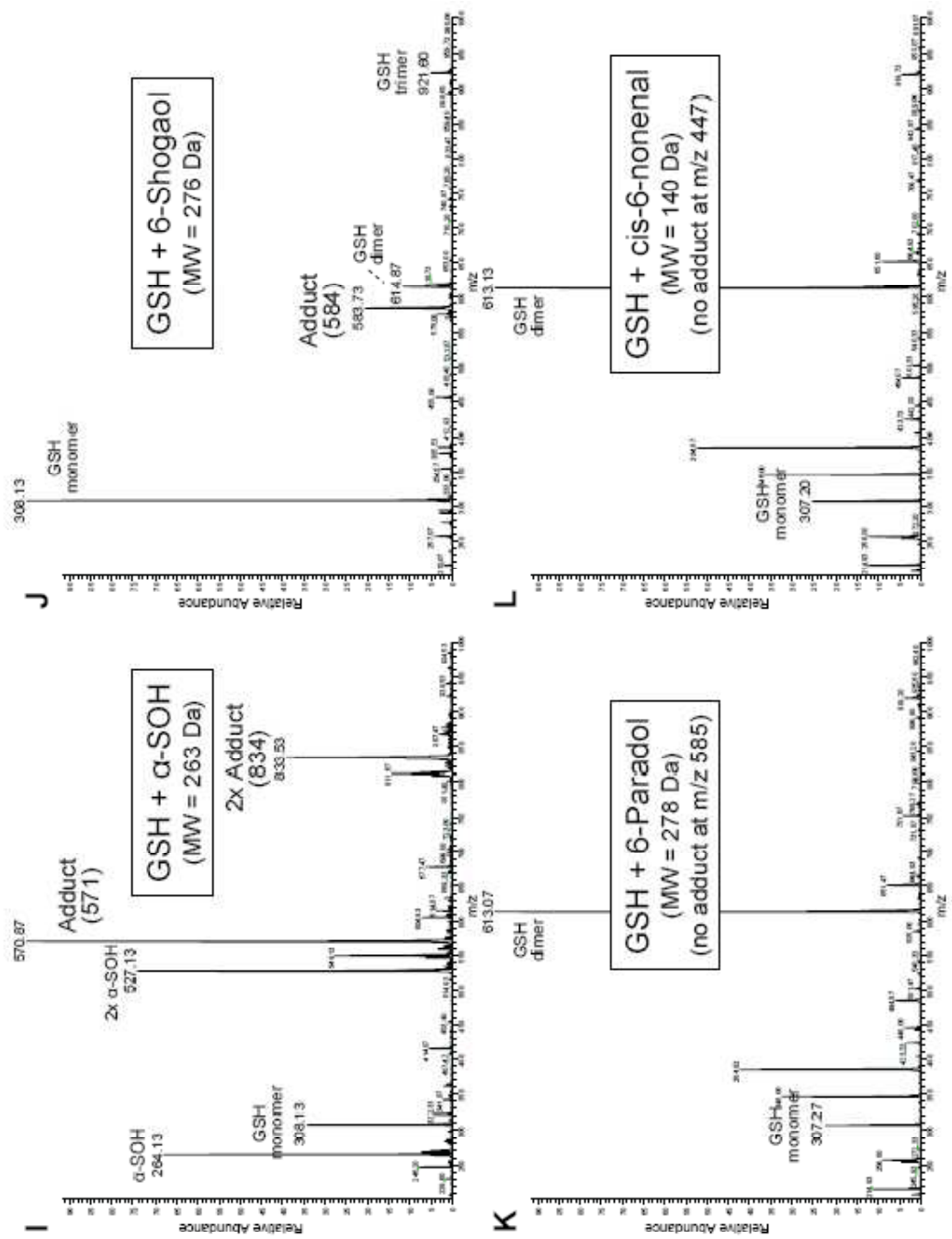
To a solution of 6-Shogaol (80 mg, 0.289 mmol, 1 equiv) in ethanol (5 mL), Pd/C (5%, 80 mg) was added at room temperature. The solution was stirred 2 h at the same temperature under hydrogen atmosphere. After total consumption of starting materials, the reaction mixture was filtered through Celite and concentrated under reduced pressure. The liquid residue was purified by flash column chromatography on silica gel using a gradient of ethyl acetate in petroleum ether. 68 mg of 6-Paradol (0.244 mmol, 84%) were isolated as a colourless oil; R_f = 0.42 (Petroleum Ether/ Ethyl acetate 80/20); ^{1}H NMR (360 MHz, CDCl3, TMS as reference) δ 6.82 (d, *J* = 8.3 Hz, 1H), 6.69-6.65 (m, 2H), 3.87 (s, 3H), 2.84-2.80 (m, 2H), 2.71-2.66 (m, 2H), 2.36 (t, *J* = 7.8 Hz, 2H), 1.51-1.50 (m, 2H), 1.26-1.24 (m, 8H), 0.87 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (90 MHz, CDCl3, TMS as reference) δ 210.62, 146.40, 143.84, 133.17, 120.75, 114.27, 111.01, 55.86, 44.62, 43.15, 31.66, 29.53, 29.17, 29.06, 23.80, 22.60, 14.07.

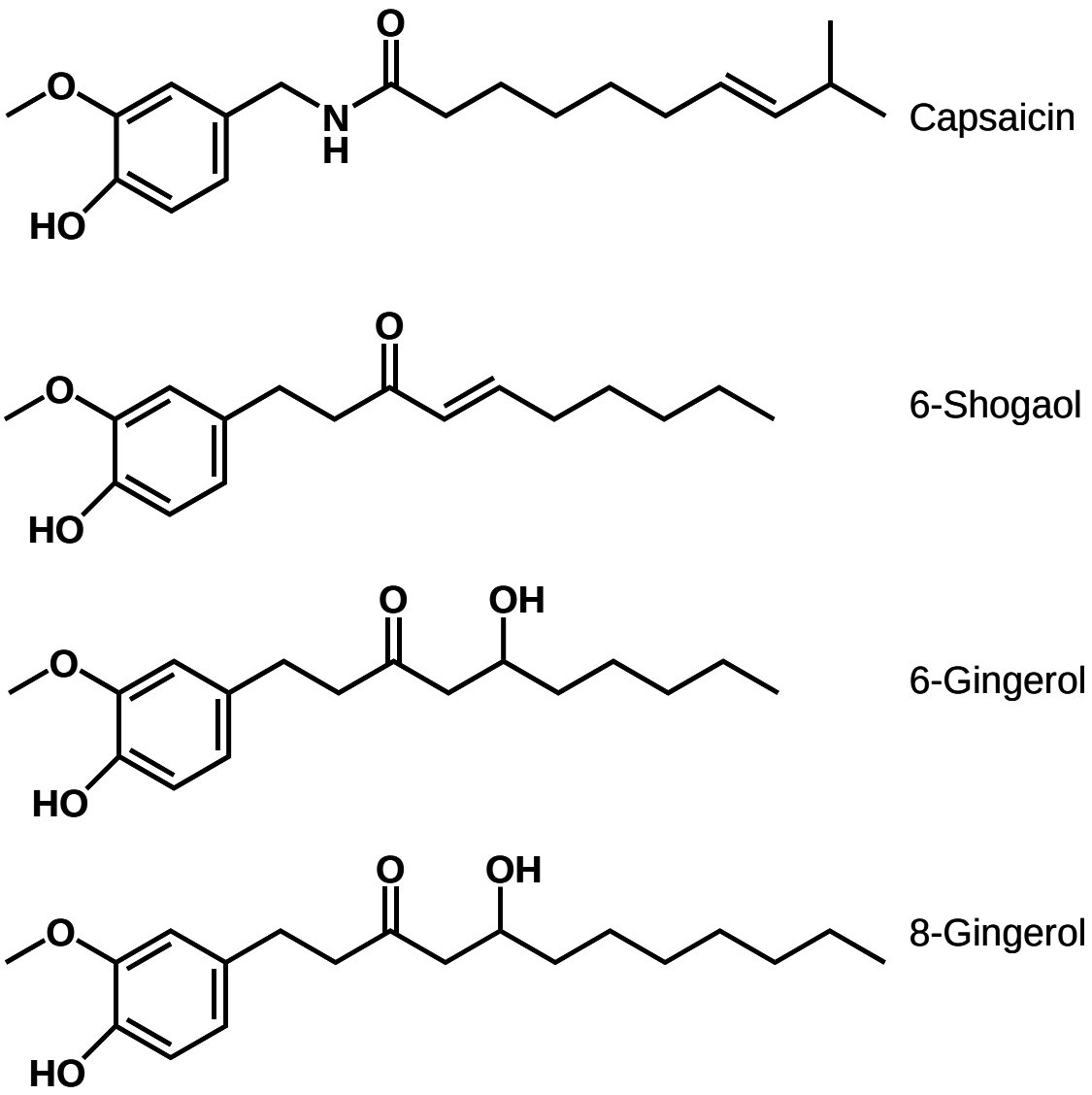
S4- ESI-MS spectra of glutathione alone or adducts formed with selected compounds. (A-L) ESI-MS spectra of GSH alone (A) or products of reactions of GSH and each molecule (B-L). GSH alone exists as a monomer, dimer and trimer in solution MH+, m/z (308, 614, 921). GSH forms no adduct with several TRPA1 agonists (2-APB, Linalool, *cis*-6-nonenal and 6-Paradol). When reacted with the remaining TRPA1 agonists (cinnamaldehyde, α-SOH, **I, II, III, IV**, 6-shogaol), adducts of predicted mass

are observed as outlined on each spectrum. Note that GSH does not form adduct with *cis*-6-nonenal whereas they are observed for compounds **I** and **IV,** suggesting that the carbonyl of the amide is the electron acceptor for the nucleophile.




E
GSH monomer 308.13
I 272.27
2x I 543.20
Adduct (579)
578.80
GSH dimer 614.80
GSH + I (MW = 271 Da)
2x Adduct (850)
848.13
GSH trimer 921.60
F
GSH monomer 308.13
II 270.27
2x II 539.13
Adduct (577)
576.80
GSH dimer 614.80
GSH + II (MW = 269 Da)
2x Adduct (846)
845.47
GSH trimer 921.60
G
GSH monomer 308.13
III 268.27
2x III 535.07
Adduct (575)
574.87
GSH dimer 614.80
GSH + III (MW = 267 Da)
2x Adduct (842)
841.40
GSH trimer 921.53
H
GSH monomer 308.13
IV 270.27
2x IV 539.13
Adduct (577)
576.80
GSH dimer 614.87
GSH + IV (MW = 269 Da)
2x Adduct (846)
845.40
GSH trimer 921.60
Relative Abundance
m/z


I
Relative Abundance
GSH + α-SOH
(MW = 263 Da)
Adduct
(571)
570.87
2x α-SOH
527.13
2x Adduct
(834)
833.53
α-SOH
264.13
GSH
monomer
308.13
m/z
613.07
J
Relative Abundance
GSH + 6-Shogaol
(MW = 276 Da)
Adduct
(584)
583.73
GSH
dimer
614.87
GSH
trimer
921.60
GSH
monomer
308.13
m/z
613.13
K
Relative Abundance
GSH + 6-Paradol
(MW = 278 Da)
(no adduct at m/z 585)
GSH
dimer
GSH
monomer
307.27
m/z
L
Relative Abundance
GSH + cis-6-nonenal
(MW = 140 Da)
(no adduct at m/z 447)
GSH
dimer
GSH
monomer
307.20
m/z



S5- Chemical structures of capsaicin, 6-gingerol, 6-shogaol and 8-gingerol. The vanilloids capsaicin, 6-gingerol, 6-shogaol and 8-gingerol stimulate TRPV1 and share a common phenol core. However only capsaicin among all these compounds does not activate TRPA1.

DISCUSSION AND FUTURE PERSPECTIVES

In the present thesis, the peripheral sensory targets of complex tasting molecules including metallic divalent salts, artificial sweeteners and pungent spices were studied using a combination of *in vitro* and *in vivo* experiments. In particular, I was interested in the physiological role of several membrane proteins being members of the family of TRP channels to mediate the perception associated with these molecules. In this chapter, the results obtained in the above research articles will be discussed and their contribution to the scientific literature will be evaluated. Potential improvements to these studies and future directions to extend the findings of this work will be discussed.

1. Is metallic taste a unique sensory feature?

1.1 What are the chemicals that cause metallic taste?

The sense of taste comprises five basic modalities described by unique descriptors (sweet, sour, umami, salty and bitter) that are transduced at the taste cell level by unique pathways. In contrast, the so-called metallic taste is never reported as a single descriptor to describe the sensory characteristics of the chemicals that elicit it. Among the compounds that produce metallic taste, the most obvious comprise the metals such as $FeSO_4$, $CuSO_4$, $ZnSO_4$ and $MgSO_4$. The prototypical metallic tasting compound is $FeSO_4$, however even this salt possesses complex sensory features: it is described as rusty iron, “penny like”, fishy and rancid (Civille GV and Lyon BG, 1996;Lawless et al., 2004;Lim and Lawless, 2005b;Lim and Lawless, 2005a;Yang and Lawless, 2005) but is also perceived as astringent/drying, with some sour and bitter attributes (Lawless et al., 2004;Lim and Lawless, 2005b;Lim and Lawless, 2005a). Zinc salts are characterized by astringency with some other qualities such as umami, burning, bitter, sour, salty and metallic (Keast et al., 2003;Yang and Lawless, 2005;Lim and Lawless, 2005a). Copper salts evoke a strong bitter, astringent and metallic taste sensations (Lawless et al., 2004;Lawless et al., 2005). Magnesium salts are primarily bitter tasting with additional sensations described as salty, metallic, astringent, sour and sweet (Lawless et al., 2004;Lim and Lawless, 2005b;Yang and Lawless, 2005;Schiffman and Erickson, 1971). Therefore, despite a common metallic denominator, the sensory profiles of the above named divalent salts are complex, vary with the concentration and involve not only taste, but also tactile properties such as astringency and, in the case, of $FeSO_4$, retronasal smell (Lim and Lawless, 2005a;Yang and Lawless, 2005).

Other molecules often cited for their metallic off-taste are the artificial sweeteners perceived as sweet in the low concentration range but their taste perception shifts from pleasant (sweet) towards unpleasant (bitter and/or metallic) (HELGREN et al., 1955;Schiffman et al., 1995;Horne et al., 2002). Metallic off-taste is also reported as a classical aversive taste together with bitterness of many drugs that are chewed rather than swallowed. In addition, metallic taste is observed during loss of taste (Dysgeusia), caused by drugs to treat hypertension and anxiety including lithium (Ackerman and Kasbekar, 1997) or is associated with infections of the mouth or teeth, skin conditions that affect the mucous membranes in the mouth such as lichen planus, or other problems, such as Crohn's disease and Burning Mouth Syndrome (Grushka et al., 1987).

To make the picture even larger, metallic taste is reported during bipolar electrical stimulation of the tongue (Lawless et al., 2005;Stevens et al., 2008). From all of these examples, it arises that the "term" metallic is widely employed to cover a broad range of sensations elicited by chemicals, tactile and electrical stimuli and observed in many conditions. Therefore, to narrow this study, I focussed on divalent salts and artificial sweeteners.

1.2 What are the molecular components underlying perception of CTDS and AS?

The results of the second study show that metal salts possess complex sensory descriptors and also present complex sensory targets during their concentration-profile. In addition, metal salts elicit remarkably dissimilar sensory response profiles which is consistent with their dissimilar sensory characteristics. $FeSO_4$ and $ZnSO_4$ evoke a biphasic response profile in mice as they produce hedonically positive sensations that stimulate T1R3-TRPM5 pathways, as demonstated by sweet and umami stimuli. When concentration increases, these salts produce aversive responses through TRPM5 pathways possibly involving T2Rs, but also sour pathways, TRPV1 stimulation (either somatosensory or in TRCs) and additional unpleasant sensations possibly tactile cues and/or astringency together with low pH. At low concentrations, $MgSO_4$ causes neutral responses to mice (it is not preferred over water), however when T1R3 is suppressed mice are attracted to low concentrations of the salt supporting the idea that T1R3 is present in a subset of taste cells that do not convey hedonically positive messages (Tordoff et al., 2008). Increasing concentrations produce marked aversion through TRPM5 pathways (possibly through T2Rs). Finally, $CuSO_4$ presented a robust aversive response that was partially attenuated when TRPM5 and TRPV1 were suppressed, suggesting that the cause of the strong aversion was due to other factors such as tactile cues or

cellular toxicity. The mechanisms of action of CTDS are summarized in figure 6 of the second publication in the results section.

Artificial sweeteners exhibited a biphasic response profile with a hedonically positive phase involving sweet pathways (T1R3+T1R2) followed by a negative phase recruiting bitter pathways (T2Rs-TRPM5) and somatosensory pathways (TRPV1), (Riera et al., 2007). Knocking out T1R3 leads to total absence of positive response where the contributions of T2Rs and TRPV1 dominate in the high concentrations whereas knocking out TRPV1 partially eliminates negative sensations and corresponds to a gain of preference from the WT in the high concentrations (third publication).

1.3 How can the mice behaviors rationalize human psychophysics?

For metals, one surprising observation is that single concentrations are usually selected in sensory studies to characterize metallic salts profiles without taking in account a possible concentration-dependent profile rendering difficult the interpretation of psychophysical results. Nevertheless, several findings retained our attention. In addition to their metallic or astringent qualities, $ZnSO_4$ and to a lesser extent $FeSO_4$, evoke savory oral sensations associated with the sensation evoked by the prototypical umami tastant MSG (Keast et al., 2004;Yang and Lawless, 2005;Stevens et al., 2006). It follows therefore that low concentrations of these salts could produce a “umami-like” taste that would offset any potentially negatively hedonic metallic or astringent component and thus rationalize the positive sensations of these two salts. The difference in metallic, astringent and other tastant qualities may explain why these salts can be distinguished. Intriguingly, psychophysical experiments revealed that 25mM $ZnSO_4$ suppresses the response to sweet tastants (Keast et al., 2004) whereas we found using brief-access tests that at concentrations <50 mM, $ZnSO_4$ produces an hedonically positive phase that could be a sweet or umami component. Of course, humans and mice may not perceive this stimulus in the same manner but taken together these findings are consistent with a modulation of T1R3 by $ZnSO_4$. One possibility is that $ZnSO_4$ may act as an allosteric modulator of T1R3, as observed with lactisole, a T1R3 modulator (Galindo-Cuspinera et al., 2006;Jiang et al., 2005) and act as a ligand at low concentration and becomes a competitive antagonist when concentration increases. This hypothesis is strengthened by the fact that $ZnSO_4$ does not inhibit cyclamate sweetness (Keast et al., 2004), a T1R3 specific ligand and they may compete for the same regulatory site.

1.4 What are the putative molecular mechanisms of metallic taste experienced with CTDS?

Role of T1R3. Together with Tordoff *et al*. the results of the second publication highlight an unexpected role for T1R3 in sensing divalent salts (Tordoff et al., 2008). From our data, T1R3 behaves as an hedonically positive target for $FeSO_4$ and $ZnSO_4$ whereas Tordoff *et al*. found that it was acting as a aversive detector for Mg^{2+} and Ca^{2+}. Two scenarios are to be envisaged in order to rationalize T1R3 contribution in CTDS perception.

In a first scenario, T1R3 convey sweet and/or umami responses when stimulated with CTDS through activation of T1R1 and/or T1R2 expressing cells. In this model, the particular taste of metals is an integration of many modalities, in the same way umami taste was believed to integrate salty and sweet (Yamaguchi, 1979;Yamaguchi S and Takahashi C, 1984). As reported in psychophysical data, low concentrations of these salts could produce a "umami-like" taste that would offset any hedonically negative component and thus explain the preference that we observed for these two salts in mice. The additional sensory components such as bitterness, astringency, saltiness, sourness, burning beside umami that contribute to the sensory perception of CTDS may explain why these salts can be distinguished from other tastants. One attractive idea is that the combination of these sensations would generate a confusion at the perceptual level by stimulating taste cells coding for opposed modalities in addition to the somatosensory system and the resulting perception would be the so-called metallic taste.

It is also possible, alternatively, that the T1R3-dependent taste response to metals underlies a unique taste quality (described in human psychophysical studies as metallic), which would be encoded by a discrete set of taste receptor cells expressing T1R3 and (an)other unknown GPCR(s). For the T1R3-dependant aversive taste of Mg^{2+} and Ca^{2+}, this receptor is not T1R1 or T1R2, as activation of T1R3 heterodimers containing these receptors leads to preference. Tordoff *et al*. suggested that the T1R3 partner might be the calcium sensing receptor CaSR. For $FeSO_4$ and $ZnSO_4$ although it is conceivable that the partner of T1R3 is T1R1 or T1R2, it is also possible that it is another as yet undiscovered receptor.

Role of TRPM5. From the behavioral data of the second study, TRPM5 appears to be the pivotal element from its implication in both hedonically positive and negative responses. When analyzing the sensory responses of the TRPM5 mutant mice it is seen that they lack the T1R3-dependent hedonically positive phases of $FeSO_4$ and $ZnSO_4$ and the aversions to $CuSO_4$ and $MgSO_4$ are respectively decreased and abolished. Several lines of evidences indicate that the role of TRPM5 is to be a downstream signaling element activated by calcium release from stores following activation of

PLCβ2 by the action of CTDS ions on T1R3 and possibly T2Rs, not through direct interaction between metals and TRPM5.

By compiling these behavioral results with the findings of Tordoff *et al.* (2008), several TRPM5-containing subgroups of TRCs to be implicated in the transduction of the taste of CTDS can be described. These results are illustrated in the publication 2, figure 6.

A first subgroup of TRPM5-TRCs expresses T1R3, respond to $FeSO_4$ and $ZnSO_4$ and are wired to the areas in the brain underlying preference. As a residual preference response is observed for $FeSO_4$ and $ZnSO_4$, it is unlikely that T1R3 responds alone to CTDS in these cells. The partner of T1R3 in those cells could be either or both T1R2 and T1R1 or another GPCR.

A second subgroup of TRPM5-TRCs expresses T1R3, responds to magnesium and calcium and is wired to the taste areas in the brain that underlie aversion. The receptors for sweet and umami are heterodimers of T1R3-T1R2 and T1R3-T1R1, respectively, and T1R3 alone responds very weakly to sweeteners (Zhao et al., 2003). Furthermore some taste cells express T1R3 but neither T1R1 nor T1R2 (Nelson et al., 2001). Therefore it is conceivable that the cells responsive to Mg^{2+} and Ca^{2+} express another receptor that dimerizes with T1R3 to form a Mg^{2+} and Ca^{2+} responsive receptor. The partner of T1R3 in the heterodimer is unlikely to be T1R2 or T1R1 since activation of T1R3 heterodimers containing these receptors leads to preference. Tordoff *et al*. suggested that the T1R3 partner might be the calcium sensing receptor CaSR.

A third and more hypothetical subgroup of TRPM5-TRCs virtually does not express T1R3 and is responsible for the preference for Mg^{2+} and Ca^{2+} and part of the preference for Fe^{2+} and Zn^{2+}. This is based on the observations that T1R3 KO mice prefer Mg^{2+} and Ca^{2+} (Tordoff et al., 2008), the preference being masked in WT animals by the aversive contribution of T1R3 expressing cells, and that T1R3 KO mice are less aversive to Fe^{2+} and Zn^{2+} than TRPM5 KO animals. Based on the latter, we can also conclude that these cells express TRPM5 also because TRPM5 KO mice are indifferent to concentrations of Mg^{2+} aversive to WT mice.

Role of TRPV1. Based on the *in vitro* findings (Publications 1 and 2) showing that TRPV1 was responding specifically to sulfate solutions of CTDS, TRPV1 was initially considered as a candidate receptor for metallic taste. Consistent with the role of TRPV1 as a sensor of aversive compounds, it was found that CTDS were agonists for heterologously expressed TRPV1 and their effects occured via electrostatic interaction with the glutamates E600 and E649 identified as proton binding residues (Jordt et al., 2000) as previously observed in the direct gating by Mg^{2+} and Ca^{2+} (Ahern et al., 2005). However in the behavioral results, the TRPV1 contribution to the aversive responses of CTDS was observed exclusively in $FeSO_4$ and $CuSO_4$, which seriously challenged the idea that TRPV1 might play the role of a “universal metallic receptor”. Interestingly no TRPV1 contribution was found to the

behavioral response to $MgSO_4$ despite its established role in pain via TRPV1 receptors (Ahern et al., 2005), likely because $MgSO_4$ unlike the other salts has a limited diffusion into the epithelium (Powell et al., 1999;Tang and Goodenough, 2003). Surprisingly, beside a marked spicy note (Yang and Lawless, 2005), $ZnSO_4$ evoked-responses to mice did not contain a TRPV1 component. These results suggest that perhaps another noxious sensor is recruited such as TRPA1. This is consolidated by the findings that concentrations of $ZnSO_4$ inferior to 1 mM induced activation of heterologously expressed TRPA1 whereas increasing concentrations had an inhibitory effect (unpublished results). These findings confirm the role of TRPV1 as a sensor of $FeSO_4$ and $CuSO_4$ recruited in the aversive responses of the compounds, however the agonistic activity of $MgSO_4$ and $ZnSO_4$ on heterologous TRPV1 *in vitro* does not appear to be physiologically relevant for perception. It is therefore unclear what sensation is mediated by TRPV1 in CTDS perception.

1.5 What is the sensory role of the TRPV1 component in the artificial sweeteners off-tastes?

For sweeteners, it appears that the off-taste has been characterized as bitter and metallic (Schiffman et al., 1995;Helgren et al., 1955). Concentration-sensory profiles of sweeteners are well established (DuBois et al., 1977) as one key concern of the sweetness of these molecules is their lingering taste. Indeed, it is accepted that sucrose will elicit a sharp sweetness followed by a quick off-set whereas AS will exhibit a slow onset and offset with additional unpleasant tastes. One explanation for the lingering comes from the elegant work of Naim and colleagues (Zubare-Samuelov et al., 2005;Zubare-Samuelov et al., 2003;Peri et al., 2000). They showed that saccharin can diffuse across the plasma membrane of the TRCs and accumulate, at relatively high concentrations, in the cytoplasm where it may interact with and subsequently delay signal-termination components located downstream of the sweet or bitter responding GPCRs (depending on the concentration). This inhibition resulting in a delay in signal termination would therefore extend the taste response thereby giving rise to the sweet and bitter lingering tastes associated with AS. However, this hypothesis can hardly explain the nature of the metallic sensation. In contrast, sweeteners stimulation of TRPV1 provides a putative rationalization of the so called metallic off-taste. The behavioral data demonstrated that artificial sweeteners stimulate TRPV1 in the aversive sensory phase thus it is surprising that these molecules have never been reported as pungent. One recurrent remark regarding TRPV1 activation is that it must underlie pungency. To explain this, several possibilities must be examined.

A first seducing hypothesis would be to consider the findings from Lim & Green (Lim and Green, 2007) who explored at the perceptual level whether bitter and burning/irritating attributes are

related. They found, under several conditions, that the bitter taste sensation of quinine and the burning/irritating sensation from capsaicin are extremely similar and even non-distinguishable. This suggests that AS at high concentrations activate T2Rs and TRPV1 which may confuse the sensations. This would provide an explanation for the unclear bitter / metallic aftertaste. This also raises the possibility of some overlap at the receptor level and it would be expected that bitter molecules may stimulate TRPV1. To test this hypothesis, a library of prototypical bitter stimuli was challenged on TRPV1 but none of these chemicals stimulated TRPV1 by calcium imaging (Riera et al., 2007). However, it cannot be excluded that capsaicin may modulate one or several T2Rs even though such data are critically missing to validate this hypothesis.

A second hypothesis is to consider the TRPV1 splice variant (TRPV1t) identified and characterized by Lyall and colleagues in rodent TRCs (Lyall et al., 2004;Lyall et al., 2005b;Lyall et al., 2005a). These researchers have shown that TRPV1t, like TRPV1, is activated by capsaicin and temperature and is modulated by ethanol. In this regard, sweeteners activation of TRPV1t could influence the taste sensation in a manner that is dependent on what other taste receptors are present in TRCs containing TRPV1t. However, TRPV1t expression profile in TRCs is unknown and its putative role is largely controversed: subsequent behavioral studies using mice lacking TRPV1 did not reveal a suppression of salt taste (Ruiz et al., 2006) and lack of data on the cloning and functional expression on this splice variant seriously challenge this hypothesis.

A third hypothesis particularly retained the attention. One important observation from the third study's results shows that TRPV1 suppression in mice highlights a minor role of this channel in the aversive response of sweeteners. Indeed, TRPV1 deletion accounts for a improved acceptance of aversive AS concentration. Heterologously expressed TRPV1 respond to sweeteners, with saccharin being the most potent agonist however all of these molecules are only partial agonists. In addition, because of their amphiphilic nature, their diffusion into epithelium to reach trigeminal afferents is slower than lipophilic compounds like capsaicin. Taken together, these results suggest that their action on somatosensory TRPV1 may elicit a relatively different temporal and intensity firing pattern of afferent neurons which may code for a different sensation. Acute burning is caused by potent agents such as capsaicin, piperine, resiniferatoxin at TRPV1 sensory fibers whereas moderate stimulation of these nerves may result in sensations that are more difficult to interpretate such as metallic off-taste.

1.6 Further perspectives: CTDS detection at the periphery

These results together with the study conducted by Tordoff *et al*. (2008) present for the first time a role for T1R3 probably independant as classical sweet and umami receptor. Further clarification of

T1R3 expression in taste cell subsets would shed light on its putative partners to encode aversive or metallic response. To this extent, the generation of transgenic mice expressing GFP under the control of T1R3 would be a useful tool: as a matter of fact, one critical issue in studying the expression pattern of this protein is the lack of an efficient antibody.

An elegant experiment to answer the question of which TRCs subsets detect CTDS would consist into measuring calcium transients directly from dissociated taste cells or lingual slices preparations of intact taste buds loaded with a calcium sensitive dye (Caicedo and Roper, 2001). This approach, developed in the Roper laboratory, has provided fundamental understanding of cellular tuning within the taste bud by showing that type II cells (expressing gustducin) were responding to unique tastant stimuli whereas type III were broadly tuned within tastants (Roper, 2007). It also allowed to show that bitter cells are not broadly tuned across bitter stimuli with the presence of distinct subpopulations of bitter-sensitive cells (Caicedo and Roper, 2001). Combined with transgenic GFP markers of cell types such as TRPM5-GFP (Bezencon et al., 2008) and T1R3-GFP mice, this approach would clarify whether specific TRCs subsets are dedicated to sense CTDS.

Certainly, the ability to study the heterologously expressed receptor would provide a model to probe its sensitivity to CTDS by assessing whether T1R3 is sufficient for CTDS sensation (through the formation of homodimers) or if it requires T1R1, T1R2 or CaSR as hypothesized by Tordoff *et al*. (2008).

To identify putative partners of T1R3 other than T1R1 or T1R2 in taste cells, a yeast two-hybrid screening may be used to discover protein-protein interactions (Deane et al., 2002) followed by protein co-immunoprecipitation to confirm the observed interactions. To do this, a cDNA library from taste cells (prey plasmids), produced by reverve-transcription of mRNA (Rossier et al., 2004), is challenged with the bait (T1R3).

Taken together, these results show that the taste of CTDS and AS underlies complex interactions between gustatory, somatosensory pathways including chemesthesis and orosensory stimuli. The biphasic taste responses obtained with most of the metallic tasting compounds underlie cooperation of these physiological systems. In particular, these data highlight a role for T1R3 in divalent salts sensing, that may be independent of sweet and umami signalling, which deserves further investigation. To conclude, these findings provide a novel vision of metallic taste by assimilating this sensation to the cooperation of several senses rather than to one characteristic modality.

2 What is the sensory coding at TRPV1 and TRPA1 level?

2.1 How are TRPA1 sensory signals dissociated from those of TRPV1?

At the mRNA level, TRPA1 is found expressed in the DRG but not in other tissue, suggesting that primary sensory neurons specifically express this channel (Story et al., 2003a). A similar pattern of expression is observed for TRPM8 (Peier et al., 2002a). However, the distribution of TRPA1 among other subpopulations of DRG neurons is quite different that of TRPM8. Almost all TRPA1-expressing neurons coexpress TRPV1 and there is no overlap between the TRPM8 positive and TRPA1 positive subpopulations (Story et al., 2003a). The coexpression of TRPA1 and TRPV1 in the same neurons raises the question of the transmission of specific TRPA1 signals. As a matter of fact, TRPA1 is believed to sense noxious cold below 17°C whereas TRPV1 responds to noxious burning. It may be interpretated as one of the molecular mechanisms responsible for noxious cold being paradoxically perceived as burning pain. However, this explanation does not clarify how cinnamaldehyde or mustard pungency are discriminated from capsaicin.

One possible explanation would be to envisage an across fiber pattern by analogy with the taste system where sensory afferents expressing TRPV1 and TRPA1 detecting directly the stimuli would be broadly tuned across the channels ligands. In this model, stimulus identity and intensity are specified by a unique combination of activity distributed across neurons and would allow a qualitative discrimination between stimuli.

Another possibility is that TRPA1 is not the only sensory receptor for cinnamaldehyde and mustard oil based pungency despite its specific role in MO-induced pain (Bautista et al., 2006;Kwan et al., 2006). If the role of this channel in detecting environmental irritants and hyperalgesia induced by noxious compounds is not questionable, several lines of evidences challenge the hypothesis that TRPA1 participates in sensory perception in contrast to TRPV1. TRPV1 mutant mice drink capsaicin as water whereas wild-type animals avoid it strongly (Caterina et al., 2000). TRPA1 knockout mice consume more MO-containing water than wild type or heterozygotes, mutants consume more than one third of the normal amount at the highest concentration (Kwan et al., 2006). This suggests that TRPA1 accounts only partially to MO induced aversion. One reason could be that mice learn to avoid the oil because of its volatile component or that another receptor is involved in the detection. Another study (Bautista et al., 2006;Bautista et al., 2008) reported that double mutant TRPV1/TRPA1

mice are completely indifferent to MO in a drinking test. To analyse the role of TRPA1 in sensory perception, the points discussed in the two following paragraphs must be examined.

2.2 How do we link sensory effects of cinnamaldehyde and mustard oil with TRPA1 activation mechanisms?

Let's now consider the temporal properties of Cinna and MO associated perceptions. Repeated applications of these substances are known to elicit sharp pungency followed with quick desensitization in contrast with capsaicin evoked burning and sensitization properties (Green, 1989;Green, 1991). Interestingly, two recent studies have shown that Cinna and MO share a similar activation mechanism on TRPA1, as they possess an electrophilic carbon or sulfur that is subject to nucleophilic attack by the sulfur in cysteines side chains of TRPA1 (Hinman et al., 2006;Macpherson et al., 2007a). This "novel" method of activation raises the issue in terms of receptor kinetics. TRPV1 recognizes its ligand capsaicin via aromatic interactions within a more traditional binding pocket (Jordt and Julius, 2002). TRPA1 modification by its ligands, resulting in the formation of covalent adducts on the receptor, persists for hours as monitored by reacting fluorescent tags with alkyne groups engineered into the electrophile (Macpherson et al., 2007a). The consequence of this binding is that heterologously expressed TRPA1 remains activated after stimulation with these covalent modifiers. The elevation of intracellular calcium in response to a Cinna analog only diminishes gradually over an hour (Macpherson et al., 2007a). However, paradoxally this phenomenon is not observed in DRG cultures where elevation of intracellular calcium displays fast desensitization after Cinna or MO treatment (Bandell et al., 2004b;Macpherson et al., 2007a). In DRGs, it is also possible that modifiers coming-off does not happen or is not fast enough and the channel is desensitized: either by calcium in short term and may be even recycling (such as internalization) in the long term. The difficulty to answer this question arises from the issue of variability of electrophysiological recordings from TRPA1. In whole cell, versus detached patches, versus calcium imaging all give different readouts with respect to desensitization. Despite these technical difficulties, several endogenous factors have been shown to play a role in the regulation of TRPA1 response to chemical irritants. Kim & Cavanaugh (Kim and Cavanaugh, 2007) showed that pungent chemicals are unable to activate TRPA1 in excised patch membranes, although strong activation is present in cell-attached patches and found a soluble cytosolic factor (polyphosphates) to be required for pungent molecules to activate TRPA1 heterologously expressed and in trigeminal neurons. In addition, these researchers have shown recently that PIP2 inhibits TRPA1 and reduces the sensitivity of TRPA1 to AITC (Kim et al., 2008). Intracellular calcium also plays a role in TRPA1 response as mutations in the EF-hand domain resulted in a decrease in mustard oil responses and a loss of cold and extracellular calcium activation (Zurborg et al., 2007;Doerner et al., 2007). Thus the interpretation of TRPA1 kinetics remains unclear

and further studies will be needed to evidence what is the native state and to clarify the role of endogenous factors.

2.3 Is there a TRPA1-based pungency?

From our results (publication 4), it is seen that alkylamides, 6-shogaol, 6-paradol and linalool contained in pungent Sichuan and Melegueta spices stimulate heterologously expressed TRPA1 and activate Cinna-sensitive neurons. Among these compounds, only two (the synthetic sanshool analogue **I** and linalool) were ineffective at activating TRPV1.

Among the alkylamides in Sichuan pepper, α-sanshools and β-sanshools differ only by the configuration of their C6 double bond, but their sensory evaluations are significantly different. The α-sanshools are characterized as tingling and burning and β-sanshools as bitter and numbing. Interestingly, hydroxy-β-sanshool evoked a moderate TRPV1 activation (Sugai et al., 2005a) and did not activate TRPA1 (Koo *et al.*, 2007) suggesting that the *cis* configuration of this specific C6 double bond is required to produce the tingling of α-SOH. Interestingly, even though only TRPA1 was found to be stereospecific to the *cis* isomer (publication 4), it is unlikely that the molecular basis of the α-SOH tingling sensation is due to TRPA1 stimulation as many TRPA1 agonists do not produce this sensation. Other sanshools (δ,γ) stimulate TRPV1 and cause burning sensations (Sugai et al., 2005a) independently of their chain length and the configuration of their unsaturations indicating a clear TRPV1 contribution to the burning sensation. Based on these assumptions, we speculate the sensory properties of the synthetic sanshool analogues **I**-**IV** to elicit burning whereas only compounds **III** and **IV** might be perceived as tingling.

To clarify the roles of TRPA1 and TRPV1 in the pungency elicited by these agents, taste preferences assays were performed in mice lacking the capsaicin receptor to assess if other aversive detectors were remaining. A recent publication (Bautista et al., 2008) has shown that two-pore potassium channels were involved in the detection of α-SOH at the DRG level and that aversive taste responses to the compound were conserved in double TRPA1-TRPV1 mutant mice. In their assay, consisting in a timed drinking test when presented 3 hours to the solutions, the preference over water was not assessed and mice which were not water deprived presumably lost motivation to drink. In our brief access paradigm, the observed behavioral responses of the TRPV1 KO mice show that TRPV1 accounts fully for the aversive response of analogue **I** and α-SOH with no apparent support to another aversive component, excluding two-pore potassium channels and TRPA1 as participating in the unpleasant effect. In addition, the responses profiles observed in vitro on TRPA1

and TRPV1 was absent at the hedonic level where the structure-activity study showed the synthetic analogue **I** of α-SOH was ineffective at stimulating TRPA1 but retained activation of TRPV1. However, the qualitative effects that may be attributed to two-pore potassium channels and TRPA1 cannot be assessed in such a test. Regarding the numbing sensation, these effects were most likely masked by the temporal latency of the numbness which prevents the mice from identifying the origin of the stimulus but also meaning that numbness probably does not affect taste preference.

Therefore, the qualitative role of TRPA1 in Sichuan pepper taste is unclear, as alkylamides turn out not to be the best model to study TRPA1-mediated taste from their multiple targets at the DRG level and are partial agonists of TRPA1. On the contrary, linalool and 6-shogaol provide a more adequate model.

Using a simple technical sensory trial, it was observed that despite a strong retronasal smell, linalool, which stimulates TRPA1 but not TRPV1 in HEK293 cells, did not elicit a pungent sensation even when tested at high concentrations (1 mM). Clearly, the absence of pungency of this compound raises the question as to why linalool which activates TRPA1 is not pungent. It is well documented that the pungent chemicals Cinna, AITC and garlic derivatives potently target TRPA1 (Bandell et al., 2004a;Jordt et al., 2004;Macpherson et al., 2005;Bautista et al., 2005). Behavioral data on TRPA1 KO mice presented with MO suggest that TRPA1 is partially involved in the aversion to AITC (Kwan *et al.*, 2006) while TRPV1 contribution to capsaicin pungency is well documented (Caterina *et al.*, 2000).

However, in the behavioral results (Fig. 7B, paper 4), it is observed a residual aversive response to the taste of 1mM 6-shogaol when TRPV1 is suppressed which could be accounted to a TRPA1 stimulation. It is striking from these results that among the tested molecules, 6-shogaol exhibits the highest affinity with TRPA1 with an EC50 of 11 µM. Obviously, to fully assess the role of TRPA1 in 6-shogaol aversion, a direct way would be to explore the taste preferences of TRPA1 KO mice. TRPA1-mediated aversive responses are observed when animals are presented by potent and selective TRPA1 agonists (MO) but taken together, these findings suggest that TRPA1 may convey noxious perceptual information but its qualitative nature remains unclear.

2.4 Further perspectives

Obvious limitations to the study of TRPA1- mediated perception(s) are arising from the sole use of animal model. Certainly, a greater understanding of the role of the channel in the aversion induced by its ligand would be achieved by studying behavioral responses of TRPA1 KO mice that unfortunately could not be obtained for these studies. However, these data would not provide information on the qualitative perception mediated by TRPA1. Many efforts have been made to

obtain specific antagonists of the channel to get a better understanding of its putative mechanical and nociceptive properties. Indeed, compensatory mechanisms are likely to occur in the TRPA1 KO mice and account for MO remaining sensitivity through upregulation of another protein that plays a similar role as TRPA1. TRPA1 is the only member of the TRPA family in mammals, and TRPV1 does not appear to be upregulated in TRPA1-deficient mice, although the two channels functionally interact (Kwan et al., 2006;Bautista et al., 2006). Two recent publications have isolated two specific TRPA1 antagonists in the objective to treat pain (McNamara et al., 2007;Petrus et al., 2007). The development of orally available antagonists of TRPA1 would provide an efficient tool for further understanding of the role of the channel at the psychophysical level.

To conclude, with opposition to TRPV1, TRPA1 channels implication in sensory perception is still poorly understood with both solid arguments in favour and against the hypothesis. Previous controversies are still vivid regarding the role in cold and mechanical sensing. For cold, several laboratories are unable to observe cold responses in TRPA1 expressing cells (Dhaka et al., 2006;Lumpkin and Caterina, 2007) but recent evidences involving intracellular calcium support the role of TRPA1 in cold sensing (Zurborg et al., 2007;Doerner et al., 2007). In contrast, a role of mammalian TRPA1 in mechanosensation is poorly probable as the analyses of TRPA1-deficient mice seriously mitigate this possibility (Bautista et al., 2006;Kwan et al., 2006). An emerging role for this channel is its expression in airway sensory neurons, and its activation by toxicants from cigarette smoke and polluted air resulting in neurogenic inflammation and respiratory hypersensitivity (Bessac et al., 2008;Gerhold and Bautista, 2008;Simon and Liedtke, 2008) in agreement with its well documented role in nociception. A major gap in the comprehension of TRPV1 and TRPA1 relies in their broadly tuned response to multiple agents and the understanding of signal coding within sensory neurons to distinguish temperature, pain and taste information.

3 General Conclusion

In this thesis work, it was provided novel evidences of the fundamental roles of TRP channels in sensory perception. In the first part of the study, it was shown that the multisensory perception of CTDS and AS underlies cooperation between gustatory, somatosensory pathways including chemesthetic and orosensory stimuli. It was observed that these compounds either evoke a biphasic response or a fully aversive trend, with both sensory profiles involving cooperation of these gustatory and somatosensory systems. For the first time, it is shown that metal cations possess a complex taste that is transduced at the level of the taste bud via different TRCs subtypes. The behavioral results have evidenced a multifunctional role of the downstream signalling element TRPM5. The TRPM5-expressing TRCs provide CTDS (iron and zinc) hedonically positive responses in T1R3 cells and in the absence of T1R3 (magnesium) but also aversive responses in T1R3 cells (magnesium) and possibly in T2Rs cells (copper, magnesium).

In particular, these data highlight a role for T1R3 in divalent metallic salts sensing, that may be independent of sweet and umami signalling, which requires further attention. The role of TRPV1 in the sensory properties of these compounds opens a new perspective to address chemosensory perception: for the first time, this channel is shown to be involved in the detection of tastant molecules encoding for a qualitative sensation dissociated from pungency. In the case of AS, this channel participates in the aversive sensations and may convey the metallic off-taste whereas its qualitative contribution to CTDS perception may be multiple and code for some of the sour, tactile or aversive attributes.

Taken together, these findings rationalize the so-called metallic taste by two putative explanations. On the one hand, this sensation may arise from the sensory confusion generated by the cooperation of several senses rather than to one characteristic modality as it is expected from the recruitment of the multisensory pathways associated with CTDS and AS. On the other hand, the emerging sensory roles of T1R3 and TRPV1, respectively independant of sweetness and pungency in the perception of CTDS and AS, may convey unique taste qualities such as metallic taste.

In the second part of the study, it was illustrated how TRPA1 and TRPV1 cooperate to detect pungent molecules from culinary spices using Sichuan and Melegueta peppers. More specifically, the molecules require covalent binding at the TRPA1 cysteines, via electrophilic attack of the cysteine sulphur on the electrophilic carbon of the ligand, to mediate their activation of the channel whereas TRPV1 is presumably stimulated in a more traditional binding mechanism. These results argue for a partial role of TRPA1 in sensing pungency from these spices as mice models show that TRPV1 was

sufficient to account for the aversive responses. The enigmatic role of TRPA1 channels in sensory perception deserves further investigation and in particular how chemical information is processed, terminated and qualitatively evaluated at the TRPV1 sensory neuron level.

Abbreviations

AceK, acesulfame-K

AITC, allyl isothiocyanate

2-APB, 2-aminoethyl diphenyl borate

AS, artificial sweeteners

Asp, aspartame

BA, brief-access test

2BP, 2-bottle preference test

Cap, capsaicin

CDD, charged-coupled device

Cinna, cinnamaldehyde

CGRP, calcitonin gene related protein

CNS, central nervous system

CPZ, capsazepine

CTA, conditioned taste aversion

CTDS, complex tasting divalent salts

$CuSO_4$, copper sulfate

DAG, diacylglycerol

DRG, dorsal root ganglia

ENAC, amiloride sensitive epithelial sodium channel

ESI-MS, electrospray ionization mass spectrometry

$FeSO_4$, ferrous sulfate

FLEX, fluorescence plate reader

GC, gustatory cortex

GPCR, G-protein coupled receptor

GSH, glutathione

HBSS, Hank's balanced salt solution

HEK, human embryonic kidney

IP3, inositol-1,4,5-triphosphate

KO, knockout mice

Lina, linalool

$MgSO_4$, magnesium sulfate

MO, mustard oil

MSG, mono sodium glutamate

MTS, metallic tasting salts (= CTDS)

MTSEA, 2-aminoethyl methanethiosulfonate hydrobromide

NaCyc, sodium cyclamate

NGF, nerve growth factor

NT, non transfected cells

NTS, nucleus of the solitary tract

OFC, orbitofrontal cortex

P2X, purinergic receptor

PIP2, phosphatidylinositol-4,5-bisphosphate

PKC, protein kinase C

PKD, polycystic kidney disease

PLC, phospholipase C

PR, preference ratio

PUFA, poly-unsaturated fatty acid

RR, ruthenium red

Sac, saccharin

SP, substance P

α-SOH, hydroxy-α-sanshool

T1R, taste receptor 1

T2R, taste receptor 2

TRC, taste receptor cell

TRP, transient receptor potential

TRPV1, transient receptor potential vanilloid 1

TRPV3, transient receptor potential vanilloid 3

TRPV4, transient receptor potential vanilloid 4

TRPA1, transient receptor potential ankyrin 1

TRPA1-3C, transient receptor potential ankyrin 1, triple mutant (C621S-C641S-C665S)

TRPM5, transient receptor potential melastatin 5

TRPM8, transient receptor potential melastatin 8

VGL, voltage-gated like

WT, wild type

$ZnSO_4$, zinc sulfate

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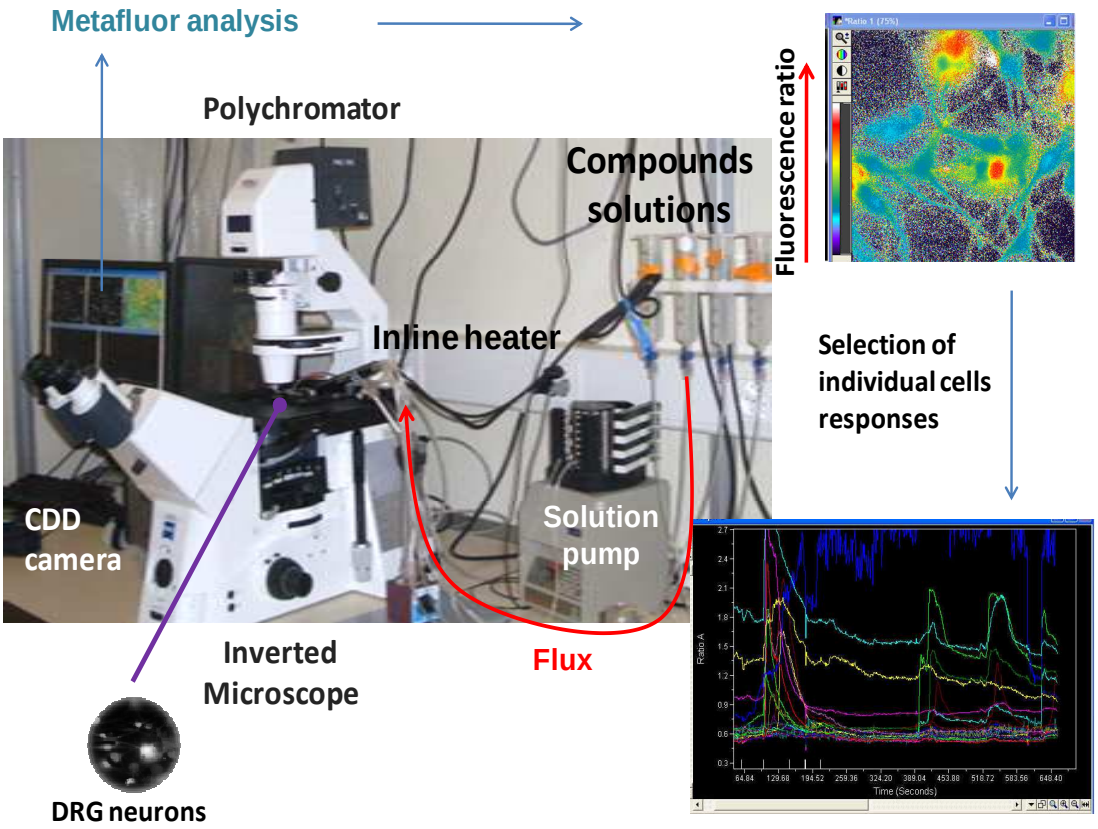
Appendix

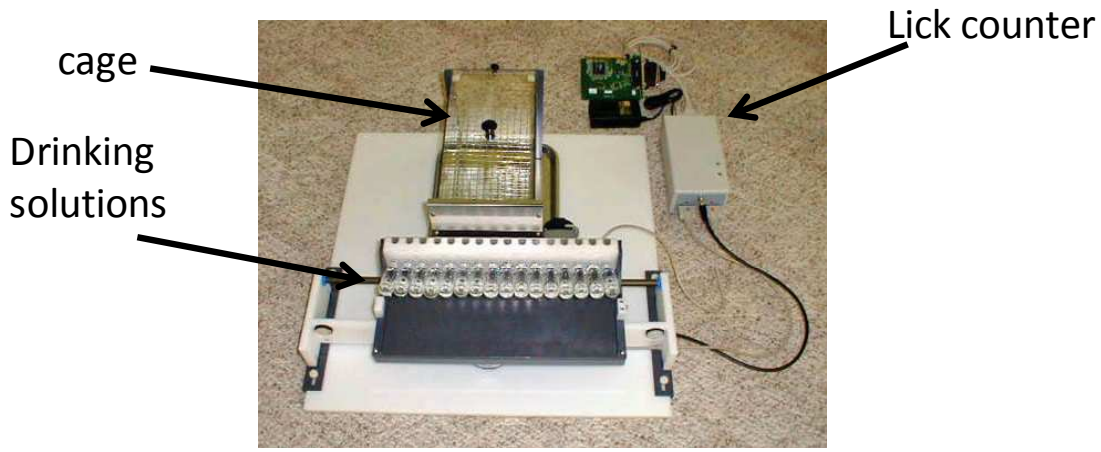
Appendix 1: the DRG imaging set-up



Schematic representation of the single cell calcium imaging set-up used to image DRG neurons. Coverslips are placed in a laminar flow perfusion chamber and constantly perfused with HBSS (supplemented with HEPES and 2mM $CaCl_2$) via a local perfusion pipette through which buffer and compounds solutions were applied. Temperature was kept at 33°C with an inline heater and perfusate temperature was monitored in the chamber with a thermocouple. Changes in $[Ca^{2+}]_i$ were measured using ratiometric digital fluorescence imaging using Fura-2/AM. Images of individual neurons were acquired with a cooled, charge-coupled device (CDD) camera (Cascade II, Photometrics, USA) mounted to an AxioObserver D1 inverted microscope. Autofluorescence was negligible and with illumination times of 100-300 ms, F340/F380 remained stable. Coverslips with attached neurons were placed in a chamber with continuous flow of supplemented HBSS. Chemical stimuli present in HBSS were applied to the flow chamber for 5s and cells were rinsed in supplemented HBSS between stimuli using a solution changer automated system. $[Ca2+]_i$ transients are represented as an increase in the fluorescence ratio of Fura-2 loaded DRG neurons. Using a 20x objective, between 30 and 50 neurons could be imaged in one experiment. Cinnamaldehyde (TRPA1), capsaicin (TRPV1) and menthol (TRPM8) are applied to determine TRPA1, TRPV1 and TRPM8 expressing DRGs. For each neuron the average fluorescence ratio F340/F380 was calculated using Metafluor software (Universal Imaging Corp.).

Appendix 2. Brief-access taste test, THE GUSTOMETER

Brief-access taste tests were conducted in a gustometer (Davis MS160-Mouse gustometer; Dilog Instruments, Tallahassee, FL). The training and testing procedures were conducted as described (Damak et al., 2006;Glendinning et al., 2002). The mice were presented alternatively with two bottle, one containing the tastant and the other distilled water for 30 minute testing periods. Each time the mouse licks it starts a 5 second trial after which the shutter is closed and the other bottle is presented. The number of licks per trial is recorded. The lick ratio was calculated as the mean number of licks per trial for the tastant (L_1) divided by the mean number of licks per trial for tastant plus mean number of licks per trial for water (L_t).



The gustometer delivers tastants in a brief-access manner.
After food or water deprivation, the mouse is placed into the cage. A shutter will open at one side of the cage and the animal will be presented to tastant versus water solutions. Each lick and the interval between licks are recorded to assess the preference of the mouse.

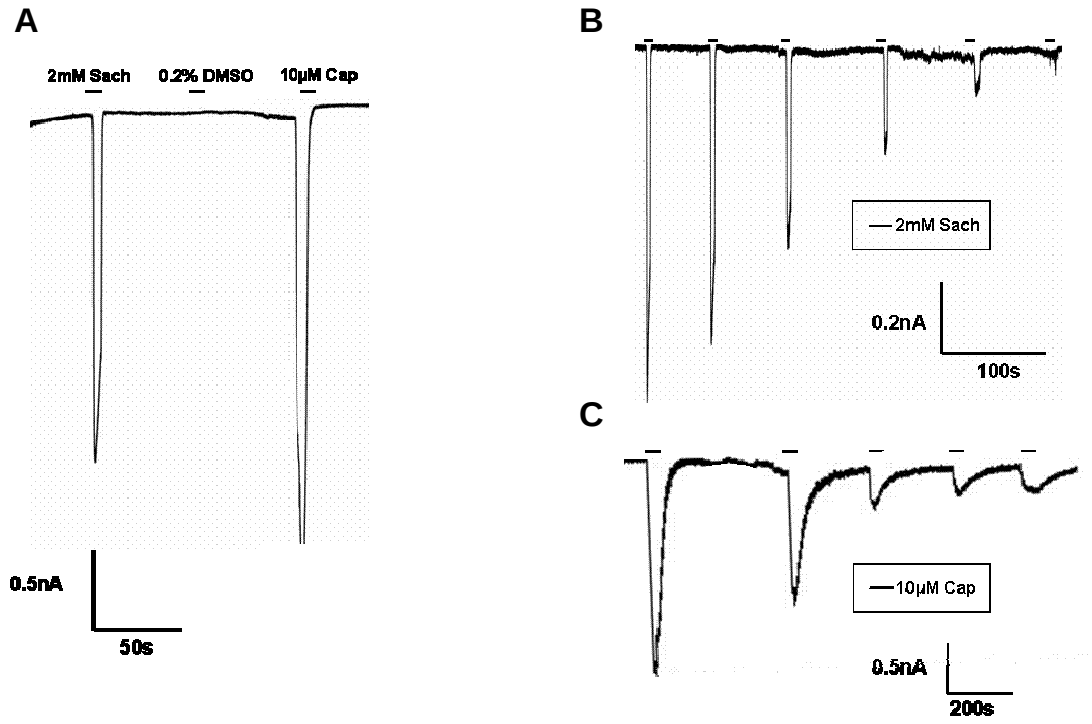
Intervention on the mice:

Animals are weighted before each deprivation or restriction period and before starting each lickometer session.

Training sessions: 23.5 hours of water deprivation, 0.5 hours of measurements in lickometer, 24 hours rehydratation.

Experimental sessions: food and water restriction for 23.5 hours, 0.5 hours of measurements in lickometer, 23.5 hours of rehydratation and renutrition.

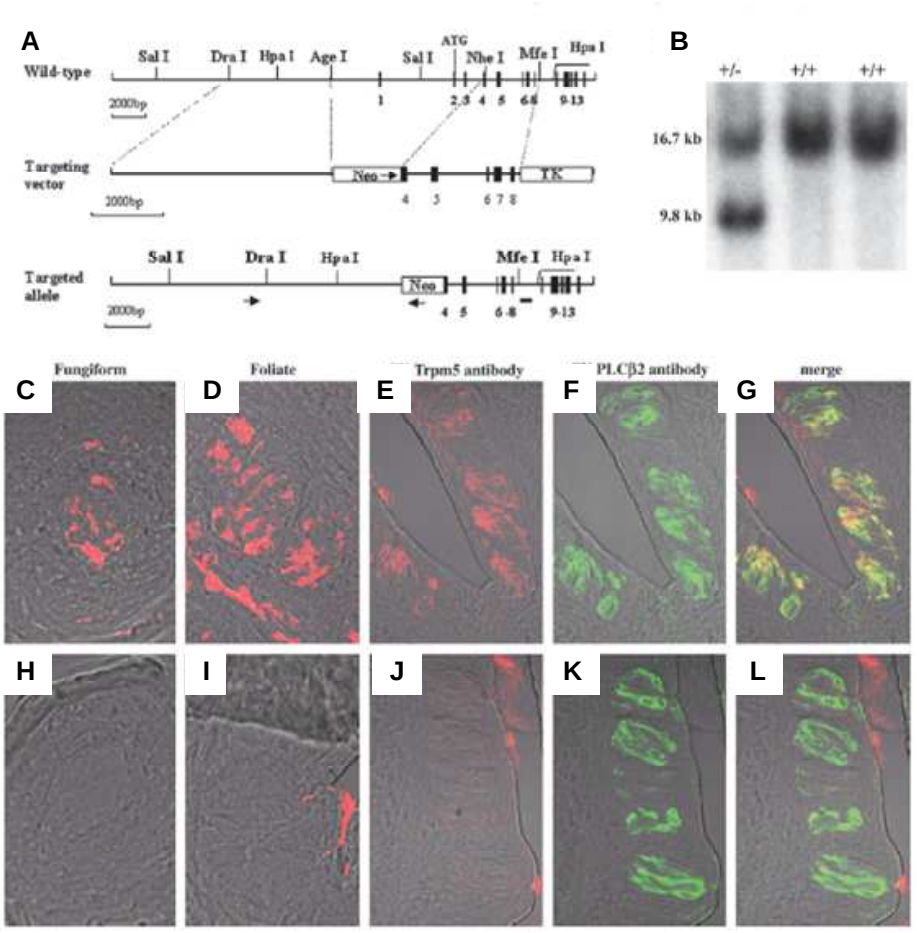
Appendix 3. Whole-cell patch-clamp electrophysiology



Saccharin produces strong currents in TRPV1-HEK293 cells. **A.** Application of 2 mM Sac to a cell expressing TRPV1, resulting membrane currents were measured under voltage clamp at -60 mV. Bars denote the duration of agonist application. Equivalent content of DMSO was used as a negative control and capsaicin was applied to determine the positive expression of TRPV1. Non-transfected cells were devoid of membrane currents when stimulated with a similar sequence (unshown). Cap currents (2-3 nA) were stronger than Sac currents (1-2 nA) **B.** TRPV1 diplayed tachyphylaxis, in which repeated short-duration applications of Sac lead to smaller responses. **C.** Cap produces fast desensitization of TRPV1 where repeated applications lead quickly to small currents.

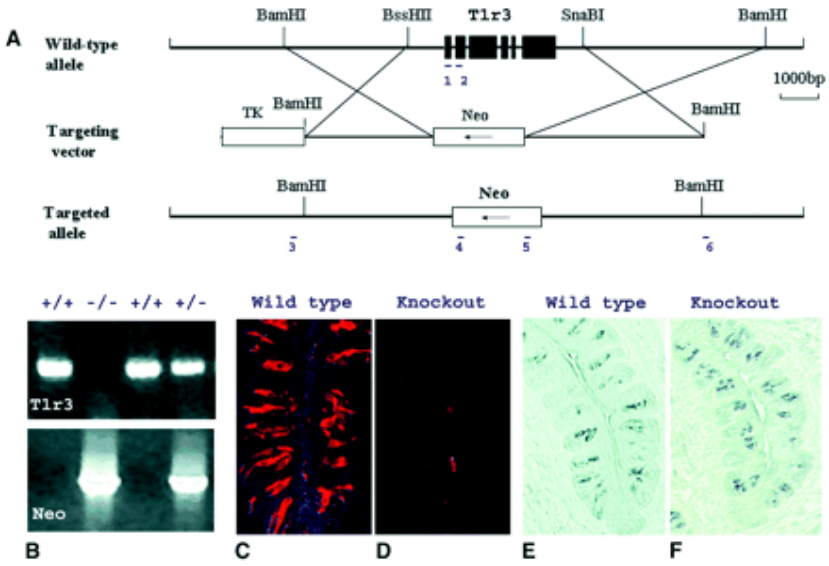
Patch-clamp recordings were performed with hTRPV1-HEK293 cells at room temperature. Standard bath solution for whole-cell recordings contained (in mM) 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 glucose, pH 7.4 (adjusted with NaOH). All recordings were performed using an Axopatch 200B amplifier controlled via the pClamp8/pClamp9 software suite (Axon Instruments Inc., Union City, CA) using standard whole-cell patch-clamp methods. Patch pipettes (resistance, 2–5MΩ) were fabricated on a Sutter instruments P-87 electrode puller (Sutter Instrument Company, Novato, CA) and filled with intracellular solution of the following composition: in mM) 140 CsCl (or 130 CsAspartate and 10 NaCl), 5 EGTA, 10 HEPES, pH 7.4 (adjusted with CsOH). Antagonist solutions were prepared as 10 mM stock solutions in dimethyl sulfoxide before dilution in extracellular solution. Data were acquired at 8.3 to 10 kHz and filtered at 2 to 5 kHz. Series resistance compensation of up to 80% was used where appropriate. Agonists were applied to cells in a controlled fashion via an automated fast-switching solution exchange system (SF-77B; Warner Instrument, Hamden, CT). Data were analyzed using the Clampfit (Axon Instruments) and Origin (Origin LabCorp, Northampton, MA) software packages.

Appendix 4. Generation of TRPM5 Knockout mice



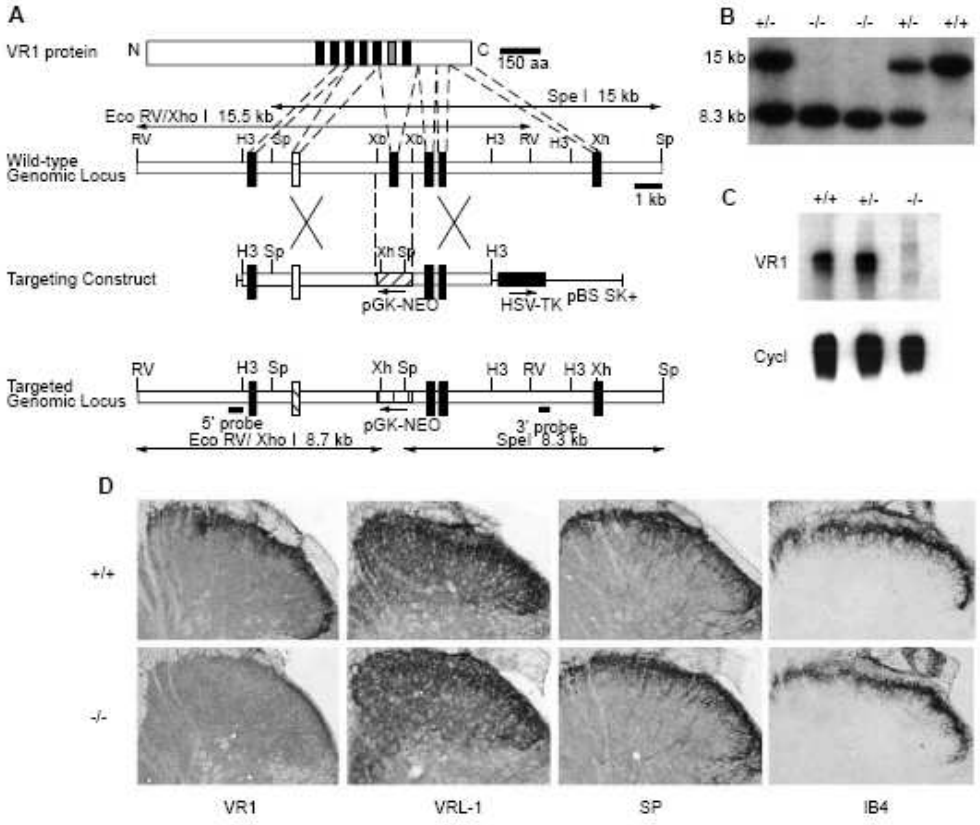
Generation of Trpm5 null mice. A. Top, map of the mouse Trpm5 locus showing the gene's first 13 exons (filled boxes), intervening, and flanking sequences (horizontal lines). Middle, map of the targeting vector showing PGKneo (Neo) and PGKTK (TK) cassettes (open boxes) and the long and short arms of the targeting vector (within the dotted lines, which indicate homologies with the Trpm5 locus). PGKneo is flanked with Lox P sites for removal of neo by Cre recombinase. The arrow indicates the direction of transcription of Neo. The targeting vector was designed to remove 2.4 kb of Trpm5 5'-flanking region containing the promoter and exons 1–4, including the translation start site in exon 2. Bottom, diagram of the Trpm5 targeted allele. The primers and probe used to screen for G418 resistant ES colonies are indicated, respectively, by the arrows and short horizontal line below the map. **B.** Southern blot of DNA from G418 resistant ES cell colonies digested with HpaI, using the probe shown in the bottom line of panel **A**. The first lane shows 16.7 and 9.8 kb bands, corresponding to the WT and targeted alleles, respectively. The next two lanes were from colonies in which the targeting vector had integrated non homologously. (**C-L**) Photomicrographs of frozen sections of taste bud–containing sections from fungiform (**C,H**), foliate (**D,I**) and circumvallate (**E-G**, **J-L**) papillae from WT (**C-G**), and Trpm5 null (**H-L**) mice imaged by indirect immunofluorescence with specific antibodies directed against Trpm5 (**C-E**, **H-J**, Cy3, red) or PLCb2 (**F,K**, fluorescein, green). Panels **G** and **L** are merged double images of panels **E** and **F**, and **J** and **K**, respectively. Note that Trpm5 protein is present in the WT mice (**C-E,G**), but absent in the Trpm5 null mice (**H-J,L**). From Damak *et al.*, 2005.

Appendix 5. Generation of T1R3 KO mice



Generation of T1R3 knockout mice. A. (Top) A map of the murine *T1r3* locus showing the six exons (filled boxes), and intervening and flanking sequences. The polymerase chain reaction (PCR) primers that were used to detect the wild-type *T1r3* allele are indicated by the numbered short lines below the map. (Middle) A map of the targeting vector showing *PGKneo* (*neo*) and *PGKTK* (*TK*) genes (open boxes) and the long and short arms of the targeting vector. *PGKneo* was flanked with LoxP sites for removal of *neo* by Cre recombinase. The arrow indicates the direction of transcription of *neo*. The targeting vector was designed to remove the entire T1r3 coding region. (Bottom) A diagram of the *T1r3* targeted allele. The primers that were used to screen the G418-resistant ES colonies are indicated by numbered short lines below the map. **B**. The offspring from the cross of two *T1r3* heterozygotes (+/–) were genotyped by PCR amplification of mouse tail DNA. (Top) PCR amplification of *T1r3* exons 1 and 2 with primers 1 and 2 [indicated in the top line of (A)]. (Bottom) PCR amplification of upstream *T1r3* sequences and *neo* with primers 3 and 4 [indicated in bottom line of (A)], indicative of successful targeting. **C** and **D**. Photomicrographs of frozen sections of taste bud–containing circumvallate papillae from wild-type (C) and T1R3 KO (D) mice, stained with T1R3-specific antibodies (red), show that T1R3 protein is absent in the T1R3 KO mice. **E** and **F**. Photomicrographs of frozen sections of taste bud–containing circumvallate papillae from wild-type (**E**) and T1R3 KO (**F**) mice, labeled by in situ hybridization with a *T1r2* probe (blue), show that expression of *T1R2* is not affected by the absence of T1R3. From Damak *et al.,* 2003.

Appendix 6. Generation of TRPV1 KO mice



Generation of TRPV1 (VR1) KO mice. A. Strategy for VR1 gene disruption. Black and gray vertical bars on the VR1 protein diagram indicate transmembrane and pore-loop domains, respectively. Exons encoding the C-terminal portion of VR1 are indicated by vertical bars on the genomic maps. RV, Eco RV; H3, Hind III; Xb, XbaI; Xh, Xho I; Sp, Spe I; aa, amino acid. **B**. Southern blot of genomic DNA derived from the progeny of two VR11/2 mice. The 15- and 8.3-kb Spe I bands, identified with the 39 probe illustrated in (**A**), indicate wildtype and targeted alleles, respectively. **C**. Northern blot analysis of VR1 mRNA expression in DRG from VR11/1, VR11/2, and VR12/2 mice. Cyclophilin (Cyc) was used as a loading control. **D**. Immunohistochemical staining of lumbar spinal cord sections from wildtype (top) and VR1-null mutant (bottom) mice. SP, substance P. From Caterina *et al*., 2000.

Appendix 7. Synthesis of new TRPA1 agonists derived from α-hydroxy-sanshool

Synthesis and evaluation of new TRPA1 agonists derived from α-hydroxy-sanshool.

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Abstract

Szechuan pepper is widely used in Asia as a spice for its pleasant pungent and tingling taste, produced by natural alkylamides called sanshools. α-Hydroxy-sanshool, the main alkylamide found in the pericarp of the fruit, stimulates sensory neurons innervating the mouth by targeting two chemosensitive members of the transient receptor potentials (TRP), TRPV1 and TRPA1. As we previously found that configuration of the unsaturations in the α-hydroxy-sanshool alkyl chain is required for TRPA1 but not TRPV1 selectivity, we aimed at obtaining more potent and selective TRPA1 agonists using α-hydroxy-sanshool as a starting material. Here we reported the preparation of new alkylamides derived from sanshool and their efficacy in stimulating TRPA1 and TRPV1 receptors. Our data provide knowledge of the main sanshools chemical functionalities required for TRP channels activation but they also evidenced new selective and potent TRPA1 agonists based on α-hydroxy-sanshool.

Keywords

TRPA1 and TRPV1 agonists, α-hydroxy-sanshool, synthesis, chemoreceptors.

Introduction

Szechuan pepper (*Zanthoxylum piperitum*), which is commonly used in Asia as a spice and traditional medicines as well, is particularly appreciated for its pleasant pungent and tingling taste. Recent studies showed that α-hydroxy-sanshool, the major alkylamide in the pepper was involved in these sensations by gating TRPV1 and to a lower extent TRPA1 channels (1-3). TRPV1 receptors have been implicated in sensing a myriad of noxious stimuli with the most notable being capsaicin, the burning molecule in red hot chilli pepper (4) but also common vanilloids such as zingerone and gingerol from ginger plant (5), eugenol from clove (6) or piperine contained in black and white pepper (7). TRPA1 receptors are found in a subset of nociceptive sensory neurons where they are coexpressed with TRPV1 (8) and respond specifically to noxious cold and pungent compounds such as isothiocyanate (mustard oil, wasabi and horseradish) and cinnamaldehyde from cinnamon.(9-12)

The main sanshools found in Szechuan pepper are α–, β–, γ–, δ–sanshools and their analogues possessing one hydroxyl group on the amide moiety (Figure 1).(13-17) They differ from the configuration of one double bond (α- and β-sanshools for instance) and the length of the polyenic system (12 carbons for α–, β–sanshools versus 14 carbons for γ-and δ-sanshools). Interestingly, sensory evaluations brought to the fore that α-hydroxy-sanshool produced tingling sensation, while δ–, γ– and α-sanshools were perceived as burning and the β-sanshools rather numbing.(2-18) One common characteristic of all theses natural compounds is their agonistic activity on TRPV1, consistent with their burning properties (2). Moreover, the sanshool pungent “sharp” and “biting” sensations may originate from TRPV1 and TRPA1 stimulation but their contribution to its numbing effects has been controversial. Very recently, two-pore potassium channels sensing volatile anaesthetics (19,20) were found to be stimulated by applications of α-hydroxy-sanshool, rationalizing some of the complex numbing and long lasting perceptions associated with the spice (3). In our previous work (21), we observed that α-hydroxy-sanshool is likely to gate TRPA1 via covalent interaction on its reactive cysteines whereas this mechanism is unlikely to occur for TRPV1, highlighting that the two channels behave differently in their response which is may account for dissociated sensory roles of the channels in their response to this compound. If TRPV1’s crucial role in pungency mediated by burning

molecules is well established (22), the physiological implication of TRPA1 in sensory perception remains so far partially elucidated (23). Therefore, to better understand the differences between TRPV1 and TRPA1 in sensing these compounds, we aimed at developing new synthetic molecules based on α-hydroxy-sanshool structure possessing rather a TRPA1 than TRPV1 selectivity.

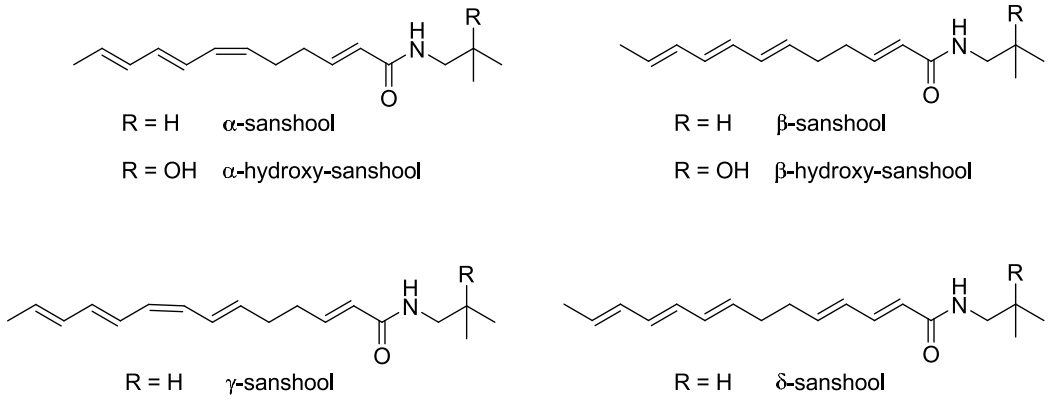


Figure 1. Structure of main sanshools found in Szechuan pepper.

Here we reported the elaboration of new alkylamides which are able to activate selectively expressed TRPA1 receptors. We first focused on sanshool derivatives having various alkyl chains. These analogues would be very useful to identify main chemical functionalities for the TRP channels activation. The second point was to evaluate the influence of the amide part replacing the 2-methylpropan-2-ol moiety by various amino acids. This study finally led to potentially new selective TRPA1 agonists more accessible and more potent than the natural α-hydroxy-sanshool (Figure 2).

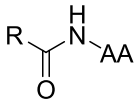
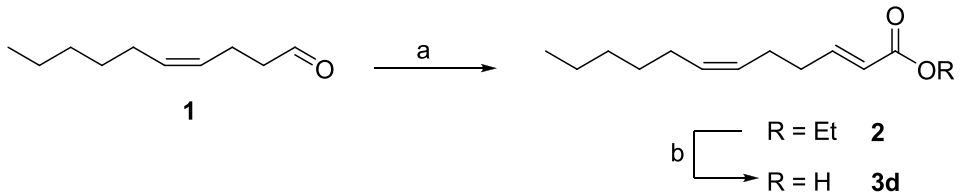


Figure 2. Elaboration of new TRPA1 agonists.

Result and Discussion

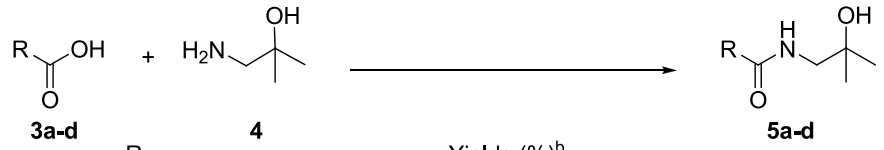
Chemistry. The synthesis of four α-hydroxy-sanshool derivatives **5a-d** with various alkyl chains were achieved starting from the corresponding carboxylic acids **3a-d** and the 1-amino-2-methylpropan-2-ol **4**. Carboxylic acids **3a-c** are commercially available while the polyene **3d** were synthesized in a 2 step-sequence from the 4-(*Z)*-decenal **1** (Scheme 1). Wittig olefination, using the (ethoxycarbonylmethyl) triphenylphosphorane in anhydrous THF, provided the diene **2** in 12 h at room temperature (54% yield). The ester function in **2** was then hydrolysed under standard saponification conditions (LiOH, THF/H_2O) to afford the carboxylic acid **3d** in 60% yield. The coupling reaction of carboxylic acids **3a-d** and the 1-amino-2-methylpropan-2-ol **4**, activated by the (benzyltriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate, led after 12 h at room temperature to the α-hydroxy-sanshool derivatives **5a-d** respectively in 57, 66, 57 and 77% yields (Table 1). (24) The alkylamide **5d** was isolated as a mixture of (6*Z*-2*E*) and (6*E*-2*E*) diastereoisomers due to the commercial 4-(*Z)*-decenal **1** which contains 10% of (*E*)-isomer. The ratio (6*Z*-2*E*)/(6*E*-2*E*) was 88/12 and was established by HPLC.

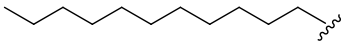
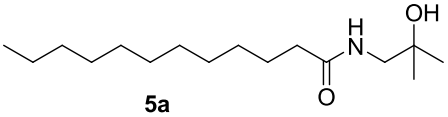
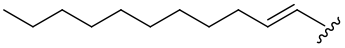
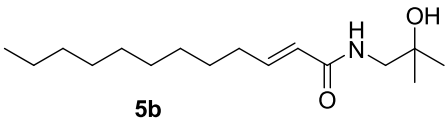
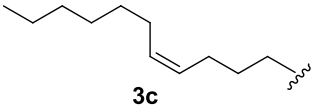
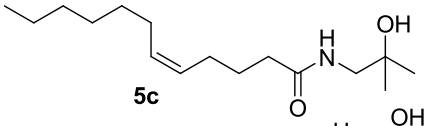
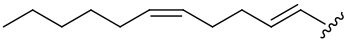
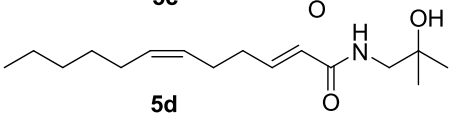
Scheme 1[a]



[a]*Reagents and conditions:* a) $Ph_3PCHCOOEt$, THF, rt, 12 h, 54%; b) LiOH, THF/H_2O, rt, 12 h and then 80 °C, 4 h, 60%.

Table 1[a]

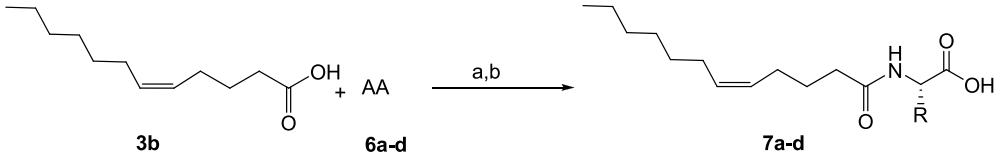


R	Yields (%)[b]	
 **3a**	57	 **5a**
 **3b**	66	 **5b**
 **3c**	57	 **5c**
 **3d**	77	 **5d**

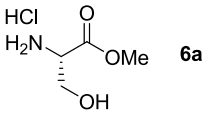
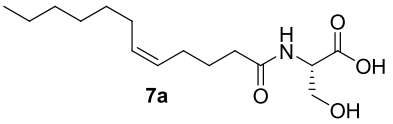
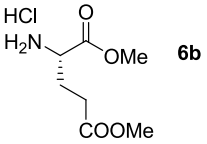
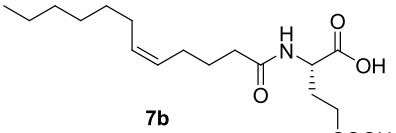
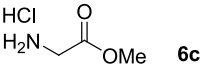
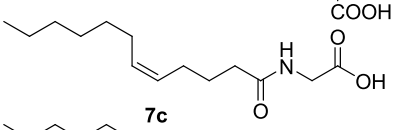
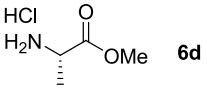
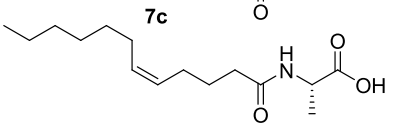
[a]*Reagents and conditions:* a) (benzyltriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate, iPr_2EtN, DMF, rt, 12 h.[b]Isolated yields.

The synthesis of alkylamides **7a-d** was achieved from the commercially available 5-(*Z*)-dodecenoic acid **3b** and various aminoacid methyl esters **6a-d**. Polar (glycine, serine) and non-polar (alanine) as well as acidic aminoacids (glutamic acid) have been used to prepare new components **7a-d**. The previous coupling reaction ((benzyltriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate, iPr_2EtN, DMF) was followed by a saponification step under standard conditions (LiOH, THF/H_2O). Alkylamides **7a-d** were finally isolated respectively in 78%, 69%, 64% and 56% yields over the 2 steps (Table 2). Moreover, various carboxylic acids containing at least one *Z*-insaturation in the alkyl chain were coupled with alanine hydrochloride methyl ester **6d** using the same procedure as previously. The amidation reaction on (2*E*-6*Z*)-dodecadienoic **3d**, linolenic and palmitoleic acids **3e-f** following by the hydrolysis of the methyl ester under basic conditions, afforded the carboxylic acids **8a-c** respectively in 73%, 33% and 76% yields over the 2 steps (Table 3).

Table 2[a]

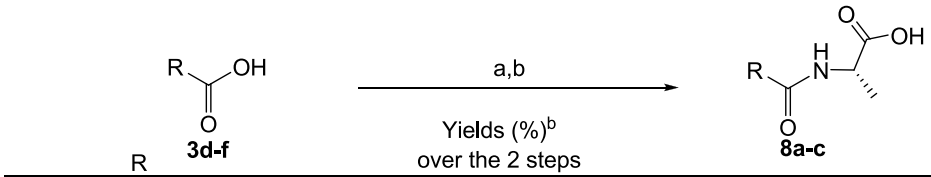


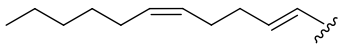
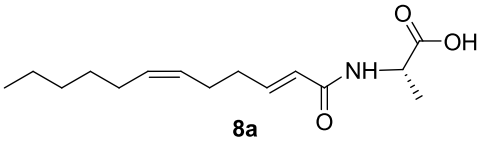
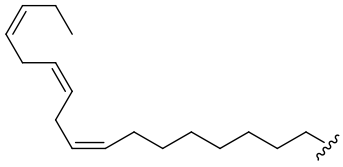
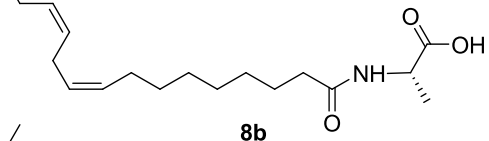
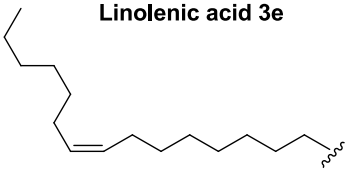
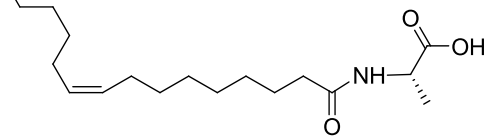
3b **6a-d** **7a-d**

AA	Yields (%)[b] over the 2 steps	Products
 **6a**	78	 **7a**
 **6b**	69	 **7b**
 **6c**	64	 **7c**
 **6d**	56	 **7d**

[a]*Reagents and conditions:* a) (benzyltriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate, iPr_2EtN, DMF, rt, 12 h; b) LiOH, THF/H_2O, rt, 2 h. [b]Isolated yields.

Table 3[a]



 **3d**	73	 **8a**
 **Linolenic acid 3e**	33	 **8b**
 **Palmitoleic acid 3f**	76	 **8c**

[a]*Reagents and conditions:* a) Alanine chloride methyl ester **6d**, (benzyltriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate, iPr_2EtN, DMF, rt, 12 h; b) LiOH, THF/H_2O, rt, 2 h. [b]Isolated yields.

In vitro Pharmacology and SAR. The natural α-hydroxy-sanshool provided the framework for establishing more potent and selective TRPA1 agonists. Despite the fact that the importance of the polyenic chain and particularly the olefins configuration in the activation of TRP channels has been already reported,(2) the role of each specific double bond had never been clarified. Hence, the activation of transient receptor potentials TRPV1 and TRPA1 by the four sanshool analogues **5a-d**, which possess various alkyl chains, was first evaluated (Figure 3). To assess TRPV1 and TRPA1 induced activities by our compounds, intracellular calcium $[Ca^{2+}]_I$ increases in HEK 293 cells were monitoring using Fura-2 based calcium imaging. Responses were expressed relative to the maximum responses evoked by 150 μm cinnamaldehyde for TRPA1 and 1 μm capsaicin for TRPV1 receptor (assessed independently to be saturating concentrations at room temperature). Normalized maximum induced responses of compounds **5a-d** were compared with that of a mixture of natural hydroxy-sanshools **SOH** extracts. The latter was obtained by purification of green Szechuan oil as described in the experimental section. The resulting purified extract contained α–, β-hydroxy-sanshool and a non-

identified isomer (m) with a α/β/m ratio of 76/21/3.(21) Since previous studies demonstrated that only α-hydroxy-sanshool was able to excite sensory neurons,(1) the response elicited from the mixture containing predominately the α-hydroxy-sanshool was assimilated to that induced by the α-hydroxy-sanshool itself. As shown Figure 3, activation of TRPV1 channels by all four sanshool analogues **5a-d** were quite similar to that induced by natural α-hydroxy-sanshool. Component **5c** was even slightly more potent. Consequently, modification of the alkyl system seemed to have very low influence on the TRPV1 activation meaning that this channel does not exhibit selectivity relatively to the unsaturations. On the contrary, TRPA1 activation by 500 μM sanshool analogues **5a-d** was dependent on the alkyl moiety. We observed weak responses induced by the unsaturated compound **5a** and the α, β-unsaturated component **5b** while analogues **5c-d** were active on TRPA1 and evoked similar increase of the intracellular calcium in HEK 293 cells than the α-hydroxy-sanshool **SOH**. These results point towards the crucial role of olefins in the alkyl chain and particularly that of the *Z*-olefin in the activation of expressed TRPA1 receptors.

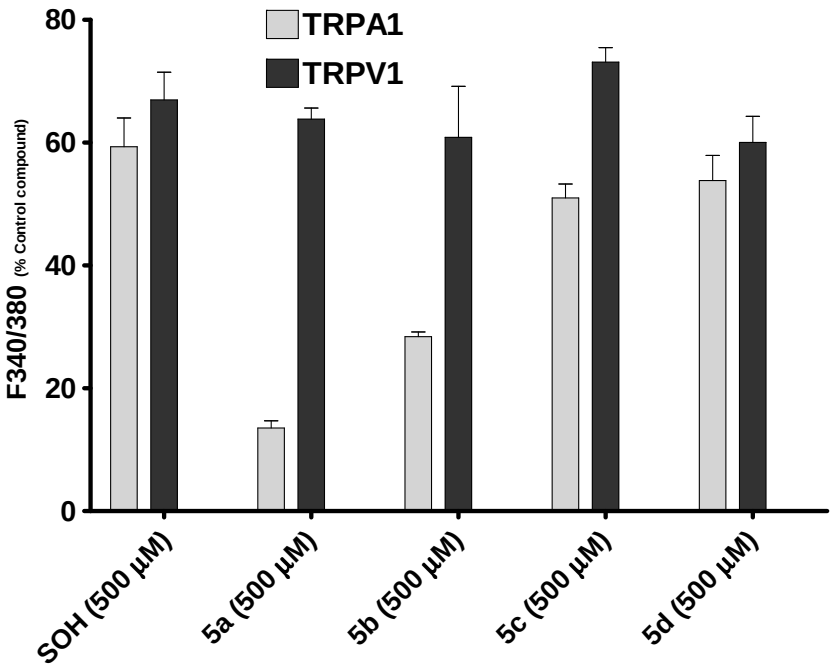


Figure 3. Normalized maximum induced responses of four sanshool analogues **5a-d**. Values are presented as means +/- SEM, *n* = 4.

While fixing the alkyl moiety as being 5-(*Z*)-undecenyl, similar SAR study was initiated modifying the amide part. Thus, the 2-methylpropan-2-ol was replaced by various aminoacids in order to develop more selective and potent TRPA1 agonists. Normalized maximal activation of TRPA1 and TRPV1 channels by new alkylamides **7a-d** was evaluated and compared with that induced by 500 μM compound **5c**. As shown in Figure 4, changing the 2-methylpropan-2-ol part of **5c** by serine or glutamic acid moieties (compounds **7a** and **7b**) resulted in a significant decrease of TRPA1 activation. Interactions between theses molecules and the TRPA1 receptors could be either disfavored by steric hindrance or by an electronic repulsion due to the lateral chain present in serine (hydroxymethanyl) and glutamic acid (propanoic acid group). Responses of expressed TRPV1 receptors induced by 500 μM monoacid **7a** and 1 mM diacid **7b** were slightly lower compared with that evoked by the alkylamide **5c**. 500 μM component **7c** having a glycinyl unit activated predominately TRPA1 channels. Response was even superior to that of produced with the molecule **5c**. However, acid **7c** was not able to discriminate significantly the TRPA1 and TRPV1 receptors. Interestingly, when glycine was replaced with alanine, alkylamide **7d** exhibited a stronger TRPA1 activation that caused by sanshool analogue **5c** and even the mixture of natural hydroxy-sanshools **SOH**. Furthermore, this component **7d** induced a maximal response very specific to expressed TRPA1 receptors since it had only a low effect on TRPV1 channels.

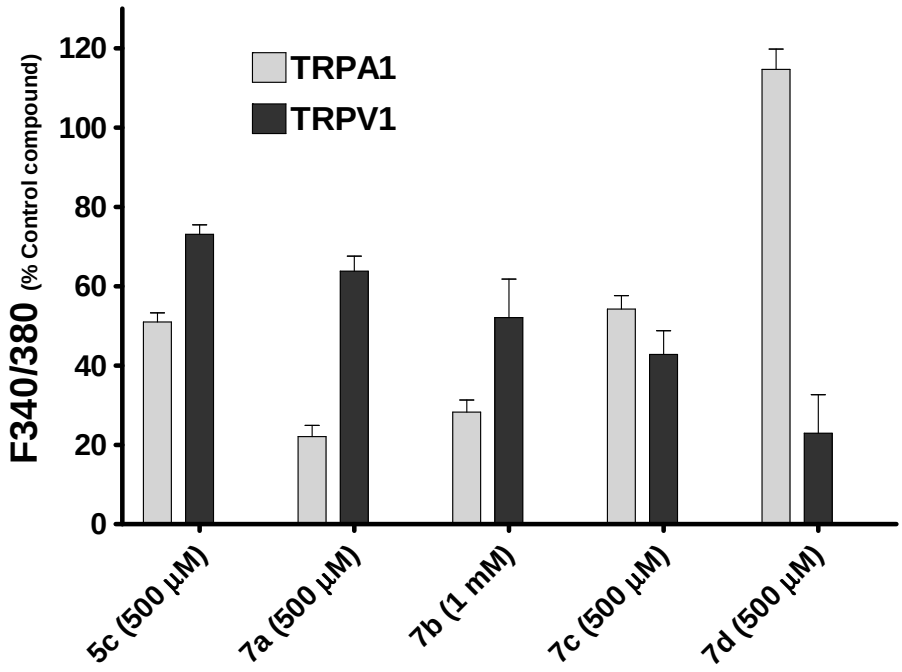


Figure 4. Normalized maximum induced responses of alkylamides **5c** and **7a-d**. Values are presented as means +/- SEM, *n* = 4.

Having identified alanine as an optimal amide substituent, an additional SAR study was carried out with alkylamides **8a-c** containing at least one *Z*-insaturation and various length of alkyl chain. As shown in Figure 5A, TRPA1-expressing cells respond to all four alkylamides **7d** and **8a-c** in a dose-dependent manner. Responses (0.001-1 mM) were measured as peak increase in fluorescence, expressed relative to the maximun 150 µM cinnamaldehyde response. Interestingly, the gain in activity and specificity obtained from **5c** to **7d**, when the 2-methylpropan-2-ol was replaced by the alanine, was not observed in the alkylamides **5d** to **8a**. Therefore, the presence of the α,β-unsaturation does not significantly modify the TRP activity induced by the α-hydroxy-sanshool analogue **5d** compared to that obtained with **5c** but negatively impacts on the alanine conjugate **8a**. These effects underlie the fact that both the alkyl chain tail and the polar amide head of the components play a role in TRPA1 activation. The best synergy between the head and the tail of the molecule to produce maximum responses at TRPA1 was obtained with 7d. From these results, it is difficult to weight for the TRPA1 activation firstly the importance of each part of the molecule (tail versus head) and

secondly the importance of the intrinsic molecular shape versus the self-aggregation of these components in solution. Based on these findings, we selected mono- and pluri-Z unsaturated moieties to be coupled to Ala. Consistent with the results obtained with 7d, we observed that the mono-unsaturated acid **8c** derived from the palmitoleic acid induced a relatively high TRPA1 activation but still exhibited an important TRPV1 response (Fig 5A and B). In addition, the obtained half maximum activation concentration EC_{50} (25 μM) was much more superior to that obtained with **7d** (263 μM). Finally, best results were observed with the alkylamide **8b** which contained 3 Z-olefins in its C18 carbon chain. Actually, 100 μM linolenic acid derivatives **8b** induced significant and selective TRPA1 activation, meaning its effect on TRPV1 was relatively low. From all tested molecules, the compound **8b** is the most potent and selective TRPA1 agonist. Normalized maximum value was similar to that evoked by 500 μM compound **7d** but its half maximum activation concentration EC_{50} was smaller than **8a** and **7d**, with only 20.3 μM versus 25 μM and 263 μM for **8a** and **7d** respectively. Since the components **8a** and **8b** which differ only from the number of insaturations in their alkyl system (1Z for **8a** versus 3Z olefins in **8b**), had different reactivity towards TRPA1 channels, this result reinforced the importance of Z-olefins to interact strongly with TRPA1 receptors.

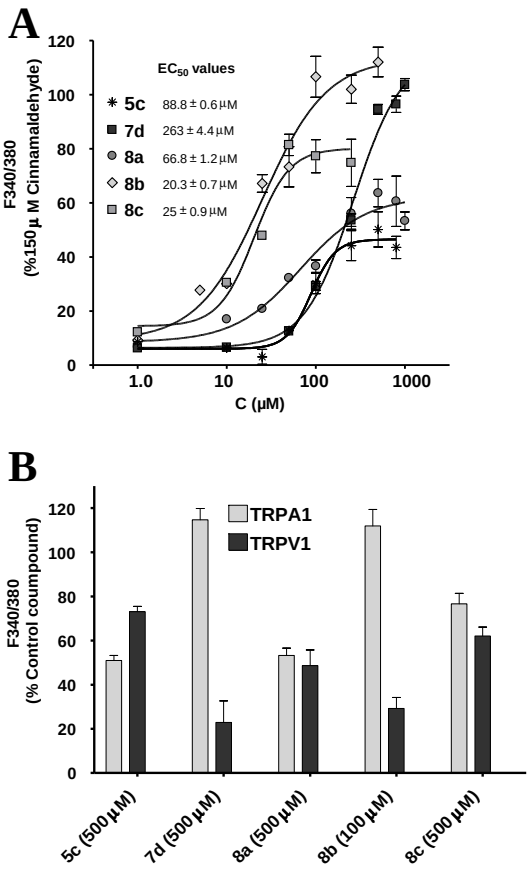


Figure 5. *A:* Alkylamides **7d** and **8a-c** induced dose-dependent increases in $[Ca^{2+}]_i$ rise in TRPA1 expressing in HEK 293 cells. Doses responses were monitored until reaching saturating concentrations and were as follows: 7d, 8a (0.001-1 mM), 8b (1-250 µm), 8c(1-500 µM) *B:* Normalized maximum induced responses of alkylamides **7d** and **8a-c**. Values are presented as means +/- SEM, $n = 4$.

Conclusion

We reported the preparation of new alkylamides designing from sanshools, a class of natural compounds responsible for the burning and the tingling taste of Szechuan pepper. Their evaluation on TRPA1 and TRPV1 channels, two transient receptor potentials involved in the trigeminal perception, led to the elaboration of new selective and more potent TRPA1 agonists **7d** and **8b**. In the future, the alkylamide **8b**, which is easily accessible from the natural linolenic acid, would be prepared in food grade quality so as to evaluate its sensory characteristics and correlate them with its TRPA1 activity.

Experimental section

Chemistry. All commercially available reagents were used as received, from their respective suppliers. Water and air sensitive reactions were carried out under argon atmosphere. Analytical thin layer chromatography (TLC) was carried out on Silica 60 F254 (Merck) and RP-18 F254s (Merck) plates. The TLC plates were visualized by shortwave UV light, ceric ammonium molybdate stain. Flash chromatography was performed using a Biotage SP1 HPFC system and FLASH cartridge (25+M and 40+M KP-SIL). 1H NMR (360.13 MHz) and 13C NMR (90.56 MHz) spectra were recorder on a Bruker DPX-360 spectrometer equipped with a broadband multinuclear z-gradient probehead. The chemical shifts (in ppm) were expressed with respect to an internal reference (TMS or TSP). Multiplicities are reported as follows: s= singlet, d= doublet, t= triplet, q= quatruplet, m= multiplet, bs= broad singlet. Melting points were recorded on Buchi Melting point B-545 apparatus and are uncorrected. Optical rotations were measured with a JASCO P2000 polarimeter. Elementary analyses were performed at the University of Geneva (Service de microanalyse). HPLC chromatogram of purified green Sichuan extract was carried out with a column N-Nucleosil 100 (Macherey-Nagel, 5 um, C-18, 250 x 4 mm), with UV detection at 254 nm and a flow rate of 1 mL/min. Solvents used are A = 0.1% (g/vol) TFA in H_2O, and B = Acetonitrile. HPLC/DAD/ESI-TOF-MS analyses were performed on an Agilent-1200 Series Rapid Resolution LC System including Binary Pump SL, High Performance Autosampler, Diode-array Detector SL and Thermostatted Column Compartment SL with an Agilent 6210 Time-of-flight Mass Spectrometer.

Purification of Green Sichuan Extract: 800 mg of Green Sichuan extract were purified by chromatography column on silica gel using a gradient of ethyl acetate in petroleum ether. Column *FLASH* 40+M was used on a Biotage HPFC Purification system with a flowrate of 40 ml/min. The hydroxy-sanshools were detected at 275 nm and collected in 21 ml fractions. Fractions were concentrated under reduced pressure to give finally 200 mg of hydroxy-sanshools as a mixture of 3 isomers which are not separable by this technique of purification (25% yield in weight). The ratio after purification was established by HPLC and stayed unchanged (*m*/α/β 3.7/75.4/20.8); R_f = 0.35 (Ethyl acetate); *α-Hydroxy-sanshool* ^{1}H NMR (360 MHz, $CDCl_3$, TMS as reference) δ 6.88 (dt, *J* = 15.3, 6.7 Hz, 1H), 6.36-5.92 (m, 5H), 5.84 (dt, *J* = 15.3, 1.3 Hz, 1H), 5.74 (dt, *J* = 13.8, 6.5 Hz, 1H), 5.37 (dt, *J* = 10.8, 6.7 Hz, 1H), 3.32 (d, *J* = 6.1 Hz, 2H), 2.54 (s, 1H), 2.36-2.27 (m, 4H), 1.78 (d, *J* = 6.7 Hz, 3H), 1.23 (s, 6H); ^{13}C NMR (90 MHz, $CDCl_3$, TMS as reference) δ 166.95, 144.47, 133.51, 131.77, 130.2, 129.64, 129.53, 125.44, 123.70, 71.06, 50.41, 32.07, 27.34, 26.46, 18.34; *β-Hydroxy-sanshool* ^{13}C NMR (90 MHz, $CDCl_3$, TMS as reference) δ 166.93, 144.35, 132.03, 131.63, 131.57, 131.44, 130.07, 129.39, 123.71, 71.06, 50.42, 31.90, 31.37, 27.34, 18.29.

Ethyl (2*E*-6Z)-dodeca-2,6-dienoate 2.(25) To a solution of the (ethoxycarbonylmethyl) triphenylphosphorane (9.40 g, 27 mmol, 2 equiv) in anhydrous THF (40 ml), the 4-(*Z*)-decenal **1** (2.46 ml, 13.5 mmol, 1 equiv) was added dropwise and the mixture was stirred overnight at room temperature. The solution was then concentrated to 25 ml and petroleum ether was added to precipitate the phosphonium salt. The white solid was filtered and the filtrate was concentrated under reduced pressure. The resulting residue was purified by flash chromatography column on silica gel using a gradient of ethyl acetate in petroleum ether. Ethyl (2*E*-6Z)-dodeca-2,6-dienoate **2** was isolated as a colorless oil (1.65 g, 7.35 mmol, 54%); R_f = 0.62 (Petroleum ether/Ethyl acetate 95/5); ^{1}H NMR (360 MHz, $CDCl_3$, TMS as internal reference) δ 6.96 (dt, *J* = 15.6, 6.6 Hz, 1H), 5.83 (dt, *J* = 15.6, 1.5 Hz, 1H), 5.45-5.30 (m, 2H), 4.18 (q, *J* = 7.0 Hz, 2H), 2.25-2.18 (m, 4H), 2.04-1.98 (m, 2H), 1.36-1.26 (m, 6H), 1.28 (t, *J* = 6.7 Hz, 3H), 0.88 (t, *J* = 6.7 Hz, 3H); ^{13}C NMR (90 MHz, $CDCl_3$, TMS as internal reference) δ 166.67, 148.64, 131.64, 127.74, 121.57, 60.15, 32.30, 31.49, 29.27, 27.23, 25.75, 22.56, 14.28, 14.06.

(2*E*-6Z)-Dodeca-2,6-dienoic acid 3d.(26) To a solution of ethyl (2*E*-6Z)-dodeca-2,6-dienoate **2** (800 mg, 3.56 mmol, 1 equiv) in THF/water (v/v 1/1, 40 ml), LiOH (512 mg, 21.4 mmol, 6 equiv) was added and the mixture was stirred 12 h at room temperature and 4 h at 80 °C. The medium was then acidified with HCl 1N to pH 5. The organic was dried over sodium sulfate, filtered and concentrated under reduced pressure. The resulting oil was purified by flash chromatography column on silica gel using a gradient of ethyl acetate in petroleum ether. 420 mg of (2*E*-6Z)-dodeca-2,6-dienoic acid **3d** were isolated as a colorless oil (420 mg, 2.13 mmol, 60%); R_f = 0.35 (Petroleum ether/Ethyl acetate 2/1); ^{1}H NMR (360 MHz, $CDCl_3$, TMS as internal reference) δ 7.07 (dt, *J* = 15.7, 6.4 Hz, 1H), 5.84 (dt, *J* = 15.6, 1.6 Hz, 1H), 5.46-5.29 (m, 2H), 2.31-2.20 (m, 4H), 2.04-1.96 (m, 2H), 1.38-1.21 (m, 6H), 0.88 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR (90 MHz, $CDCl_3$, TMS as internal reference) δ 171.55, 151.75, 131.56, 127.49, 120.83, 32.49, 31.49, 29.26, 27.24, 25.60, 22.56, 14.20.

1-Amino-2-methylpropan-2-ol 4.(27) To a solution of 1,1-dimethyl-epoxide (4 ml, 44.98 mmol, 1 equiv) and benzylamine (5.39 ml, 49.42 mmol, 1.1 equiv) in 250 ml water, triethylamine (0.062 ml, 0.449 mmol, 0.01 equiv) was added dropwise at room temperature. The mixture was stirred overnight at the same temperature and then concentrated under reduced pressure. The resulting oil was purified by flash chromatography column on silica gel using a gradient of methanol in dichloromethane. 7.95 g of a colorless oil were isolated as a mixture of 1-(benzylamino)-2-methylpropan-2-ol and benzylamine. The oil was dissolved in methanol (130 ml) and Pd/C (5%, 3.00 g) was added. The mixture was successively degassed and submitted to hydrogen atmosphere. The same operation was performed three times and then the mixture was stirred 1 h 30 min at room temperature under hydrogen atmosphere. The suspension was then filtered over Celite and

concentrated under reduced pressure to give the 1-(amino)-2-methylpropan-2-ol **4** as a colourless oil (2.54 g, 28.49 mmol, 62% from the 1,1-dimethyl-epoxide); ^{1}H NMR (360 MHz, $CDCl_3$, TMS as internal reference) δ 2.60 (s, 2H), 2.04 (s, 2H), 1.16 (s, 6H); ^{13}C NMR (90 MHz, $CDCl_3$, TMS as internal reference) δ69.76, 52.37, 26.74.

General procedure for the preparation of sanshool analogues 5a-d (Table 1): To a solution of acid **3a-d** (1 equiv), 1-amino-2-methylpropan-2-ol **4** (1 equiv) and di-*iso*propylethylamine (2 equiv) in anhydrous DMF (0.084 M), the (benzyltriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate (1 equiv) was added at 0 °C. The solution was stirred 12 h at room temperature and then diluted with water and ethyl acetate. The organic layer was washed with water, dried over sodium sulphate, filtered and concentrated under reduced pressure. The resulting oil was purified by flash chromatography column on silica gel using a gradient of ethyl acetate in petroleum ether.

***N*-(2-Hydroxy-2-methylpropyl)-dodecanamide 5a.** 57% yield, white solid; R_f = 0.51 (ethyl acetate); ^{1}H NMR (360 MHz, $CDCl_3$, TMS as internal reference) δ6.48 (s, 1H), 3.28 (d, *J* = 5.7 Hz, 2H), 3.17 (s, 1H), 2.28 (t, *J* = 7.5 Hz, 2H), 1.67-1.61 (m, 2H), 1.34-1.23 (m, 16H), 1.23 (s, 6H), 0.87 (t, *J* = 6.6 Hz, 3H); ^{13}C NMR (90 MHz, $CDCl_3$, TMS as internal reference) δ 177.83, 73.71, 53.41, 39.28, 34.71, 32.41, 32.29, 32.14, 32.10, 30.05, 28.74, 25.49, 16.92; LC/ESI-TOF-MS: obs. *m/z* [M-1]-: 270.24; Anal. cald for $C_{16}H_{33}NO_2$: C 70.80, H 12.29, N 5.12 ; found: C 70.83, H 12.29, N 5.12.

(2*E*)-*N*-(2-Hydroxy-2-methylpropyl)-dodec-2-enamide 5b. 66% yield; pale orange oil; R_f = 0.60 (ethyl acetate); ^{1}H NMR (360 MHz, $CDCl_3$, TMS as internal reference) δ6.79 (dt, *J* = 15.3, 7.0 Hz, 1H, H-10), 6.03 (s, 1H), 5.75 (dt, *J* = 15.2, 1.5 Hz, 1H), 3.26 (d, *J* = 6.0 Hz, 2H), 2.06 (qd, *J* = 7.7, 1.4 Hz, 2H), 1.40-1.34 (m, 2H), 1.24-1.17 (m, 12H), 1.16 (s, 6H), 0.81 (t, *J* = 6.7 Hz, 3H); ^{13}C NMR (90 MHz, $CDCl_3$, TMS as internal reference) δ167.35, 145.79, 123.23, 71.14, 50.57, 32.21, 32.00, 29.93, 29.55, 29.42, 29.33, 28.36, 27.42, 22.79, 14.23; LC/ESI-TOF-MS: obs. *m/z* [M-1]-: 268.23; Anal. cald for $C_{16}H_{31}NO_2$ + 0.2H_2O: C 70.39, H 11.59, N 5.13; found: C 70.36, H 11.47, N 5.08.

(5*Z*)-*N*-(2-Hydroxy-2-methylpropyl)-dodec-5-enamide 5c. 57% yield; pale orange oil; R_f = 0.60 (ethyl acetate); ^{1}H NMR (360 MHz, $CDCl_3$, TMS as internal reference) δ5.87 (s, 1H), 5.37-5.22 (m, 2H), 3.19 (d, *J* = 6.0 Hz, 2H), 2.16 (t, *J* = 7.4 Hz, 2H), 2.01 (q, *J* = 6.9 Hz, 2H), 1.93 (q, *J* = 6.4 Hz, 2H), 1.64 (q, *J* = 7.5 Hz, 2H), 1.27-1.15 (m, 8H), 1.15 (s, 6H), 0.81 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR (90 MHz, $CDCl_3$, TMS as internal reference) δ 176.95, 133.96, 131.21, 73.73, 53.13, 39.94, 34.57, 32.48, 31.80, 30.10, 30.09, 29.48, 28.56, 25.45, 16.91; LC/ESI-TOF-MS: obs. *m/z* [M-1]-: 268.23; Anal. cald for $C_{16}H_{31}NO_2$ + 0.2H_2O: C 70.39, H 11.59, N 5.13; found: C 70.58, H 11.56, N 5.16.

(2*E*-6*Z*)-*N*-(2-Hydroxy-2-methylpropyl)-dodeca-2,6-dienamide 5d. 77% yield; (ratio (6*Z*-2*E*)/(6*E*-2*E*) 87.5/12.4, 77%); colourless oil; R_f = 0.51 (ethyl acetate); ^{1}H NMR (360 MHz, $CDCl_3$, TMS as internal reference) δ 6.87 (dt, *J* = 15.3, 6.7 Hz, 1H), 5.95 (s, 1H), 5.83 (d, *J* = 15.4 Hz, 1H), 5.44-5.30 (m, 2H), 3.33 (d, *J* = 5.9 Hz, 2H), 2.59 (s, 1H), 2.27-2.15 (m, 4H), 2.04-1.97 (m, 2H), 1.36-1.21 (m, 6H), 1.23 (s, 6H), 0.88 (t, *J* = 6.5 Hz, 3H); ^{13}C NMR (90 MHz, $CDCl_3$, TMS as internal reference) δ 166.99, 144.81, 131.21, 127.89, 123.34, 71.04, 50.41, 32.19, 31.47, 29.27, 27.31, 27.22, 25.91, 22.54, 14.05; LC/ESI-TOF-MS: obs. *m/z* [M-1]-: 266.18; Anal. cald for $C_{16}H_{29}NO_2 + 0.3H_2O$: C 70.44, H 10.94, N 5.13; found: C 70.53, H 10.97, N 5.10.

Typical procedure for the preparation of compounds 7a-d and 8a-c (Table 2 and 3): To a solution of carboxylic acid **3b-f** (1 equiv), aminoacid methyl ester hydrochlorid **6a-d** (1 equiv) and di-isopropylethylamine (2 equiv) in DMF (0.084 M), the (benzyltriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate (1 equiv) was added at 0 °C. The solution was stirred 12 h at room temperature and then diluted with water and ethyl acetate. The organic layer was washed with water, dried over sodium sulphate, filtered and concentrated under reduced pressure. The resulting oil was purified by flash chromatography column on silica gel using a gradient of ethyl acetate in petroleum ether. The resulting methyl ester (1 equiv) was dissolved in THF/H_2O (0.084 M, v/v, 3/1) and LiOH (3 equiv) was added at room temperature. The mixture was stirred 2 h and then acidified with 0.5 N HCl to pH 6. Ethyl acetate was added, the aqueous phase was concentrated under reduced pressure, purified by flash chromatography column on reverse phase using a gradient of methanol in water and finally freeze-dried to give a white foam.

(*S,Z*)-2-Dodec-5-enamido-3-hydroxypropanoic acid 7a: 78% yield; white foam; R_f = 0.55 (Methanol/Water/AcOH 80/20/1); $[\alpha]^{24}_D$= +24.07° (*c* 1.08 mg/ml, MeOH); ^{1}H NMR (360 MHz, MeOD-d^4, TMS as internal reference) δ 5.45-5.34 (m, 2H), 4.31 (t, *J* = 4.8 Hz, 1H), 3.81 (d, *J* = 4.8 Hz, 2H), 2.29 (t, *J* = 7.1 Hz, 2H), 2.11 (q, *J* = 6.9 Hz, 2H), 2.06 (q, *J* = 6.3 Hz, 2H), 1.71 (dq, *J* = 7.9, 7.9 Hz, 2H), 1.36-1.30 (m, 8H), 0.91 (t, *J* = 6.3 Hz, 3H); ^{13}C NMR (90 MHz, MeOD-d^4, TMS as internal reference) δ 178.16, 176.96, 133.11, 131.27, 65.75, 59.43, 38.28, 34.34, 32.24, 31.48, 29.60, 29.19, 28.34, 25.11, 15.83; LC/ESI-TOF-MS: obs. *m/z* [M-1]-: 284.16 (M-H); Anal. cald for $C_{14}H_{26}NO_4Li + 0.4H_2O$: C 60.35, H 9.05, N 4.69; found: C 60.22, H 8.83, N 4.62.

(*S,Z*)-2-Dodec-5-enamidopentanedioic acid 7b: 69% yield; white solid; R_f = 0.50 (Methanol/Water/AcOH 60/40/1); $[\alpha]^{24}_D$= -4.90° (*c* 1.02 mg/ml, MeOH); ^{1}H NMR (360 MHz, MeOD-d^4, TMS as internal reference) δ 5.45-5.33 (m, 2H), 4.43 (dd, *J* = 8.9, 5.0 Hz, 1H), 2.40 (td, *J* = 7.9, 1.2

Hz, 2H), 2.27 (t, *J* = 7.4 Hz, 2H), 2.17-1.89 (m, 6H), 1.68 (dq, *J* = 7.3, 7.3 Hz, 2H), 1.35-1.30 (m, 8H), 0.91 (t, *J* = 6.3 Hz, 3H); ^{13}C NMR (90 MHz, MeOD-*d*^{4}, TMS as internal reference) δ 177.88, 177.56, 176.74, 133.18, 131.20, 54.66, 37.79, 34.34, 32.79, 32.22, 31.46, 29.59, 29.46, 29.10, 28.38, 25.11, 15.83; LC/ESI-TOF-MS: obs. *m/z* [M+1]+: 345 (M+ H_2O); Anal. cald for $C_{17}H_{29}NO_5$+ 0.3H_2O: C 61.35, H 8.96, N 4.21; found: C 61.47, H 8.81, N 4.18.

(*Z*)-2-Dodec-5-enamidoacetic acid 7c: 64% yield; white foam; R_f = 0.42 (Methanol/Water/AcOH 80/20/1); ^{1}H NMR (360 MHz, MeOD-*d*^{4}, TMS as internal reference) δ 5.44-5.33 (m, 2H), 3.37 (s, 2H), 2.26 (t, *J* = 7.8 Hz, 2H), 2.10 (q, *J* = 7.0 Hz, 2H), 2.06 (q, *J* = 6.3 Hz, 2H), 1.64 (qd, *J* = 7.5, 7.5 Hz, 2H), 1.38-1.30 (m, 8H), 0.91 (t, *J* = 6.3 Hz, 3H); ^{13}C NMR (90 MHz, MeOD-*d*^{4}, TMS as internal reference) δ 177.86, 177.03, 133.11, 131.26, 45.92, 38.06, 34.34, 32.23, 31.48, 29.60, 29.17, 28.32, 25.11, 15.84; LC/ESI-TOF-MS: obs. *m/z* [M-1]-: 254.20 (M-H); Anal. cald for $C_{14}H_{24}NO_3Li$ + 0.35H_2O: C 62.42, H 9.32, N 5.20; found: C 62.28, H 9.27, N 5.19.

(*S,Z*)-2-Dodec-5-enamidopropanoic acid 7d: 56% yield; white foam; R_f = 0.39 (Methanol/Water/AcOH 80/20/1); $[\alpha]^{24}_D$= + 14.17° (*c* 1.20 mg/ml, MeOH); ^{1}H NMR (360 MHz, MeOD-*d*^{4}, TMS as internal reference) δ 5.41-5.33 (m, 2H), 4.21 (q, *J* = 7.1 Hz, 1H), 2.22 (t, *J* = 7.5 Hz, 2H), 2.06 (q, *J* = 6.8 Hz, 2H), 2.02 (q, *J* = 6.3 Hz, 2H), 1.70-1.61 (m, 2H), 1.36-1.28 (m, 8H), 1.32 (d, *J* = 7.1 Hz, 3H), 0.91 (t, *J* = 6.3 Hz, 3H); ^{13}C NMR (90 MHz, MeOD-*d*^{4}, TMS as internal reference) δ 179.87, 174.91, 131.71, 129.86, 51.83, 36.82, 32.94, 30.83, 30.08, 28.20, 27.73, 27.01, 23.71, 19.45, 15.84; LC/ESI-TOF-MS: obs. *m/z* [M-1]-: 268.20 (M-H); Anal. cald for $C_{15}H_{26}NO_3Li$ + 0.35H_2O: C 63.97, H 9.56, N 4.97; found: C 64.14, H 9.48, N 4.94.

(*S,E,Z*)-2-[Dodeca-2,6-dienamido]propanoic acid 8a: 73% yield; white solid, R_f = 0.71 (Methanol/Water 80/20); mp = 95-97 °C; $[\alpha]^{25}_D$= -7.84° (1.02 mg/ml, MeOH); ^{1}H NMR (360 MHz, MeOD-*d*^{4}, TMS as reference) δ 6.78 (dt, *J* = 15.4, 6.5 Hz, 1H), 5.95 (d, *J* = 15.3 Hz, 1H), 5.44-5.34 (m, 2H), 4.40 (qd, *J* = 7.2, 7.2 Hz, 1H), 2.26-2.15 (m, 4H), 2.03 (q, *J* = 6.9 Hz, 2H), 1.39 (d, *J* = 7.2 Hz, 3H),1.39-1.30 (m, 6H), 0.88 (t, *J* = 6.5 Hz, 3H); ^{13}C NMR (90 MHz, MeOD-*d*^{4}, TMS as reference) δ 177.03, 168.04, 145.40, 132.01, 129.22, 124.85, 49.97, 33.26, 32.66, 29.27, 28.19, 27.13, 23.64, 18.23, 14.43; LC/ESI-TOF-MS: obs. *m/z* [M-1]-: 266.19; Anal. cald for $C_{15}H_{24}LiNO_3$ + 0.5H_2O: C 63.82, H 8.93, N 4.96; found: C 63.93, H 8.97, N 4.81.

(*Z,Z,Z*)-2-[Octadeca-9,12,15-trienamido]propanoic acid 8b: 33% yield; white solid, R_f = 0.24 (Methanol/Water 80/20); mp =100-102 °C; $[\alpha]^{25}_D$= +5.32° (0.94 mg/ml, MeOH); ^{1}H NMR (360 MHz, MeOD-*d*^{4}, TMS as reference) δ 7.27 (d, *J* = 6.3 Hz, 1H), 5.39-5.26 (m, 6H), 3.70 (qd, *J* = 6.8, 6.8 Hz, 1H),

2.77 (t, *J* = 5.8 Hz, 4H), 2.06-1.99 (m, 6H), 1.45 (qd, *J* = 7.3, 7.3 Hz, 2H), 1.32-1.20 (m, 8H), 1.12 (d, *J* = 6.9 Hz, 3H), 0.92 (t, *J* = 7.5 Hz, 3H); ^{13}C NMR (90 MHz, MeOD-*d*^{4}, TMS as reference) δ 178.10, 174.64, 135.57, 133.99, 132.02, 131.98, 131.61, 131.06, 54.01, 36.48, 29.76, 29.46, 29.43, 29.32, 27.36, 26.12, 25.91, 25.82, 20.76, 20.17, 14.85; LC/ESI-TOF-MS: obs. *m/z* [M-1]-: 348.28; Anal. cald for $C_{21}H_{34}NO_3 + 0.6H_2O$: C 68.87, H 9.69, N 3.82; found: C 69.02, H 9.54, N 3.74.

(*Z*)-2-[Hexadeca-9-enamido]propanoic acid 8c: 76% yield; white solid, R_f = 0.24 (Methanol/Water 80/20); mp = 56-58 °C; $[\alpha]^{25}_D$= -14.42° (1.04 mg/ml, MeOH); ^{1}H NMR (360 MHz, DMSO-*d*^{6}, TMS as reference) δ 8.04 (d, *J* = 7.2 Hz, 1H), 5.32 (t, *J* = 5.3 Hz, 2H), 4.17 (qd, *J* = 7.3, 7.3 Hz, 1H), 2.07 (t, *J* = 7.3 Hz, 2H), 1.98-1.96 (m, 4H), 1.48-1.45 (m, 2H), 1.39-1.21 (m, 19H), 0.85 (t, *J* = 6.2 Hz, 3H); ^{13}C NMR (90 MHz, DMSO-*d*^{6}, TMS as reference) δ 174.52, 172.12, 129.82, 47.85, 35.17, 31.33, 29.30, 29.28, 28.88, 28.79, 28.74, 28.47, 26.79, 25.37, 22.28, 17.42, 14.12; LC/ESI-TOF-MS: obs. *m/z* [M-1]-: 324.29; Anal. cald for $C_{19}H_{35}NO_3 + 0.3H_2O$: C 68.97, H 10.84, N 4.23; found: C 68.96, H 10.74, N 3.99.

Molecular biology

Cloning and expression of human TRPV1 and TRPA1 receptors in HEK293 cells: Cloning and expression of these receptors was performed following previously published protocols (21). Briefly, cloned human TRPV1 cDNA was obtained from RZPD (Germany) and hTRPA1 cDNA from OriGene (Rockville, MD). Genes were subcloned into pcDNA5/FRT (Invitrogen, Carlsbad, CA) to generate stable cell lines using the Flp-In system (Invitrogen) after sequencing verification.

Measurement of intracellular calcium levels [Ca2+]$_i$ and membrane potential variation in HEK 293 cells using a fluorescent plate reader: Cell-lines stably expressing TRP channels were seeded into 96 well plates previously coated with poly-D-lysine. Cells were incubated in Hank's Balanced Salt Solution (HBSS) supplemented with 2mM $CaCl_2$ and 20mM HEPES (pH 7.4), containing the cytoplasmic calcium indicator Fura-2/AM at 2 μM (Molecular Devices, Sunnyvale, CA). For membrane potential assays, cells were loaded with a voltage-sensitive dye according to protocol (Red dye, Molecular Devices) and fluorescence changes were measured upon application of compounds (λ_{ex1}=530 nm, λ_{em}=565 nm). Experiments were conducted at room temperature. [Ca2+]$_i$ fluxes from an homogenous cell population (approximately 100'000 cells) were measured as changes in fluorescence intensity when stimulated with agonists using a FLEXstation (Molecular Devices). Cells expressing TRP channels were then challenged with the different sanshools derivatives.

Data analysis: Responses of molecules in HEK 293 cells were expressed as a percentage of maximum responses evoked by 150µM cinnamaldehyde for TRPA1 and 1µM capsaicin for TRPV1 (these concentrations were assessed independently to be saturating under these conditions). For all experiments, calcium fluxes were measured as changes in fluorescence intensity, before and after the addition of agonists. The peak response was taken to be the characteristic value and was obtained by substracting the peak value from the baseline (value before injection). Doses response curves were fitted using the Hill equation (GraphPad Prism Software, San Diego, CA) to obtain EC_{50} values and Hill coefficients. Data obtained from this study were expressed as mean ± S.E.M.

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2004 **Master thesis**, Aroma and taste impact group, Nestlé Research Center, Lausanne, Switzerland. Supervisor: Dr Fabien Robert
Study of physico-chemical interactions between low molecular weight molecules and macromolecules of a food model.

2001-2004 **Master degree** (Engineering degree), Chemistry and Chemical Engineering, French National Institute of Applied Sciences (INSA), Rouen, France.

2004 **Exchange programme** in biochemistry, molecular biology, biotechnology, environmental biology, EPFL.

2003 **Research project**, Laboratory of Macromolecular Materials, INSA. Supervisor: Dr Fabrice Burel
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1999-2001 **Preparatory cycle** at INSA Rouen, Bilingual International English section (SIB-IBIS programme).

1999 French Scientific **Baccalaureate,** first class Honors, Lycée Charles Renouvier, Prades, Pyrénées Orientales, France.

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Riera C.E., Vogel H., Simon S.A., Damak S. and le Coutre J. Submitted to J. Neuroscience, 2008 aug.

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PATENTS

Tingling flavour composition (2007) European Patent Application N° 07120825 (first Filling).
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The EPFL Ph.D student in industry: serving two masters.
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Artificial sweeteners stimulate sensory neurons through activation of TRPV1 receptors.
29th annual Association for Chemoreception Sciences (AChemS) meeting, Sarasota, USA, 2007

Food flavours and the sweetener saccharin activate the transient receptor potential vanilloid subtype I (TRPV1) channel.
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