

A transient receptor potential channel expressed in taste receptor cells

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We used differential screening of cDNAs from individual taste receptor cells to identify candidate taste transduction elements in mice. Among the differentially expressed clones, one encoded *Trpm5*, a member of the mammalian family of transient receptor potential (TRP) channels. We found *Trpm5* to be expressed in a restricted manner, with particularly high levels in taste tissue. In taste cells, *Trpm5* was coexpressed with taste-signaling molecules such as α -gustducin, $G\gamma_{13}$, phospholipase C- β_2 (PLC- β_2) and inositol 1,4,5-trisphosphate receptor type III (IP₃R3). Our heterologous expression studies of *Trpm5* indicate that it functions as a cationic channel that is gated when internal calcium stores are depleted. *Trpm5* may be responsible for capacitative calcium entry in taste receptor cells that respond to bitter and/or sweet compounds.

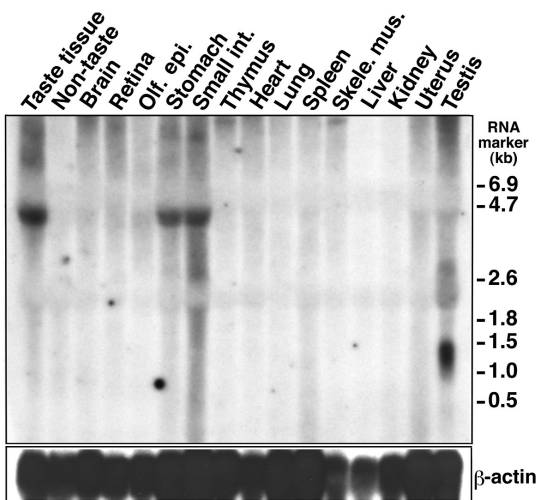
The sense of taste is critical for the nutrition and survival of humans and other organisms. There are four widely accepted human taste qualities—sweet, bitter, salty and sour—and two more debated qualities: fat and umami (the taste of glutamate). The ability to identify sweet-tasting food is particularly important, as it provides the means to seek out nutritive carbohydrates. The perception of bitter taste, on the other hand, is important for its protective value, enabling the avoidance of harmful and potentially deadly plant alkaloids and other environmental toxins.

Taste is mediated by multiple signaling cascades present in specialized taste receptor cells (TRCs) that are found within taste buds in the lingual epithelium (for review, see refs. 1,2). Salty and sour tastes are elicited by cations such as Na⁺, K⁺ and H⁺, which pass through and/or modulate ion channels such as ASIC (acid-sensitive ion channel), HCN (hyperpolarization-activated cation channel), MDEG1 (mammalian degenerin channel) and ENaC (epithelial sodium channel)². The detection of bitter, sweet and umami compounds depends on signaling cascades initiated by G-protein-coupled receptors (GPCRs). The T2R and TRB receptors comprise a gene family encoding ~25 GPCRs presumed to be responsive to bitter compounds^{2–5}. *Tas1r3* encodes the taste receptor T1r3, is allelic with *Sac* (the rodent sweet-sensitivity locus) and is a component of the sweet-responsive receptor^{6–11}. Glutamate and other umami

compounds may be detected by the taste receptor mGluR4, a variant metabotropic glutamate GPCR¹², or by coexpressed T1r1 and T1r3 receptors¹³.

There are several known downstream signaling components that are responsive to bitter and sweet compounds^{1,2}, including α -gustducin^{14,15}, $G\gamma_{13}$ ¹⁶, $G\beta_3$ ¹⁷, PLC- β_2 ¹⁸, phosphodiesterase 1A (PDE 1A; M. Bakre, R. Lupi and R.F.M., unpub. observ.), IP₃R3^{19,20} and cyclic nucleotide-regulated channels²¹. Release of calcium ions from internal stores has been implicated in TRC responses to both bitter and sweet compounds^{22,23}. The generation of diacyl glycerol (DAG) and IP₃ by bitter compounds such as denatonium depends on activation of T2R/TRB receptors coupled to PLC- β_2 by heterotrimeric gustducin^{5,16–18,24}. Many of these candidate transduction elements, such as IP₃R3, PLC- β_2 and $G\gamma_{13}$, are coexpressed in a subset of TRCs^{19,20}.

Additional unknown signaling molecules may be involved in Ca²⁺-related TRC signal transduction as well. In other cell types, capacitative calcium entry (CCE)—Ca²⁺ influx subsequent to Ca²⁺ release from internal stores—is thought to be mediated by TRP channels²⁵. Mammalian TRP channels have been implicated in diverse physiological processes²⁶, including several sensory responses, but not gustation. On the basis of their sequence length and relatedness, TRP channels can be subdivided into three subgroups: canonical (TRPC), vanilloid (TRPV) and melastatin (TRPM)²⁶.



Here we describe the molecular cloning from mouse TRCs of *Trpm5*, a long TRP channel belonging to the TRPM subfamily. *Trpm5* is selectively expressed in TRCs, where it is coexpressed with other molecules involved in taste transduction. Our characterization of heterologously expressed *Trpm5* suggests that it may function in TRCs to mediate CCE.

RESULTS

Identification of a TRP channel in TRCs

To identify genes preferentially expressed in the α -gustducin-positive (α -gus⁺) subset of TRCs, we generated cDNA probes and libraries from individual TRCs by RT-PCR¹⁶. These α -gus⁺ TRCs from transgenic mice expressing green fluorescent protein (GFP) from the α -gustducin promoter²⁷ were readily distinguished from α -gus⁻ TRCs by their green fluorescence and bipolar morphology. cDNA libraries from single α -gus⁺ TRCs were screened by hybridization with probes from single α -gus⁺ ('self') and α -gus⁻ ('non-self') TRCs. Of 40,000 bacteriophage clones from an α -gus⁺ cDNA library differentially screened, 60 clones were positive with the self-probe and negative with the non-self probe (see Supplementary Fig. 1 online). By DNA sequencing, we determined that one of these clones, *lqseq91*, did not have significant homology to any entries in the GenBank database (dots B3 and B'3 in Supplementary Fig. 1). A full-length clone was isolated by screening 500,000 plaques from a mouse taste tissue cDNA library using DNA from the *lqseq91* clone as the probe. Sequencing of multiple clones identified an open reading frame of 4,077 bp predicted to encode a protein of 1,158 amino acids (later determined to correspond to GenBank protein record AAF98120). A BLAST search of the GenBank database indicated that the predicted protein was a novel member of the TRP family of ion channels. A BLAST search of the finished and unfinished human genomic sequences identified the human ortholog, which mapped to chromosome 11p15.5.

Fig. 2. Expression of *Trpm5* mRNA in taste receptor cells. Sections of mouse lingual epithelia containing circumvallate and foliate papillae were hybridized with ³³P-labeled antisense RNA probes for *Trpm5* (a and c) or *Gnat3* (α -gustducin) (d), and then visualized by autoradiography. Hybridization controls with sense probes showed the absence of non-specific binding of the *Trpm5* probe (b) or the *Gnat3* probe (e).

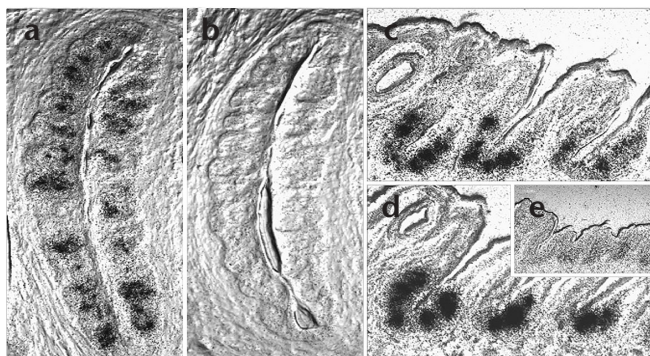


Fig. 1. Distribution of *Trpm5* mRNA in mouse tissues. Top, autoradiogram of a northern blot hybridized with a *Trpm5* cDNA probe. Each lane contained 25 μ g total RNA isolated from the following mouse tissues (from left to right): circumvallate and foliate papillae-enriched taste tissue (taste tissue), lingual tissue devoid of taste buds (non-taste), brain, retina, olfactory epithelium, stomach, small intestine, thymus, heart, lung, spleen, skeletal muscle, liver, kidney, uterus and testis. A 4.5-kb transcript is present in taste tissue, stomach and small intestine, and, to a much lesser extent, in uterus and testis. Bottom, to control for mRNA quantity, the same blot was stripped and reprobbed with a β -actin cDNA probe.

After our cloning and preliminary characterization of this TRC-expressed TRP channel, two groups independently identified its mouse^{28,29} and human³⁰ forms and named it Mtr1 (melastatin and TRP-related protein 1). These reports identify Mtr1 in the context of imprinting associated with human chromosome 11p15.5 and mouse distal chromosome 7, but do not address Mtr1 expression in TRCs or its potential function in taste transduction. According to recently proposed nomenclature for TRP channels, Mtr1 has been renamed *Trpm5* (as the fifth member of the melastatin-related TRP channel subfamily)³¹.

***Trpm5* is selectively expressed in taste tissue**

Although we identified *Trpm5* by differential screening of clones expressed in α -gus⁺ versus α -gus⁻ TRCs, we also investigated whether *Trpm5* is more broadly expressed in other taste cells and tissues. To determine the tissue distribution of *Trpm5* mRNA, we carried out northern blotting with multiple mouse tissues. A *Trpm5* 3' untranslated region (UTR) probe hybridized predominantly to a transcript of 4.5 kb in taste tissue, with no detectable expression in lingual tissue devoid of taste buds. Lower expression was also detected in stomach and small intestine, and very weak expression was seen in uterus and testis (Fig. 1). This contrasts with previous studies²⁹ in which an RT-PCR probe that amplified the 3' portion of the *Trpm5* coding region detected highest expression in liver and low-level expression in other tissues such as heart, brain, kidney and testis. As this probe was generated by RT-PCR of total RNA, it may have been contaminated by unspliced *Trpm5* RNA. An examination of the mouse *Trpm5* genomic sequence shows stretches of intronic sequences homologous to what seems to be an expressed pseudogene with wide tissue distribution in the expressed sequence tag (EST) databases. Such elements are also present in the human *Trpm5* gene and may provide an explanation for the appearance of a broad tissue distribution on northern and dot blots despite the very low representation of both mouse and human *Trpm5* genes in the EST databases (Supplementary Fig. 2). Alternatively, the RT-PCR



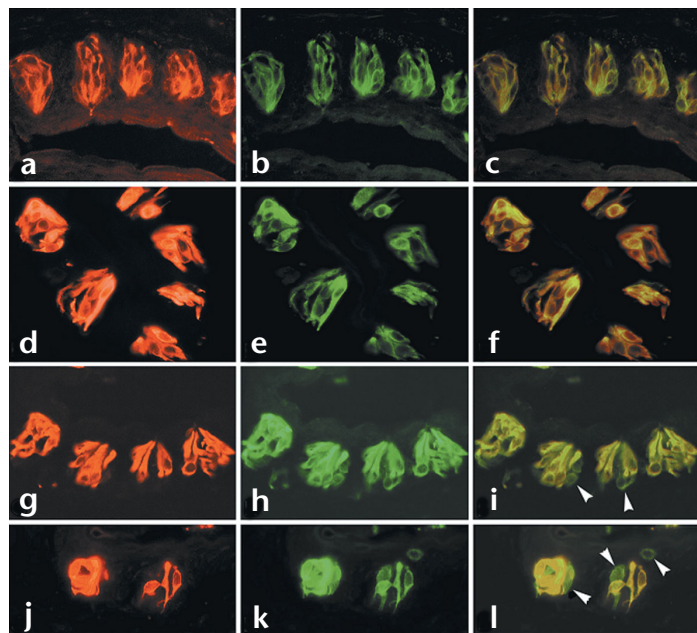


Fig. 3. Coexpression of Trpm5, $G\gamma_{13}$ and PLC- β_2 in taste receptor cells. Immunofluorescence of $G\gamma_{13}$ (a), Trpm5 (b) and their overlay (c) in the same longitudinal section of mouse circumvallate papillae. Immunofluorescence of PLC- β_2 (d), Trpm5 (e) and their overlay (f). Immunofluorescence of α -gustducin (g, j), Trpm5 (h, k) and their overlays (i and l). Most TRCs that were positive for Trpm5 also expressed α -gustducin; arrowheads indicate TRCs that expressed Trpm5 but not α -gustducin. Magnification, 200 \times .

probe may have detected other TRP-encoding mRNAs by cross-hybridization. Although there are reports³² of alternative splice sites in mouse *Trpm5*, our 3' UTR probe would have detected all of these variants, as the 3' UTR is common to all of the predicted mRNAs.

Trpm5 is expressed in a subset of TRCs

We used *in situ* hybridization to determine the cellular pattern of expression of Trpm5 in mouse TRCs. Trpm5-encoding mRNA was found in TRCs in circumvallate and foliate papillae, but not in the surrounding non-gustatory epithelia (Fig. 2a and c). Trpm5-expressing TRCs were present in most of the taste buds, although not all TRCs were positive, suggesting that expression was restricted to a subset of TRCs. The general pattern of Trpm5 expression in taste buds was comparable to that of α -gustducin (Fig. 2d). Sections hybridized with control sense probes showed minimal labeling of TRCs and the surrounding tissue by either the Trpm5 (Fig. 2b) or the α -gustducin probes (Fig. 2e).

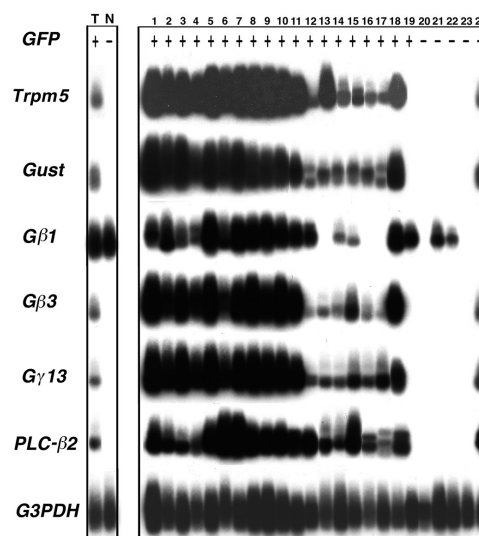
To determine whether Trpm5 is coexpressed in TRCs with signal transduction elements that might be involved in its activation, we did double immunostaining of sections containing TRCs using antisera against pairs of signaling molecules. Trpm5 protein was coexpressed absolutely with $G\gamma_{13}$ (Fig. 3a–c) and PLC- β_2 (Fig. 3d–f). We have previously noted that TRCs positive for $G\gamma_{13}$ and PLC- β_2 also express IP₃R³¹⁹, suggesting that these four molecules might be part of a common signal trans-

duction pathway. For the most part, Trpm5 was coexpressed with α -gustducin (Fig. 3g–i), although a subset of the Trpm5⁺ TRCs were negative for α -gustducin and all α -gus⁺ TRCs expressed Trpm5 (Fig. 3i and l, arrowheads). This pattern is consistent with our observations that α -gus⁺ TRCs constitute a subset of TRCs that express $G\gamma_{13}$, $G\beta_3$, PLC- β_2 and IP₃R³^{16,19} (L.H. and R.F.M., unpub. observ.). We also confirmed the expression of human TRPM5 in human fungiform taste buds by immunohistochemistry (data not shown).

To independently monitor coexpression of Trpm5 in TRCs with the above-mentioned signal transduction molecules, as well as with $G\beta_1$ and $G\beta_3$, we carried out single-cell expression profiling¹⁶. In this way, we determined that expression of α -gustducin, $G\beta_3$, $G\gamma_{13}$, PLC- β_2 and Trpm5 was restricted to taste tissue (Fig. 4, left), and that in this particular set of 24 TRCs, Trpm5 was coexpressed with α -gustducin, $G\beta_3$, $G\gamma_{13}$ and PLC- β_2 (Fig. 4, right). Expression of Trpm5 largely overlapped with that of $G\beta_1$ (15 of 19 Trpm5⁺ cells were also $G\beta_1$ ⁺). The coincident expression of these various signal transduction molecules with Trpm5 and IP₃R³¹⁹ could provide the physical opportunity for activation of Trpm5 by IP₃/IP₃R³-mediated depletion of internal calcium stores. This depletion would be mediated by a signaling pathway in which GPCRs couple to heterotrimeric gustducin (for example, α -gustducin/ β_3 / γ_{13}) or to other $G\alpha/\beta_1-\beta_3/\gamma_{13}$ -containing heterotrimers that might release $\beta\gamma$ to activate PLC- β_2 .

To identify other TRP channels in TRCs, we used PCR to determine whether Trpc1–6, or Trpm1 and Trpm2, are expressed in taste tissue. Amplification by PCR using specific primer pairs did not identify products for any of these eight mouse TRP chan-

Fig. 4. Single taste receptor cell expression of Trpm5, α -gustducin, $G\beta_1$, $G\beta_3$, $G\gamma_{13}$ and PLC- β_2 . Left, Southern hybridization to RT-PCR products from mouse taste tissue (T) and control non-taste lingual tissue (N). We used 3'-region probes from *Trpm5*, *Gnat3* (α -gustducin), *Gnb1* ($G\beta_1$), *Gnb3* ($G\beta_3$), *Gng13* ($G\gamma_{13}$), *Plcb2* (PLC β_2) and *G3pdh* (glyceraldehyde-3-phosphate dehydrogenase). Note that Trpm5, α -gustducin, $G\beta_3$, $G\gamma_{13}$ and PLC- β_2 were all expressed in taste tissue but not in non-taste tissue. Right, Southern hybridization to RT-PCR products from 24 individually amplified taste receptor cells from a transgenic mouse expressing green fluorescent protein (GFP) from the gustducin promoter²⁷. *G3pdh* served as a positive control to demonstrate successful amplification of products.



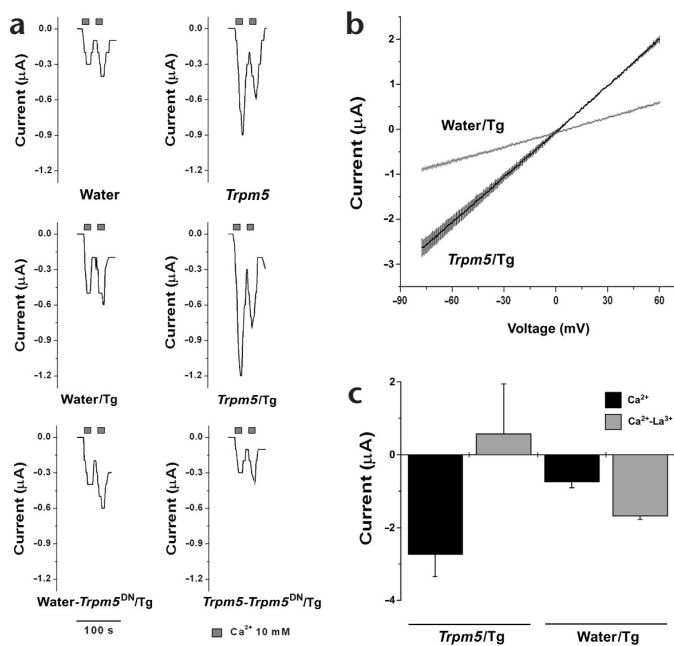


Fig. 5. Heterologous expression of Trpm5 in *X. laevis* oocytes. (a) Oocytes were injected with water or Trpm5 cRNA and/or Trpm5^{DN} cRNA. Two days after injection, oocytes were incubated in a Ca^{2+} -free ND96 solution with Tg. Recordings were started in the presence of EGTA, and Ca^{2+} was added later (filled squares). The traces represent currents elicited at a membrane potential of -80 mV. (b) I - V curves for oocytes treated with Tg, injected with water or Trpm5 cRNA ($n = 4$, mean \pm s.e.m.). (c) The average maximum inward current elicited with external Ca^{2+} (black) or present in the bathing media or after addition of La^{3+} (gray) for oocytes treated with Tg and injected with water or Trpm5 cRNA ($n = 4$, mean \pm s.e.m.; holding potential was -80 mV).

nels when taste tissue cDNA was used as the template (data not shown). In control experiments, *Trpc1-6* and *Trpm1* and *Trpm2* PCR products (confirmed by DNA sequencing) were amplified from one or more non-taste cDNA templates (brain, retina or intestine; data not shown). In additional controls, DNA sequencing confirmed that the *Trpm5* PCR product was indeed amplified from taste tissue cDNA (data not shown). These results suggest that these eight TRPs are not expressed highly, if at all, in taste tissue. Other TRP channels might be expressed in TRCs, but at present Trpm5 is the only known TRP channel highly expressed in taste tissue and, as shown here, in TRCs.

Expressed Trpm5 facilitates Ca^{2+} influx

To determine whether Trpm5 functions as a calcium channel, we expressed it in *Xenopus laevis* oocytes and CHO (Chinese hamster ovary) cells. We used the oocyte's endogenous calcium-activated chloride conductance (I_{ClCa}) as a reporter to detect changes in calcium conductance that are due to expression of Trpm5 (as has been done previously with other TRP channels³³). Mouse *Trpm5* cRNA was injected into *X. laevis* oocytes, and two-electrode voltage clamp recordings were taken two days later. To determine whether Ca^{2+} flux through Trpm5 might be activated by depletion of Ca^{2+} stores, we incubated the oocytes for two hours before the recording in $2-4 \mu\text{M}$ thapsigargin (Tg), an irreversible inhibitor of the sarco-endoplasmic reticulum Ca^{2+} -ATPase (SERCA)³⁴.

Representative recording traces of oocytes injected with Trpm5 cRNA and treated with Tg showed a distinct inward current typical of the oocyte's I_{ClCa} , which was elicited by switching the external bath solution from 1 mM EGTA to 10 mM Ca^{2+} (Fig. 5a, Trpm5/Tg trace). These traces differed significantly in magnitude from those of control oocytes injected with water and treated with Tg (Fig. 5a, water/Tg trace), indicating that Trpm5 encodes a functional protein that enhances Ca^{2+} influx, and whose activation is stimulated by emptying of the internal Ca^{2+} stores. The modest activation of I_{ClCa} in the water/Tg control oocytes was probably due to an endogenous *X. laevis* TRP chan-

nel³⁵ that can be activated by Tg. The oocytes injected with Trpm5 cRNA showed much higher baseline activity than the water-injected controls when Tg was omitted (compare Trpm5 and water traces in Fig. 5a), suggesting that the Trpm5 channel is partially activated under basal conditions.

To confirm that these effects depended on expression of Trpm5, we developed a dominant-negative construct (Trpm5^{DN}) comprised of transmembrane segments 5 and 6 and the C-terminal region of Trpm5 and monitored its effects on Trpm5 activity. Co-injection of Trpm5^{DN} and Trpm5 cRNA (1:4 ratio, respectively) abolished the Trpm5-dependent activation of I_{ClCa} (compare the Trpm5/Tg and Trpm5-Trpm5^{DN}/Tg traces in Fig. 5a). Expression of Trpm5^{DN} did not inhibit or enhance the endogenous Ca^{2+} influx seen in water-injected control oocytes treated with Tg (compare water/Tg and water-Trpm5^{DN}/Tg in Fig. 5a) or in water-injected oocytes not treated with Tg (data not shown).

Current-voltage (I - V) curves were generated by applying voltage ramps from a holding potential of 0 mV. The curves from oocytes treated with Tg and those injected with water or Trpm5 cRNA were linear and non-rectifying. The slope increase in the Tg-treated/Trpm5-expressing group reflects the enhancement of I_{ClCa} and, therefore, an increase in Ca^{2+} permeation when Trpm5 is expressed (Fig. 5b). The slopes of the I - V curves from Tg-untreated and water- or Trpm5 cRNA-injected oocytes were similar to that of the Tg-treated/water-injected group (data not shown). Tg-treated oocytes expressing Trpm5 showed greatly enhanced Ca^{2+} influx compared with water-injected oocytes, as measured by the maximum current elicited when Ca^{2+} was added to the external bath (Fig. 5c). Lanthanum (La^{3+}), a general blocker of many TRP channels³⁶, blocked the increase in the current through I_{ClCa} , indicating that calcium flux through Trpm5 channels was blocked (Fig. 5c). La^{3+} did not block, but instead partially enhanced, the endogenous current in the control Tg-treated/water-injected oocytes as previously described³⁷; the differential sensitivity to La^{3+} between control oocytes and those expressing Trpm5 is consistent with Trpm5 acting as a Ca^{2+} channel.

For an independent monitor of Trpm5 activity, we transfected CHO cells with a bicistronic construct (pTrpm5-IRES-GFP) engineered to express Trpm5 and GFP, and then compared the currents of transfected cells with those of control cells that were transfected with a vector lacking the Trpm5 coding sequence (pIRES-GFP). In the presence of external Ca^{2+} , cells transfected with pTrpm5-IRES-GFP showed a current with a linear, non-rectifying I - V curve with a steeper slope than that of control cells (compare Trpm5 and control traces in Fig. 6a). This current was reversibly inhibited by $100 \mu\text{M}$ La^{3+} (Fig. 6a and b, Trpm5/ La^{3+} trace). In a small number of untransfected cells, there was a

Fig. 6. Heterologous expression of Trpm5 in CHO cells. Trpm5 was expressed by transfection of the cells with a bicistronic vector engineered to express Trpm5 and GFP. **(a)** *I*-*V* curves determined by applying voltage ramps to control CHO cells and CHO cells expressing Trpm5. The *I*-*V* traces from control cells and those displaying the Trpm5-induced current with and without La³⁺ treatment (Trpm5/La³⁺ and Trpm5, respectively) are shown; note that the trace from control cells is indistinguishable from that of Trpm5⁺ cells treated with La³⁺. **(b)** The current trace from a Trpm5⁻ transfected cell shows the inhibitory effect of La³⁺. **(c)** Scatter plot of GFP⁺ (open circles) and GFP⁻ (open squares) cells transfected with pTrpm5-IRES-GFP and treated with Tg. Ca²⁺ influx was measured by determining the fluorescence change of X-rhod-1 AM. Values are expressed as percentage of the Ca²⁺ influx observed in the GFP⁻ group (filled square, average value is 100%).

lanthanum-inhibited rectifying current. To confirm that Trpm5 mediates Ca²⁺ flux, we used the fluorescent, calcium-sensitive dye X-rhod-1 AM to monitor calcium changes in CHO cells and oocytes expressing Trpm5. A substantial population of GFP⁺, but not GFP⁻, CHO cells that were transfected with pTrpm5-IRES-GFP and treated with Tg showed an enhanced calcium influx; furthermore, the average fluorescence of the GFP⁺ cells was 20% greater than that of the GFP⁻ cells (Fig. 6c). No differences were seen between GFP⁺ and GFP⁻ cells from pIRES-GFP-transfected cells, or when Tg treatment was omitted (data not shown).

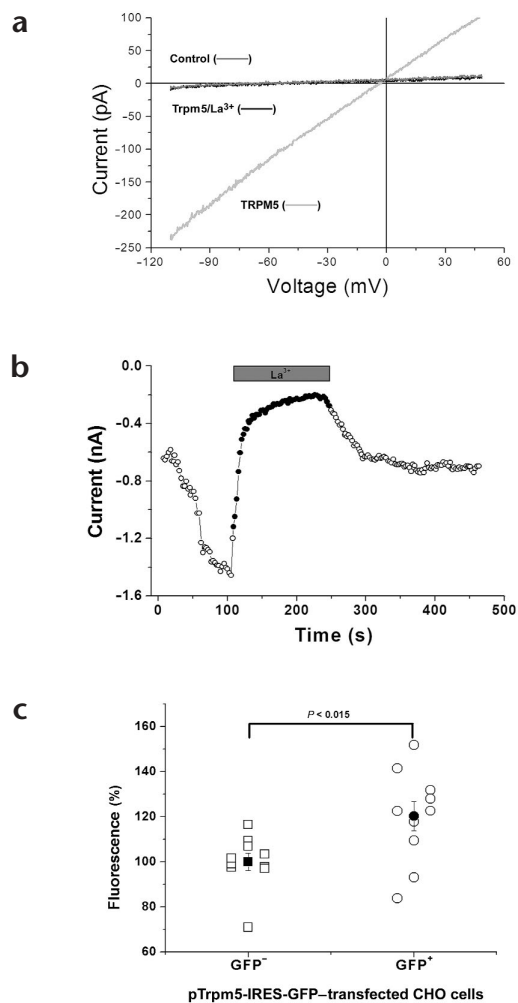
Ca²⁺ imaging of oocytes showed greater calcium permeability in oocytes injected with *Trpm5* cRNA than in those injected with water (Fig. 7). When Tg was used to deplete Ca²⁺ stores, the Trpm5-injected oocytes showed a greater influx of Ca²⁺ than did the water-injected controls (Fig. 7). This can be seen in the images acquired at several intervals after the addition of Ca²⁺ (10 mM) to the extracellular bath (Fig. 7a) and in the continuously monitored oocytes (Fig. 7b). The average fluorescent intensity of the Trpm5-injected oocytes was much greater than that of the water-injected controls and fluorescence most noticeable in the presence of Tg (Fig. 7c). These results corroborate the observations obtained from electrophysiological recordings in *Trpm5*-transfected CHO cells and *Trpm5* cRNA-injected *X. laevis* oocytes and are consistent with the potential function of Trpm5 as a calcium channel activated by the depletion of Ca²⁺ stores.

DISCUSSION

TRC responses to bitter and sweet compounds seem to involve Ca²⁺ influx as well as Ca²⁺ release from internal stores^{22,23,38,39}. To identify taste signaling elements, we used differential hybridization with probes from α -gus⁺ and α -gus⁻ TRCs (previously used to identify G β_3 and G γ_{13} as taste signaling molecules¹⁶). Using this approach, we cloned Trpm5, a TRP protein that is (i) expressed selectively in TRCs, (ii) coexpressed with other signaling elements, (iii) present in sweet- and bitter-responsive TRCs and (iv) activated subsequent to depletion of Ca²⁺ stores. Trpm5 may well be a CCE channel or calcium store-operated channel, although additional studies will be needed to confirm this.

How might TRC-expressed TRP channels be activated?

Several mechanisms for TRP channel activation have been proposed. The calcium influx factor (CIF) model posits a second-messenger signal that is released from stores in the endoplasmic reticulum upon Ca²⁺ depletion, thereby activating the TRP channel⁴⁰. Identification of the CIF is still pending. The IP₃/TRP direct coupling model proposes direct activation of TRP channels by the ligand-bound form of the IP₃ receptor. A particular domain of this receptor interacts with a region of TRP channels⁴¹. Furthermore, direct activation of TRP channels by second mes-



sengers is supported by several reports wherein native and heterologously expressed TRP channels are shown to be activated by PLC- β_2 -generated second messengers and their metabolites, such as DAG⁴². One or more of these mechanisms may hold for activation of Trpm5 in TRCs. The finding that IP₃R3 is coexpressed with G β_3 , G γ_{13} and PLC- β_2 in TRCs^{19,20} is consistent with Trpm5 channels being activated by IP₃R3 and/or DAG (or its metabolites). Our preliminary studies suggest that DAG does not activate heterologously expressed Trpm5 directly (C.A.P. and R.F.M., unpub. observ.).

What is the role of Trpm5 in TRCs?

Several signaling elements have been identified in TRCs, including G-protein subunits (α -gustducin¹⁴ and G γ_{13} ¹⁶), GPCRs (T1R^{2,6-11,13} and T2R/TRB²⁻⁵), effector enzymes (PLC- β_2 ¹⁸ and PDE 1A (M. Bakre, R. Lupi and R.F.M., unpub. observ.)) and ion channels (cyclic nucleotide-gated channels²¹). Multiple lines of evidence implicate gustducin as a key component of the responses of TRCs to bitter and sweet compounds. In TRCs, α -gustducin is coexpressed with the T1R3 receptor⁹ and the T2R/TRB receptors²⁻⁴ and has been shown to specifically couple with the cycloheximide-responsive mT2R5 receptor⁵. Mouse α -gustducin knockouts show markedly diminished behavioral and electrophysiological responses to bitter and sweet compounds¹⁵. Heterotrimeric gust-

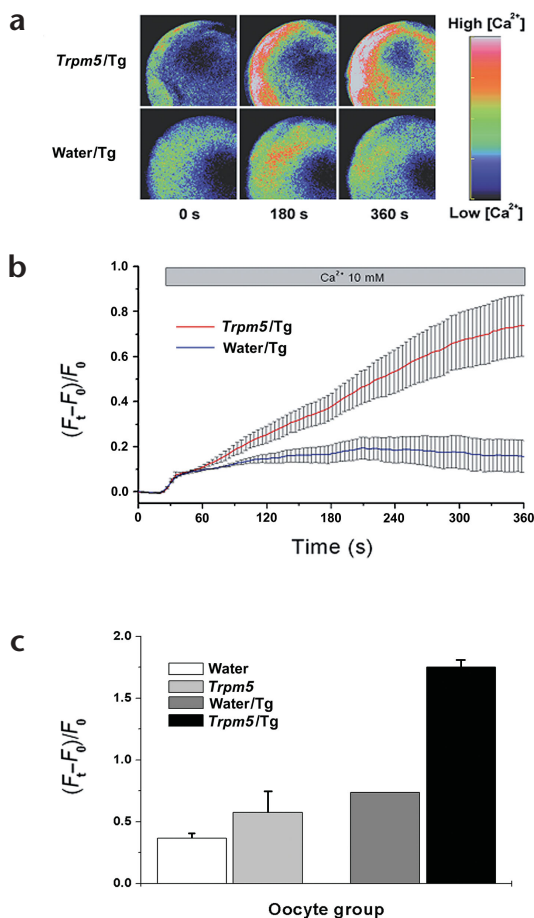


Fig. 7. Trpm5 facilitates Ca²⁺ influx in *X. laevis* oocytes. Two days after injection, oocytes were loaded with X-rhod-1 AM and treated with Tg in Ca²⁺-free solution. Fluorescent analysis of changes in relative Ca²⁺ levels revealed enhanced Ca²⁺ permeability in Trpm5-injected oocytes, a phenomenon elicited by depletion of internal Ca²⁺ stores (triggered by incubation with Tg) and dependent on the presence of extracellular Ca²⁺. **(a)** False-colored images of representative oocytes from each injected group at selected time points. **(b)** Average traces ($n = 3$, mean \pm s.e.m.) of oocytes injected with water or Trpm5 cRNA, incubated with Tg and then exposed to extracellular Ca²⁺ (horizontal gray bar). **(c)** Average fluorescent intensity (AU, arbitrary units) of the different groups of oocytes analyzed ($n = 3$, mean \pm s.e.m.).

channels argues that the expressed channels may form homomultimers or interact with other TRP channels expressed in the host cell. The fact that Trpc1–6 and Trpm1 and Trpm2 are not detectable in taste tissue, as determined by PCR, suggests that Trpm5 is the sole or major TRP in TRCs. It is possible, however, that Trpm5 forms heteromultimers with other TRP channels that may be expressed in TRCs. The fact that only a subpopulation of GFP⁺ CHO cells transfected with pTrpm5-IRES-GFP showed greater Ca²⁺ influx may indicate the involvement of a cofactor or a specific Trpm5 regulator.

Observations in *Drosophila melanogaster*, *Caenorhabditis elegans* and vertebrates indicate that TRP channels are critical to sensory modalities such as vision⁴⁵, olfaction^{46,47}, osmosensation⁴⁸ and nociception⁴⁹. Recent studies show that TRCs from mud-puppy and α -gus⁺ mice respond to depletion of Ca²⁺ stores or stimulation with acetylcholine with an influx of calcium^{38,39}. Prolonged stimulation of TRCs with the bitter compound denatonium causes a sustained increase in intracellular Ca²⁺, apparently via Ca²⁺ influx taking place after internal Ca²⁺ stores are depleted and after Ca²⁺ is added to the extracellular medium³⁹. This suggests that CCE in TRCs, particularly in those TRCs that express α -gustducin (determined by a transgenically expressed fluorescent marker²⁷) and T2R/TRB receptors (an α -gus⁺ subset of TRCs, inferred by responsiveness to denatonium), contributes to an increased internal Ca²⁺ concentration and is involved in TRC signaling. What, then, is the molecular nature of the putative CCE channel in denatonium-responsive TRCs? Our findings that Trpm5 is present in α -gus⁺ TRCs and has biophysical properties of a CCE-like channel when heterologously expressed make Trpm5 a prime candidate for this role *in vivo* in TRCs.

METHODS

All experiments were performed under NIH guidelines for the care and use of animals in research and were approved by the Institutional Animal Care and Use Committee of the Mount Sinai School of Medicine of New York University.

Filter hybridization. Individual inserts of a λ ZAPII cDNA library from a single α -gust⁺ TRC were amplified by PCR. DNA was spotted on a nylon membrane, denatured, neutralized and cross-linked by UV irradiation. The single cell probes were prepared by PCR re-amplification. After hybridization and washing, the membranes were exposed to X-ray film at -80°C for 12 h.

Northern hybridization. A northern blot with 25 μg of mouse tissue total RNA per lane was hybridized with a 480 bp random-primed ³²P-radio-labeled probe corresponding to the 3' UTR of Trpm5. The blot was exposed to X-ray film for 3 d at -80°C .

In situ hybridization. ³³P-labeled RNA probes (Trpm5, 1.7 kb and α -gustducin, 1 kb) were used for *in situ* hybridization in frozen sections (10 μm) of mouse lingual tissue as previously described¹⁶.

ducin has been shown to mediate two responses in TRCs: a decrease in cyclic nucleotide monophosphates via activation of PDE 1A by α -gustducin⁴³ (M. Bakre, R. Lupi and R.F.M., unpub. observ.) and a rise in IP₃ via activation of PLC- β ₂ by G β ₃/G γ ₁₃^{16–18}. The identification of the downstream components of these two signaling pathways should yield additional insights into taste transduction. The coincident expression of G β ₃, G γ ₁₃, PLC- β ₂ and IP₃R3 is consistent with the following signaling pathway: GPCR \rightarrow G protein \rightarrow G β ₃/G γ ₁₃ \rightarrow PLC- β ₂ \rightarrow IP₃ + DAG \rightarrow IP₃R3 \rightarrow Ca²⁺ release.

We propose that Trpm5 functions as a Ca²⁺ channel, most probably a CCE channel. First, in *X. laevis* oocytes, I_{ClCa} was markedly enhanced by Trpm5. Second, this effect depended on the presence of extracellular Ca²⁺. Third, Ca²⁺ influx in oocytes and CHO cells—as measured by the Ca²⁺-sensitive dye X-rhod-1 AM—increased when Trpm5 was present. Fourth, these various effects were seen maximally after depletion of calcium stores by Tg. It is clear that, in the presence of external calcium, Trpm5 responds to the emptying of Ca²⁺ stores by increasing Ca²⁺ influx. However, the mechanism whereby Trpm5 is activated by stored calcium depletion is unknown.

Many TRP channels form functional hetero- and homomultimers, with tetramers thought to be the predominant form^{26,44}. Heterologous expression of individual TRP channels frequently does not reproduce the properties of the native TRP-like currents²⁶. That heterologously expressed TRP proteins function as cation channels with properties distinct from those of the native

Immunocytochemistry. Polyclonal antisera against a Trpm5 peptide (residues 1028–1049) were raised in rabbits. The PLC- β_2 antibody was from Santa Cruz Biotechnologies (Santa Cruz, California); the anti- α -gustducin and anti-G γ_{13} antibodies were as described¹⁶. Frozen sections (10 μ m thick) of mouse lingual tissue (previously fixed in 4% paraformaldehyde and cryoprotected in 20% sucrose) were blocked in 3% BSA, 0.3% Triton X-100, 2% goat serum and 0.1% sodium azide in PBS for 1 h at room temperature and then double immunostained by sequential incubation with antiserum against Trpm5, anti-rabbit-Ig-Cy3 conjugate, normal anti-rabbit-Ig, anti-PLC- β_2 (or anti- α -gustducin or anti-G γ_{13}) antibody and, finally, with anti-rabbit-Ig-FITC conjugate. Control sections incubated without anti-PLC- β_2 (or anti- α -gustducin or anti-G γ_{13}) antibody did not show any fluorescence. Trpm5 immunoreactivity was blocked by pre-incubation of the antisera with the immunizing peptides at 20 μ M. Pre-immune serum did not show any immunoreactivity.

Gene expression profiling. The expression patterns of single TRCs were determined by Southern hybridization with 3'-end cDNA probes for mouse *Trpm5*, *Gnat3* (α -gustducin), *Gnb1* (G β_1), *Gnb3* (G β_3), *Gng13* (G γ_{13}), *Plcb2* (PLC- β_2) and *G3pdh* (glyceraldehyde-3-phosphate dehydrogenase) as previously described¹⁶.

Heterologous expression in *X. laevis* oocytes. General procedures for oocyte harvesting, maintenance and recording were as previously described⁵⁰. Oocytes were injected with 40–50 ng of *Trpm5* cRNA and/or 10 ng of *Trpm5*^{DN} cRNA obtained using the mMessage mMachine kit (Ambion, Austin, Texas). The *Trpm5* clone was subcloned into pTreex, a vector derived from pGMHE. The *Trpm5*^{DN} clone was derived from pTreex-*Trpm5* and encodes the last two transmembrane domains of *Trpm5* plus the C-terminal tail. Oocytes were maintained in ND96 solution at 18°C. Recordings were done two days after injection with a CA-1B High Performance Oocyte Clamp (Dagan Corp., Minneapolis, Minnesota). Immediately before recording, oocytes were incubated for 2 h in ND96 solution (with 1 mM EGTA) with or without Tg (2–4 μ M). Recordings were done with ND96 in the external bath with 1 mM EGTA, 10 mM Ca²⁺ or 1–3 mM La³⁺ at a holding potential of –80 mV.

Electrophysiological recordings from mammalian cells. CHO-K1 cells were transiently transfected with pTrpm5-IRES-GFP, a construct generated by subcloning the *Trpm5* coding region into the pIRES2-EGFP plasmid (Clontech, Palo Alto, California), using the Effectene transfection kit (Qiagen, Valencia, California). Recordings were made 2–3 d after the transfection. Cells were grown in DMEM (Invitrogen, Carlsbad, California) and supplemented with 5% FBS. Whole-cell patch clamp recordings were made using the HEKA EPC9 amplifier, and voltage protocols were generated using the PULSE-PULSEFIT software (HEKA, Southboro, Massachusetts). The standard extracellular solution contained 160 mM sodium aspartate, 10 mM HEPES, 4.5 mM KCl, 2 mM CaCl₂ and 1 mM MgCl₂ (pH 7.4). The standard pipette solution contained 150 mM potassium (or cesium) glutamate, 1 mM EGTA and 10 mM HEPES (pH 7.3–7.4). To obtain current–voltage relationships, voltage ramps (duration, 211 ms) were applied. The sampling frequency was 5 kHz. The holding potential was 0 mV.

Calcium imaging. CHO cells: 2 d after transfection with either pTrpm5-IRES-GFP or pIRES-GFP, cells were loaded with X-rhod-1 AM (10 μ M, Molecular Probes, Eugene, Oregon) by incubation in Dulbecco's PBS (DPBS). After washing in dye-free DPBS, cells were monitored using an Olympus BX50WI microscope attached to an Olympus Fluoview laser-scanning confocal unit (568 nm line for excitation, 605 nm filter for fluorescence emission) (Olympus, Melville, New York). Data acquisition and processing were done with the software provided by the manufacturer. Solutions were Tg (2 μ M in DPBS) and DPBS with 1 mM EGTA or 5 mM Ca²⁺.

***X. laevis* oocytes.** 48 h after injection with *Trpm5* cRNA, oocytes were loaded with X-rhod-1 AM (10 μ M) by incubation for 1 h followed by incubation in 2 μ M Tg for 2 h at room temperature. Data acquisition was as for CHO cells except that ND96 buffer was used.

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

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Competing interests statement

The authors declare that they have no competing financial interests.

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