TRPM5-expressing solitary chemosensory cells respond to odorous irritants

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Abstract

Inhaled airborne irritants elicit sensory responses in trigeminal nerves innervating the nasal epithelium, leading to protective reflexes. The sensory mechanisms involved in the detection of odorous irritants are poorly understood. We have identified a large population of solitary chemosensory cells expressing the transient receptor potential channel M5 (TRPM5) using transgenic mice where the promoter of TRPM5 drives the expression of green fluorescent protein (GFP). Most of these solitary chemosensory cells lie in the anterior nasal cavity. These GFP-labeled solitary chemosensory cells exhibited immunoreactivity for synaptobrevin-2, a vesicle-associated membrane protein important for synaptic transmission. Concomitantly, we found trigeminal nerve fibers apposed closely to the solitary chemosensory cells, indicating potential transmission of sensory information to trigeminal fibers. In addition, stimulation of the nasal cavity with high concentrations (0.5-5 mM) of a variety of odorants elicited event-related potentials (ERPs) in areas rich in TRPM5-expressing solitary chemosensory cells. Further, odorous chemicals and trigeminal stimuli induced changes in intracellular Ca^{2+} levels in isolated TRPM5-expressing solitary chemosensory cells in a concentration dependent manner. Together, our data demonstrate that the TRPM5-expressing cells respond to a variety of chemicals at high exposure levels typical of irritants and are positioned in the nasal cavity appropriately to monitor inhaled air quality.
**Introduction**

Odorous chemicals are irritants at high concentrations, eliciting sensations such as burning, stinging, warmth, coolness, itching or pain in the nasal cavity (Bryant and Silver 2000; Doty et al. 1978). Intranasal irritation signals poor air quality and potential health risks (Cain and Cometto-Muniz 1995). This sensation is transmitted by the trigeminal nerve innervating the nasal epithelium, as indicated by robust trigeminal responses to a large number of volatile odorous chemicals (Silver et al. 1986, 1991). It was assumed that these sensations arise from direct stimulation of intraepithelial free nerve endings. However, most of these endings are buried within the epithelial sheet making it difficult for stimuli to reach sensory endings quickly (Finger et al. 1990).

Recently, solitary chemosensory cells (also called “solitary chemoreceptor cells”) innervated by the trigeminal nerve have been identified in the anterior nasal cavity in rodents with an antibody against α-gustducin, a G protein α subunit found in taste receptor cells (Finger et al. 2003; Sbarbati and Osculati 2003; Zancanaro et al. 1999). Solitary chemosensory cells within the airways and gastrointestinal tract (Kaske et al. 2007) also were identified by an antibody directed against the transient receptor potential channel M5 (TRPM5), an ion channel critical in taste transduction (Perez et al. 2002; Zhang et al. 2003). Whether these cells are identical to the gustducin-expressing population was unclear. Interestingly, the α-gustducin positive cells of the nasal epithelium express bitter-responsive type 2 taste receptors (T2Rs) similar to bitter-responsive taste cells. On the basis of these observations and the finding that bitter stimuli applied to the respiratory mucosa elicit trigeminal nerve responses, Finger and co-
workers postulated that these α-gustducin-expressing cells are solitary chemosensory cells that mediate trigeminal responses to bitter substances (Finger et al. 2003).

Many inhaled irritants are odorous chemicals. The trigeminal nerve responds to high levels of most odorants, but the mechanism underlying this response is unclear (Bryant and Silver 2000). Here we asked whether these or other nasal solitary chemosensory cells respond to odorous irritants. We used transgenic mice, in which the TRPM5 promoter drove the expression of green fluorescent protein (TRPM5-GFP) to identify the solitary chemosensory cells in this study. Members of the TRP family, such as the thermally sensitive TRPV1, TRPV4, TRPM8 and TRPA1, are expressed in neurons of trigeminal and dorsal root ganglia and interact with volatile compounds mediating sensations such as the coolness of mint and the hotness of chili paper (Jordt et al. 2003; Silver et al. 2006; Voets et al. 2005). In addition, other TRP channels function as elements of G-protein coupled signaling cascades in chemosensory transduction. For example, TRPC2 is the transduction channel for chemical detection in the vomeronasal organ (Liman et al. 1999) and TRPM5 participates in taste transduction for sweet, bitter and amino acid substances (Perez et al. 2002; Zhang et al. 2003), as well as semiochemical transduction in the olfactory epithelium (Lin et al. 2007).

Here we found that TRPM5 is expressed in a large population of solitary chemosensory cells, a subset of which co-expresses α-gustducin. Moreover, solitary chemosensory cells expressing TRPM5 respond to odors at high concentration with increases in intracellular Ca^{2+}. This is the first demonstration at the cellular level that chemosensory cells in the respiratory epithelium respond to odorous irritants. Preliminary data were published in abstract forms (Lin et al. 2005; Ogura et al. 2006).
Materials and Methods

Animals

Adult C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). TRPM5 GFP transgenic mice contain a TRPM5-GFP construct including 11 kb of mouse Trpm5 5' flanking sequence, Trpm5 Exon 1 (untranslated), Intron 1, and the untranslated part of Exon 2, and eGFP (Clapp et al. 2006). Adult male cyclic nucleotide-gated channel A2 subunit (CNGA2) knockout mice were offspring produced by backcrossing the heterozygous CNGA2 knockout female mice (Brunet et al. 1996) (provided kindly by Dr. John Ngai) with wild-type FVB male mice. This resulted in knockout male offspring because the CNGA2 gene is X-linked and therefore hemizygous. Offspring were genotyped using the polymerase chain reaction (PCR). All animal care and procedures were in compliance with the Animal Care and Use Committees of University of Colorado at Denver and Health Sciences Center and University of Maryland, Baltimore County.

Immunocytochemistry

Tissue preparation. Mice were anesthetized with ketamine/xylazine (100 µg-20 µg/g body weight), perfused transcardially with 0.1M phosphate buffer (PB) followed by a PB buffered fixative containing 3% paraformaldehyde, 0.019 M L-lysine monohydrochloride, and 0.23% sodium m-periodate (Lin et al. 1999). The nose was harvested and post-fixed for 2 h. For direct visualization of GFP expression, the nose was split along the midline
to expose the nasal cavity. Low magnification pictures were taken using an Olympus dissecting and a Nikon E600 compound microscopes equipped with epi-fluorescence. High magnification whole-mount images on stripped epithelia containing GFP-positive cells were taken using an Olympus Fluoview confocal microscope. For immunolabeling using tissue sections, bones surrounding the nose were removed after fixation and tissues were transferred into PBS with 25% sucrose overnight and embedded with OCT. Fourteen micron transverse sections were cut using a cryostat, mounted onto Superfrost plus slides (Fisher Sci, Pittsburgh, PA) and stored in –80 C degree freezer until used.

Cell counting. For estimation of the number of TRPM5-expressing solitary chemosensory cells in respiratory epithelium, the TRPM5-GFP mice were fixed, and the nose was split along the midline. The respiratory epithelia from three hemi-noses were stripped, spread out and mounted onto microscope slides with fluoromount-G (Fisher Sci). Multiple images were taken at low magnification (4X) using an Olympus epi-fluorescence microscope and the respiratory epithelium from the hemi-noses was reconstructed. The GFP-positive solitary chemosensory cells were counted manually. To estimate the number of GFP-expressing cells that co-express α-gustducin, phospholipase C β2 (PLC β2) or the G-protein γ subunit 13 (γ13), serial coronal sections from the anterior portion of the nose of TRPM5-GFP mice were immunoreacted with antibodies raised against α-gustducin, PLC β2, or γ13. GFP positive and immunoreactive cells were counted from fluorescence images taken using an Olympus epi-fluorescence microscope.
**Immunolabeling.** Sections or stripped epithelium were rinsed and incubated in blocking solution containing 2% normal donkey serum, 0.3% Triton X-100 and 1% bovine serum albumin in PBS for 1.5 hour. Sections were then incubated overnight or 72 hours with primary antibodies against each of the following proteins: TRPM5, γ13 (1:250 or 1:500, Perez et al 2002), PLCβ2 (1:200; Cat No: sc-206, Santa Cruz Biotechnology, Santa Cruz, CA), calcitonin gene related peptide (CGRP) (1:500, Cat# IHC6006, Peninsula Lab. San Carlos, CA), Substance P (1:1000, Cat No: AB1977, Chemicon, Temecula, CA), α-gustducin (1:1000, Cat No: sc-395, Santa Cruz Biotechnology, Santa Cruz, CA) or PGP9.5 (ubiquitin carboxyl-terminal hydrolase; 1:500, Cat No: 7863-0504, Biogenesis, Sandown, NH). After incubation of the primary antibodies, sections were then washed and reacted with donkey anti-rabbit secondary antibody (Alexa 555, Probes, Eugene, OR) for 1 hour at room temperature. Sections were mounted on slides with Fluoromount-G. Controls for these experiments consisted of removing primary antibodies and using tissues from the TRPM5KO mice for the anti-TRPM5 antibody, which resulted in negative labeling. Images were taken using an Olympus Fluoview confocal microscope using the serial acquisition mode in cases involving dual fluorescent signals.

**Recordings of event-related potentials (ERPs)**

*Solutions and chemicals.* Odorous chemicals were obtained form Aldrich Chemical Company Inc (Milwauki, WI), Fluka (Ronkon Koma, NY) or Takasago Corporation (Shinagawa, Japan) at the highest purity available. Odorants were made freshly by dilution with vigorous vortexing into the Ringer’s saline containing (in mM): 145 NaCl, 5 KCl, 20 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid buffer (HEPES), 1 MgCl₂,
1 CaCl₂, 1 Na pyruvate and 5 D-glucose (pH 7.2). See Fig. 5 for the list of odorants. The phospholipase C (PLC) inhibitor U73122 (Calbiochem, San Diego, CA) was dissolved in DMSO and diluted into the bath solution at a final concentration of 5 µM.

**ERP recordings.** The method was adapted from Lin et al (2004). Mice were sacrificed by CO₂ inhalation, followed by cervical dislocation. The head was removed, and split along the midline to expose anterior nasal respiratory epithelium. The half head was mounted on a recording chamber using the dental adhesive Impregum F (ESPE, Germany). Ringer’s solution was perfused continuously over the surface and stimuli were delivered through a gravity-fed computer-controlled perfusion system with an approximate flow rate of 0.2 ml/s. Each stimulus was presented three times and the largest response was used. Following stimulation, the epithelium was washed with saline for 2 min, or until its response to Ringer’s solution was back to the basal level recorded at the beginning of the experiment. ERPs are evoked local field potential and recorded using an Axopatch 200 B amplifier controlled by a PC computer with Axon software (Clampex 8, Axon Instruments, Inc. Union City, CA). Recording electrodes were filled with 0.9% agar made in Ringer’s solution with 1% neutral red or fast green and were placed on the apical surface of the anterior mucosa at similar locations marked in Figure 1, where dense populations of TRPM5-positive cells were located. The reference Ag/AgCl electrode was connected to bath saline through an agar bridge. The recorded signals were low-pass-filtered at 20 Hz, digitized at 500 Hz and analyzed using the Axon software Clampfit.
**Removal of the main olfactory epithelium.** To determine whether olfactory sensory neurons contributed to the ERPs recorded from the reparatory epithelia, we removed the entire main olfactory epithelia and vomeronasal organ (VNO) in some animals. The nose of each mouse was split and olfactory turbinate in the dorsal-posterior region of the nasal cavity, the septum with the VNO, and the dorsal recess which extend from arterial to posterior, were removed with fine forceps and surgical blades under a dissecting microscope. The most rostral end of the nasal cavity where the Grueneberg ganglion resides (Fuss et al. 2005; Koos and Fraser 2005; Storan and Key 2006), was also removed. The half nose was then mounted and ERPs were recorded from the respiratory epithelia.

**Ca$^{2+}$ imaging**

Fura-2 imaging was used to measure intracellular Ca$^{2+}$ levels (Ogura 2002; Ogura et al. 1997). SCC isolation was adapted from the method of Rawson et al. (1997). Briefly, anterior respiratory epithelia of the TRPM5 GFP mice were removed from the nasal cavity after euthanasia, cut into small pieces and placed in Ca$^{2+}$-Mg$^{2+}$-free Ringer with 10–30 U/ml of papain (Worthington, Lakewood, N.J) and 2 mM cysteine for 30 min at room temperature. The cells were detached by gentle pipetting. The supernatant was transferred to an O-ring chamber on a cover slip pre-coated with poly-lysine (Sigma). After the cells settled, the solution was replaced with Ringer’s containing 2 μM fura-2/AM (Molecular Probes) for 20 min and washed with normal Ringer’s. Excitation wavelength was alternated between 350 and 380 nm using filter wheel (Lambda 10-2; Sutter Instruments, Novato, CA) coupled to the output of a xenon lamp (model
The ratio of fluorescence intensity at excitation wavelengths of 350 and 380 nm was used to indicate the intracellular Ca$^{2+}$ levels. Fluorescent images were obtained with a 40X oil objective lens (N.A. 1.3) with a high-pass 525 nm emission filter (Chroma Technology, Rockingham, VA). Axon Imaging Workbench software version 2.2 (INDEC BioSystems, Santa Clara, CA) was used to capture images and to change the position of the filters. Image pairs were acquired every 2 s during responses and every 5-10 s during control and wash-out periods. To determine whether the extracellular Ca$^{2+}$ influences the stimulus-induced Ca$^{2+}$ response, we omitted the extracellular CaCl$_2$ and repeated the same stimulation in the same cells after recording the control response in normal saline.

Results

**TRPM5 promoter-driven GFP expression in scattered cells throughout the respiratory epithelium lining the nasal cavity**

We determined the location of TRPM5-expressing cells by monitoring GFP expression in the nasal cavity of TRPM5-GFP mice. We found expression of GFP in a subset of cells in the respiratory epithelium of the upper respiratory tract. Similar to solitary chemosensory cells reported by Finger et al. (2003) and Sbarbati and Osculatti (2003), and the TRPM5-immunoreactive cells reported by Kaske et al. (2007), the GFP-expressing cells are scattered throughout the respiratory epithelium in the nose. In the nasal cavity, the GFP-expressing cells were concentrated anteriorly, near the entrance to the nasal cavity (Figs. 1 A and B). Figs. 1C and D show higher magnification confocal
images of GFP-expressing cells obtained from an epithelial section and stripped epithelium respectively. These cells often were elongate and slant across the respiratory epithelium with apical processes reaching the lumenal surface (arrowheads in Fig. 1C and D). In some of these cells, several processes also emanate from the soma (arrow in Fig. 1D). None of these cells displayed axonal processes visible with the fluorescence microscope. The morphological characteristics of the GFP-expressing cells were similar to those α-gustducin–positive solitary chemosensory cells (Finger et al. 2003), but differed from the olfactory neurons in the main olfactory epithelium or in cells in the Grueneberg ganglion (Farbman 2000; Fuss et al. 2005; Koos and Fraser 2005). On average, there are 5668.5 ± 1029.6 (mean ± sem, n=4) GFP-expressing solitary chemosensory cells in the respiratory epithelium on one side of the nasal cavity. The reported number of gustducin-positive solitary chemosensory cells is about one thousand per side (Finger et al. 2003). Thus using the TRPM5-GFP mice, we were able to find a large population of cells morphologically similar to presumed solitary chemosensory cells in the respiratory mucosa and regions for optimal electrophysiological recordings.

**Immunoreactivity of TRPM5 in GFP-expressing solitary chemosensory cells**

To determine whether the GFP-positive cells express TRPM5 protein we performed immunolabeling using an anti-TRPM5 antibody in stripped epithelia containing GFP cells from the TRPM5-GFP mice. Strong immunoreactivity was observed in the GFP-positive cells. We picked at random 51 GFP-positive cells from 3 animals and imaged z-stacks of GFP fluorescence and fluorescence emitted by the secondary antibody in a laser scanning confocal microscope and in a regular fluorescence
microscope. We found complete co-localization of GFP and TRPM5 immunoreactivity. A representative confocal image obtained from the whole-mount preparation is shown in Fig. 1E. The specificity of the antibody against TRPM5 has been established by other investigators in previous studies (Clapp et al. 2006; Perez et al. 2002). No specific immunoreactivity was observed in control experiments where we omitted the primary antibody against TRPM5 (not shown). We further confirmed the specificity of the TRPM5 antiserum by probing epithelia from TRPM5<sup>-/-</sup> mice. No staining of epithelial solitary chemosensory cells was observed in these knockout animals. These results demonstrate that TRPM5 was expressed in the GFP-positive cells. In the experiments described below we used GFP as a surrogate indicator of TRPM5 expression.

**α-gustducin is expressed in a subset of GFP (TRPM5)-expressing cells**

As indicated above, α-gustducin is expressed in a population of solitary chemosensory cells scattered throughout the nasal cavity (Zancanaro et al. 1999; Finger et al. 2003). In taste buds, a subset of TRPM5 expressing taste receptor cells co-express α-gustducin (Perez et al. 2002). We tested whether TRPM5 and α-gustducin are co-expressed in some solitary chemosensory cells. Immunohistochemical experiments with an antibody against α-gustducin were performed on either coronal sections or whole-mount preparations of the nasal epithelia from TRPM5-GFP mice. We found that immunoreactivity for α-gustducin was present in a subset of GFP positive solitary chemosensory cells. The percentage of GFP cells expressing α-gustducin counted from both sections and whole mount epithelial strips were similar. On average for the entire nasal cavity, 14.6 ± 1.67 % (n=5) of GFP cells expressed α-gustducin (data pooled and
averaged from the tissue sections [12.3 %, n =2] and whole mounts [16 %, n=3]). A larger fraction of solitary chemosensory cells co-expressed GFP and α-gustducin in epithelial patches of the anterior respiratory areas (Fig. 2A, insert). Thus subpopulations of solitary chemosensory cells with different sensory properties may be present in the nasal respiratory epithelium.

**Expression of signaling components of the PLC pathway**

TRPM5 is a down-stream signaling component of the PLC pathway in taste receptor cells (Liman 2007). To investigate whether other elements of the PLC pathway are present in the GFP-positive cells, we immunoreacted sections with antibodies against PLCβ2 and γ13, a G-protein γ subunit associated with PLCβ2 activation in taste cells (Huang et al. 1999). We found that 62% of the GFP positive cells expressed PLC β2 (605 cells examined from 4 animals) and 73% of the GFP positive cells expressed γ13 (903 cells examined from 4 animals). Immunoreactivity for PLCβ2 and γ13 in GFP-positive cells is shown in Figs. 2, B and C. At the level of light microscopy we could not detect differences in morphology between GFP cells immunolabeled with anti-PLCβ2 and γ13 and GFP cells that were not immunoreactive. Although a higher fraction of GFP-positive cells expressed PLCβ2 and γ13 in the anterior portion of the nose, GFP-positive cells without immunoreactivity to these antibodies can be found throughout the respiratory mucosa. These results indicate that many but not all solitary chemosensory cells expressed PLCβ2 and γ13.
Close apposition of nerve processes to GFP-positive solitary chemosensory cells

We investigated the relationship between GFP-positive cells and nerve fibers by using markers found in trigeminal nerves innervating the nose: PGP9.5, a pan neuronal marker to label all the nerve fibers (Finger et al. 2003), and substance P and calcitonin-gene related peptide (CGRP) to label trigeminal peptidergic fibers (Finger et al. 1990). In whole mount preparations, we found PGP9.5-positive nerve fibers closely apposed to the GFP-positive solitary chemosensory cells (Fig. 3A). In sections through the respiratory epithelium we found that the nerve fibers typically course along the length of the solitary chemosensory cells or wrap the basal regions of the cells (Fig 3B). Interestingly, in sections immunoreacted for substance P we found GFP-expressing cells embraced the substance P-positive fibers as they coursed across the cell body (Fig. 3C). These results indicate that GFP (TRPM5)-expressing solitary chemosensory cells very likely receive trigeminal innervation.

Expression of the vesicle-associated membrane protein synaptobrevin-2 in GFP-positive cells.

The close association of the GFP-expressing solitary chemosensory cells and the trigeminal fibers suggests that solitary chemosensory cells may relay sensory information onto nerve fibers through synaptic transmission. We determined whether the GFP-positive solitary chemosensory cells express synaptobrevin-2, a vesicle-associated membrane protein critical for synaptic vesicle docking and exocytosis. Synaptobrevin-2 is associated with synaptic vesicles at synapses in the peripheral sensory tissues, such as retina (von Kriegstein et al. 2003), cochlear hair cells (Safieddine and Wenthold 1999)
and taste receptor cells (Yang et al. 2004). We found immunoreactivity for synaptobrevin-2 in the 68 out of 70 GFP-positive solitary chemosensory cells in 12 sections from two animals randomly examined (Fig. 4). Interestingly, positive immunolabeling for synaptobrevin-2 also was observed in nerve fibers running in the basal lamina of the respiratory epithelium as well as many fibers that contact the solitary chemosensory cells. Thus, synaptobrevin-2 was present in both solitary chemosensory cells and the nerve fibers innervating them. In addition, we found 43 cells that showed immunoreactivity for synaptobrevin-2, but did not express GFP. Many of these non-GFP cells were spindle shape in the same cross epithelial sections with fine basal processes reaching the basal lamina. We found these cells often in epithelial regions with relatively fewer synaptobrevin-2 positive nerve fibers (data not shown). The fine processes of these cells and lacking GFP expression made it harder to visualize the cell boundary and determine whether they were innervated at light microscopic levels. It is likely that other potential solitary chemosensory cells, which do not express TRPM5, may be present in the nasal respiratory epithelium.

**Odorous chemicals evoked event related potentials in the respiratory epithelium.**

The ERP recording is a common method used to investigate the local field potential changes evoked by irritants in the nasal respiratory mucosa (Hummel 2000; Rombaux et al. 2006). The ERP also is called the negative mucosal potential (NMP) by other investigators (Cain et al. 2006). Since many odorants at relatively high concentrations induce a sense of irritation (Silver et al. 2000), we examined whether strong odorants induced ERP responses in the areas densely populated by the TRPM5-
expressing cells. ERPs were recorded at the location indicated by the area bounded by a red line in Fig.1A, a region rich in GFP-expressing cells. At concentrations of 100 µM and below, the eight stimuli tested did not show apparent ERP responses (3 animals for each stimulus) except nicotine, which induced robust and repeatable responses (0.74 ± 0.34 mV; n=4). At concentrations of 500 µM-5mM, all the stimuli tested (12 in total) induced repeatable ERP responses. The average amplitudes of the responses to individual stimuli ranged from 0.075 mV (kovanol) to 0.57 mV (ethyl propionate) at 5 mM. The largest amplitude traces to different stimuli selected from 3-7 animals are shown in Fig. 5A. The averaged amplitudes for each stimulus (5 mM) are shown in Fig. 5B (n=3-7). In addition, we monitored whether ERP depended on stimulus concentrations. Odorous chemicals at different concentrations were applied to different animals and averaged ERP concentration-dependent responses from 3 animals for each stimulus were shown in Fig. 5C.

To estimate whether the olfactory sensory neurons might contribute to the ERP responses, we recorded ERP from preparations in which we removed the entire olfactory epithelium, including the olfactory turbinate, septum, dorsal recess, VNO, and rostral end of the nasal cavity containing Grueneberg ganglion. The evoked ERP amplitudes (mV) under such condition were: menthone: 0.12 ± 0.04; lilial: 0.25 ± 0.07; geranial: 0.23 ± 0.04; 2-heptanone 0.14 ± 0.05; and pentyl acetate 0.15 ± 0.03 (mean ± sem; n=3; 5 mM concentration each). These ERP response amplitudes were not statistically different from the results shown in Fig.5B (two-way ANOVA, p = 0.50, F = 0.46). In addition, we recorded ERP from cyclic nucleotide-gated channel subunit A2 (CNGA2) knockout mice.
CNGA2 knockout abolishes most of the olfactory responses to common odor stimulation (Brunet et al. 1996; Lin et al. 2004). CNGA2 knockout mice displayed ERPs to odorous stimuli at the anterior respiratory areas similar to those of wild type mice (data not shown). This result indicates that the respiratory epithelia, likely the TRPM5-expressing solitary chemosensory cells mediated detection of high concentrations of odorous stimuli.

**Odorous chemicals induce changes in intracellular Ca^{2+} levels of the solitary chemosensory cells**

To determine whether the GFP (TRPM5)-positive solitary chemosensory cells were responsive to volatile irritants, we monitored intracellular Ca^{2+} levels in freshly isolated GFP-positive cells loaded with the fluorescent Ca^{2+} indicator Fura-2. A variety of odorous chemicals at 0.5 mM induced an increase in the intracellular Ca^{2+}. Fig. 6A showed representative Ca^{2+} responses in the GFP-expressing cells. A repeated response to lilial in the same cells was also shown. The amplitudes of Ca^{2+} responses to odorous stimuli in the GFP-positive cells were concentration-dependent; higher concentrations of stimuli evoked larger Ca^{2+} responses (Fig. 6B). At concentrations of 20 µM, there were little or no responses to either lilial or geraniol, but at 500 µM, both compounds produced robust responses. The responsiveness of each cell tested is shown in Fig. 7. Each cell responded to at least one stimulus. Many cells responded to high concentrations of lilial (88 % of 17 cells), geraniol (61 % of 18 cells) and citral (50 % of 18 cells). Only one of 16 cells responded to urinary components, such as 2, 5-dimethylpyrazine (DMP) and 2-heptanone. Interestingly, most cells responded to multiple, but not all stimuli tested, suggesting selectivity in these GFP-positive cells. Taken together, our data provide the
first direct evidence that the TRPM5-expressing solitary chemosensory cells respond to high concentrations of diverse volatiles.

Since TRPM5 is the down-stream effecter of the PLC signaling pathway, we tested involvement of the PLC pathway in the responses to volatile substances by using the PLC inhibitor U73122. In the presence of U73122 (5 µM), the response to lilial was reduced significantly (Fig 6C; n=5). We had attempted to wash out the U73122 effect. Three out of 5 cells tested were partially recoverable after 10 min washing with regular saline. Additionally we tested whether the intracellular Ca$^{2+}$ increase was due to a Ca$^{2+}$ influx from the extracellular medium or due to Ca$^{2+}$ release from intracellular stores. We found that the amplitudes of Ca$^{2+}$ responses to geraniol (500 µM) decreased when cells were bathed in zero Ca$^{2+}$ extracellular solution. The average amplitude of Ca$^{2+}$ responses was 62 ± 14 % of the control responses obtained in normal saline in the same cells (n=5, paired t-test, p = 0.14). The result indicates that both Ca$^{2+}$ influx and Ca$^{2+}$ release from the intracellular stores may contribute to the irritant-induced Ca$^{2+}$ responses in the solitary chemosensory cells. Our data suggest that the PLC pathway and TRPM5 may be involved in detection of volatile chemicals in the GFP-expressing solitary chemosensory cells.

Discussion

High concentrations of nearly any odorant compound produce a sensation of irritation in the nasal cavity. Previous investigators have assumed that the volatile compounds are detected directly by the free endings of the trigeminal nerve, but the recent discovery of solitary chemosensory cells in the nasal cavity offers a possible
alternative for detection of such substances. Previous study on solitary chemosensory
cells has shown that some of α-gustducin-expressing solitary chemosensory cells express
members of the T2R family of taste receptors (for bitter substances) and trigeminal fibers
respond to bitter-tasting ligands applied in solution to the nasal cavity (Finger et al. 2003).
Here we sought to investigate the population of TRPM5-expressing solitary
chemosensory cells and their chemical responsiveness to volatile irritants. We used both
transgenic animals and immunolabeling to identify cells that express TRPM5, an ion
channel that is present in taste receptor cells and belongs to the TRP family of ion
channels with members known for their role in chemosensory transduction. We found
that TRPM5 is expressed in a large population of cells in the nasal respiratory epithelia
morphologically similar to those expressing α-gustducin. Further, we demonstrated that
high levels of various odorants induced local event-related potentials and changed
intracellular Ca\(^{2+}\) levels of isolated TRPM5-expressing solitary chemosensory cells.
This is the first direct demonstration that TRPM5-expressing solitary chemosensory cells
in the nasal cavity respond to odorous chemical irritants.

**Relation with the α-gustducin expressing solitary chemosensory cells**

The TRPM5-expressing cells share similar morphology with α-gustducin
expressing solitary chemosensory cells described previously (Finger et al. 2003). In the
nasal cavity, as in taste buds (Perez et al. 2002), only a subset of TRPM5-expressing cells
expresses α-gustducin. In rats, the maximum density of α-gustducin expressing nasal
solitary chemosensory cells was \(~ 300\) cells per \(\text{mm}^2\) and the total number was estimated
at 1000 in each half of the nose (Finger et al. 2003). We found significantly more
TRPM5-positive solitary chemosensory cells in the nasal cavity of mice, roughly 11,200 per nose (5,600 in each nostril). Only about 15% of the TRPM5-positive solitary chemosensory cells also express α-gustducin in mice, so there seemed to be roughly the same number of gustducin-positive solitary chemosensory cells in the two species.

TRPM5 positive taste receptor cells express members of the PLC transduction cascade and some also express gustducin (and presumably T2R taste receptors). We found immunoreactivity for both PLCβ2 and γ13 was present in most of the solitary chemosensory cells. It is not known whether the GFP-positive cells that are not immunoreactive for PLCβ2 and γ13 represent solitary chemosensory cells at different developmental stages or different cell types. The nasal solitary chemosensory cells have a limited lifespan, turning over about every 2 months (Gulbransen and Finger 2005). If immature solitary chemosensory cells express TRPM5-driven GFP prior to PLCβ2, gustducin, or γ13, then this would account for the presence of solitary chemosensory cells that exhibit GFP fluorescence but are non-immunoreactive for these other signaling molecules. Conversely some solitary chemosensory cells may utilize other transduction cascades to trigger the TRPM5 channel.

In any event, we found that a PLC pathway blocker suppresses responses of solitary chemosensory cells to odorous chemicals, suggesting that at least some of the TRPM5-expressing solitary chemosensory cells utilize a PLC-mediated pathway in detecting odorous irritants. However, further experiments are required to determine whether TRPM5 itself is an obligatory element in the transduction of nasal irritants.
Differences between trigeminal-mediated and olfactory-mediated detection of odorous chemicals

Both the olfactory and trigeminal systems detect volatile chemicals. However, these two systems differ fundamentally. First, the olfactory system in rodents expresses approximately a thousand odor receptors, allowing detection and discrimination of a vast array of odorants with exquisite sensitivity. Odor detection by the olfactory system elicits a sense of smell for individual odorants, whereas detection by the trigeminal system provides a sense of irritation, ranging from tingling to pain with limited discrete categories (Bryant and Silver 2000). In contrast to the fine discriminative abilities for the sense of smell, quality discrimination for trigeminal irritants is quite poor. Second, there is a significant difference in the detection thresholds in the two systems. Thresholds in the olfactory system are in most cases several orders of magnitude lower than the thresholds estimated for irritation effects of the upper airways (Cometto-Muniz and Cain 1990, 1994, 1995; Cometto-Muniz et al. 2004; Doty et al. 2004). In our previous studies, we had examined odor-evoked changes in local field potential in the main olfactory epithelium using the electro-olfactograms (EOG) recording (Lin et al. 2004), a method similar to ERP recording. Both lilial and geraniol (1 µM) induced changes in EOG in the olfactory epithelia (0.44 ± 0.06mV, n=7; 0.45 ± 0.07mV, n=10, respectively, unpublished data). In contrast, we were never able to record any responses -- either ERP or Ca\textsuperscript{2+} imaging of TRPM5-expressing cells -- for these odorous stimuli at such low concentrations.
Finally, a multitude of G-protein coupled odor receptors (~1000 in mice) trigger the cAMP signaling pathway mediating olfactory function in olfactory sensory neurons which transfer the signal directly to the olfactory bulb through their axons. In contrast, here we showed that the trigeminally-mediated chemical sensation likely involves detection and neurotransmitter-mediated transmission of information to nerve endings. In addition, although TRPM5 has not been demonstrated in free nerve endings, studies on gene targeted mice strongly suggest that some chemicals interact directly with ion channels in the nerve endings (Jordt et al. 2003). Trigeminal sensory neurons receiving input from the nasal cavity show chemosensory properties and respond to volatile organic compounds (Damann et al. 2006; Inoue and Bryant 2005).

In conclusion, the TRPM5-expressing chemosensory cells found in this study provide a mechanism by which an animal can monitor quickly changes in environmental irritants. Further studies are needed to investigate signal transduction and regulation in these chemosensory cells.

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References


Ogura T, Lin W, Margolskee R.F., Finger TE, and Restrepo D. TRPM5-expressing solitary chemoreceptor cells in the mouse nasal cavity respond to odors at high


Figure Legends

**Fig. 1.** TRPM5-expressing solitary chemosensory cells in the respiratory epithelium of the mouse nasal cavity.  A. Anterior nasal cavity viewed from a hemisected head from a TRPM5-GFP mouse, showing the GFP-positive solitary chemosensory cells densely populate the anterior part of the respiratory epithelium.  A white circle indicated area where the event-related potential was recorded for Fig 5. The anterior region of the main olfactory epithelium (MOE) is outlined in white line. B. A Magnified confocal image showing that the GFP-expressing cells are solitary, somewhat spindle-shaped. On average, there are 5600 GFP-positive solitary chemosensory cells in the respiratory epithelium of a half nose. C. An image from a section through the respiratory epithelium showing that the apical processes (arrow head) of a GFP-positive cell extends to the epithelial surface. D. A high magnification confocal image from a whole mount of stripped epithelium showing several slender GFP-positive cells. Apical processes (arrow head) and basal processes (arrow) emanate from the soma of these cells. E. Anti-TRPM5 antibody (red) strongly labeled GFP-positive cells. The image was taken from a whole mount preparation of a stripped epithelium. Apical regions are marked by arrow heads. Scale bars: A, 1mm; B, 50 µm, C, D and E 10 µm.

**Fig. 2.** Co-localization of TRPM5 with α-gustducin and components of the PLC signaling pathway.  Confocal images were taken from immunoreacted whole mount stripped epithelial tissue obtained from the TRPM5-GFP transgenic mice. TRPM5
expressing cells are GFP-positive, shown in green. A. Some but not all of the TRPM5-expressing cells immunoreacted with antibody against α-gustducin (marked by asterisks), showing that the population of TRPM5-expressing cells and α-gustducin-expressing cells overlapped but were not identical. Insert, an image from the anterior area showing more solitary chemosensory cells co-expressing GFP and α-gustducin. B and C, TRPM5 expressing cells also immunoreacted with PLCβ2 (B) and γ13 (C) respectively, suggesting that these cells express signaling elements of the PLC pathway similar to those in taste cells. Scale bars: 20 µm.
**Fig. 3.** Innervation of the TRPM5-expressing cells. Trigeminal nerve fibers in the nasal cavity were visualized using antibodies against PGP 9.5 and substance P. A. A confocal image showed PGP-immunoreactive nerve fibers closely apposing GFP-expressing cells in a whole mount preparation (arrows). B. In sections through the epithelium, PGP 9.5 positive fibers can be observed wrapping the basal region of a GFP-expressing SCC and coursing along the cell body (arrow heads). C. Substance-P positive fiber contacting a TRPM5-expressing cell. Multiple button-like swellings (arrow heads) were seen. Insert: an optical section showing that in several regions the GFP-labeled cell surrounds the nerve fibers indented into the soma (arrow heads). Scales: A, 10 µm; B and C 5 µm; insert, same as C.
**Fig. 4.** Expression of a vesicle-associated membrane protein synaptobrevin-2 in TRPM5-positive cells. A. Immunoreactivity for synaptobrevin-2 was seen in the TRPM5 expressing-cells (arrows), as well as presumed peptidergic nerve fibers that run along the basal lamina of epithelium and in close apposition to the TRPM5 cells. B. Synaptobrevin-2 immunoreactivity (red) overlaid with images of TRPM5 cells (green). C. A magnified image showing synaptobrevin-2-reactive processes and TRPM5 cells. Apical processes are indicated by arrowheads. Scales: 10 µm.

**Fig. 5.** Odorous stimuli-induced changes in the event-related potential (ERP). Traces are the largest amplitude ERPs recorded in the anterior respiratory epithelia where dense TRPM5-expressing solitary chemosensory cells reside. A variety of chemicals at 0.5-5 mM concentrations induced responses, except nicotine which induced sizable responses at 50 µM. Ringers solution did not induced visible changes in ERP (control). The ERP responses were repeatable. B. The ERP amplitudes for individual stimuli (5 mM) were averaged from 3-7 traces. C. The ERP responses are concentration-dependent. At 0.1 mM, citral, lilial and valeric acid did not induce visible ERPs. At concentrations of 1 or 5 mM, these same stimuli elicited measurable ERPs.
**Fig. 6.** Odors at high concentrations induce changes in intracellular Ca\(^{2+}\) levels in isolated GFP (TRPM5)-expressing cells.  A. Typical responses to various odors at 500 µM from the GFP-expressing cells. Response to lilial was repeated in the same cells. The evoked responses were repeatable. Bars indicate the stimulation periods. GFP (TRPM5)-expressing cells also respond to CO\(_2\) a common trigeminal stimulus. 2,5-DMP: 2,5 dimethylpyrazine. B. Concentration-dependent response curves for geraniol and lilial. Plotted values are percent changes from the resting Ca\(^{2+}\) levels. GFP (TRPM5)-expressing cells only responded to concentrations higher than 100 µM. Data are obtained from four cells tested with 20, 100, and 500 µM stimuli. C. Inhibition by the PLC inhibitor U73122. C1. Lilial (500 µM)-evoked Ca\(^{2+}\) responses (left). The response was suppressed in the presence of U73122 (5 µM). These two recordings were from the same cell. The effect of U73122 was partially recoverable. C2. Summary of U73122-induced inhibition. Peaks of responses to lilial at 500 µM were measured from both control and U73122 treated conditions in the same cells. For each cell, the peak value of responses in the presence of U73122 was normalized to the value of the control response. U73122 inhibited the lilial-evoked Ca\(^{2+}\) changes in the solitary chemosensory cells significantly (Paired t-test, p=0.019, n = 5).
**Fig. 7.** Responding profile of GFP-expressing solitary chemosensory cells. GFP-expressing cells respond to volatile stimuli. A total of 18 responding cells were stimulated with four or more chemicals. (●): cell responded. (○): cell did not respond.
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