Cellular/Molecular

# Transient Receptor Potential Channel Type M5 Is Essential for Fat Taste

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Until recently, dietary fat was considered to be tasteless, and its primary sensory attribute was believed to be its texture (Rolls et al., 1999; Verhagen et al., 2003). However, a number of studies have demonstrated the ability of components in fats, specifically free fatty acids, to activate taste cells and elicit behavioral responses consistent with there being a taste of fat. Here we show for the first time that long-chain unsaturated free fatty acid, linoleic acid (LA), depolarizes mouse taste cells and elicits a robust intracellular calcium rise via the activation of transient receptor potential channel type M5 (TRPM5). The LA-induced responses depend on G-protein-phospholipase C pathway, indicative of the involvement of G-protein-coupled receptors (GPCRs) in the transduction of fatty acids. Mice lacking TRPM5 channels exhibit no preference for and show reduced sensitivity to LA. Together, these studies show that TRPM5 channels play an essential role in fatty acid transduction in mouse taste cells and suggest that fatty acids are capable of activating taste cells in a manner consistent with other GPCR-mediated tastes.

## Introduction

Obesity has become one of the most serious health concerns in the world, and it is a disease that is reaching epidemic proportions, especially in developed countries. One of the precipitating factors that has been suggested to play a role in the development of dietary-induced obesity is the increase in dietary fat intake (Bray and Popkin, 1998, 1999; Bray et al., 2004). Despite this relationship, there is little information regarding the nature of the underlying mechanisms the body uses to recognize the sensory cues in fat. Obviously, understanding the mechanisms that enable fat-responsive tissues, including the taste system, to recognize and respond to dietary fat would be of utmost importance in the fight to control fat intake and, with it, to reduce the incidence of dietary-induced obesity worldwide.

For many years, dietary fat was considered to be tasteless, and it was assumed that its primary sensory attribute was its texture (Rolls et al., 1999; Verhagen et al., 2003), primarily detected through the somatosensory system. However, when the effects of texture were markedly reduced, rats could still discriminate different oils and continued to prefer fat solutions, suggesting that fat might provide salient cues to the gustatory system as well (Fukuwatari et al., 2003). During the last decade, there has been accumulating evidence from our laboratory and others demonstrating the ability of components in fats, specifically free fatty

acids, to activate taste cells, consistent with there being a "taste of fat." Gilbertson et al. (1997, 1998, 2005) provided the first evidence that free fatty acids activate taste receptor cells by inhibiting delayed rectifying potassium channels. More recently, several additional fatty acid-responsive proteins have been identified that may play a role in initiating fatty acid transduction, including the fatty acid-binding protein, CD36 (Baillie et al., 1996; Fukuwatari et al., 1997; Laugerette et al., 2005; Sclafani et al., 2007) and several G-protein-coupled receptors (GPCRs) (Milligan et al., 2006; Rayasam et al., 2007; Matsumura et al., 2007, 2009; Hirasawa et al., 2008; Cartoni et al., 2010). Despite the identification of these putative fatty acid-responsive proteins, the underlying mechanisms for fat transduction have not been unequivocally elucidated.

In this report, we show for the first time that the prototypical polyunsaturated long-chain free fatty acid, linoleic acid (LA), depolarizes taste cells and elicits robust intracellular calcium rise. The LA-induced responses are significantly reduced when the activities of G-proteins or phospholipase C (PLC) are inhibited, suggesting the involvement of a G-protein-PLC pathway in fatty acid transduction. We also show that LA activates taste cells through the activation of a monovalent cation-selective channel, transient receptor potential channel type M5 (TRPM5). When TRPM5 is inhibited by its specific blocker, or deleted from the genome, LA-induced responses are significantly reduced. Mice lacking TRPM5 channels show no preference for and reduced sensitivity to LA, which further confirms the role TRPM5 channel plays in fatty acid transduction. Together, these studies elucidate possible mechanisms of fat taste transduction in mouse taste cells, and demonstrate the critical role TRPM5 channel plays in this process.

## Materials and Methods

*Animals.* The TRPM5 knock-out strain has been described in detail previously (Damak et al., 2006). All experiments were performed on adult (2- to 6-month-old) male C57BL/6J or TRPM5 knock-out mice that

**Figure 1.** LA-induced intracellular $Ca^{2+}$ rise was dependent on extracellular $Na^{+}$, extracellular $Ca^{2+}$, and intracellular $Ca^{2+}$ ions. LA was applied at a final concentration of 30 $\mu M$. **A**, Bath application of LA induced a robust intracellular $Ca^{2+}$ rise in single taste cells. **B**, Changes in intracellular $Ca^{2+}$ concentration are pseudocolored as depicted. Arrows indicate the taste cells that showed significant increases in intracellular $Ca^{2+}$ concentration in response to LA. **C**, Data from LA-induced changes in intracellular $Ca^{2+}$ were fit with a Boltzmann function with an $EC_{50} = 13.7$ $\mu M$ ($n = 105$). LA was applied at 1–100 $\mu M$ final concentration. **D–F**, LA-induced intracellular $Ca^{2+}$ rise in single taste cells in the absence of extracellular $Na^{+}$ ions (**D**), extracellular $Ca^{2+}$ ions (**E**), and when intracellular $Ca^{2+}$ stores were depleted with thapsigargin (**F**). **G**, Mean responses (relative change in intracellular $Ca^{2+}$ concentration) $\pm$ SEM to LA in Tyrode's (control), $Ca^{2+}$-free Tyrode's ($n = 486$), $Na^{+}$-free Tyrode's ($n = 312$), and when intracellular $Ca^{2+}$ stores were depleted with thapsigargin ($n = 152$).

were maintained on a 12 h day/night cycle with normal mouse chow and water provided *ad libitum*. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Utah State University and were performed in accordance with American Veterinary Medical Association guidelines.

*Taste cell isolation.* Individual taste buds or taste cells were isolated from the tongues using techniques previously described (Gilbertson, 1995; Gilbertson et al., 1997). Briefly, tongues were removed and immediately immersed in ice-cold Tyrode's solution. The anterior portion of the tongue containing the fungiform papillae was injected between the muscle layer and the lingual epithelium with ~0.2 ml of physiological saline (Tyrode's) containing a mixture of collagenase I (1 mg/ml), dispase II (2.45 mg/ml), and trypsin inhibitor (1 mg/ml). Between 0.2 and 0.3 ml of the same enzyme solution was also used to inject the area surrounding the two foliate papillae and the circumvallate papilla. The injected tongue was incubated in a $Ca^{2+}$-$Mg^{2+}$-free Tyrode's and bubbled with $O_2$ for 45 min at room temperature. Following incubation, the tongue was washed with saline. The lingual epithelium was removed from the underlying muscle layer with forceps, pinned out in a Sylgard-lined Petri dish containing the $Ca^{2+}$-$Mg^{2+}$-free Tyrode's, and incubated for 10 min. Then the epithelium was incubated in the same enzyme solution for appropriately 7 min. After incubation, the lingual epithelium was washed with saline and incubated in the $Ca^{2+}$-$Mg^{2+}$-free Tyrode's for 10 min. Individual taste cells were moved from the epithelium under low magnification (50×) by gentle suction from a fire-polished pipette (~100 $\mu m$ pore) and plated immediately into a recording chamber containing Tyrode's for patch-clamp recording experiments, or onto a coverslip coated with Cell-Tak Cell and Tissue Adhesive for calcium

imaging, or into a 0.5 ml microfuge tube on ice for the reverse transcriptase (RT)-PCR assays.

*Solutions.* Standard extracellular saline solution (Tyrode's) and calcium-free Tyrode's were prepared as described previously (Gilbertson et al., 1997, 2005). Solutions in which the NaCl concentration was altered (nominally sodium-free, 10 mM sodium and 60 mM sodium Tyrode's) were prepared by an equimolar substitution of mannitol for NaCl. $Ca^{2+}$-$Mg^{2+}$-free Tyrode's contained the following (in mM): 140 NaCl, 5 KCl, 2 BAPTA, 10 HEPES, 10 glucose, and 10 Na pyruvate, pH 7.40, adjusted with NaOH; 310 mOsm. A potassium-based intracellular solution was used for measurement of membrane potential, containing the following (in mM): 140 K gluconate, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 11 EGTA, 3 ATP, and 0.5 GTP, pH 7.2, adjusted with KOH; 310 mOsm. A cesium-based intracellular solution was used for recording TRPM5 currents, in which 140 mM Cs acetate was substituted for the K gluconate and pH was adjusted to 7.2 with Tris OH; 310 mOsm. Hanks' buffered salt solution with HEPES buffer and Na pyruvate with fetal bovine serum (HHP with 2% FBS) contained the following: 98% 1× HBSS, 1% HEPES, 1% Na pyruvate, and 2% heat-inactivated fetal bovine serum; stored at 4°C for up to 1 week. Tastant mixture contained the following: 20 mM saccharin, 100 $\mu M$ SC45647, 10 mM denatonium benzoate, 100 $\mu M$ cycloheximide, and 5 mM monosodium glutamate. An additional taste mixture lacking monosodium glutamate was used in some experiments and yielded identical results. The 100 mM KCl solution contained the following (in mM): 45 NaCl, 100 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, and 10 Na pyruvate, pH 7.40, adjusted with KOH; 310 mOsm.

*Patch-clamp recording.* Recordings were made from individual taste cells or taste cells maintained in the taste bud at room temperature in the
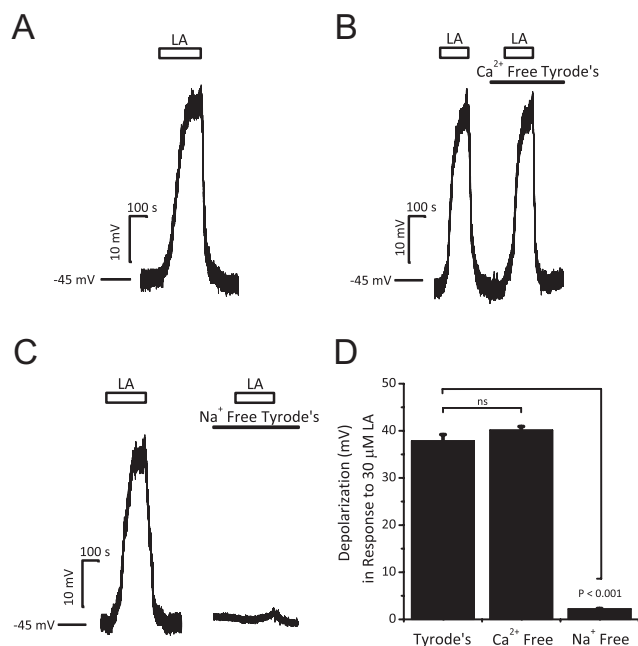
**Figure 2.** LA depolarized taste cells, and this depolarization was dependent on extracellular Na$^+$ ions but not on extracellular Ca$^{2+}$ ions. LA was applied at a final concentration of 30 $\mu$M. **A**, Bath application of LA-induced membrane depolarization in taste cells. Cells were held at 0 current level (e.g., at its resting membrane potential). **B**, **C**, LA-induced depolarization in taste cells in the absence of extracellular Ca$^{2+}$ ions (**B**) and extracellular Na$^+$ ions (**C**). **D**, Mean responses (depolarization) ± SEM to LA in Tyrode's ($n = 10$), Ca$^{2+}$-free Tyrode's ($n = 5$), and Na$^+$-free Tyrode's ($n = 5$).

whole-cell variation of the patch-clamp technique (Gilbertson et al., 1997, 2005). For membrane potential measurement, the current-clamp mode of the amplifier was used while holding the cell at its zero current level (i.e., at rest). LA was applied by bath application, and the membrane potential of taste cells was recorded continuously before, during, and after LA application. LA-induced TRPM5 currents in taste cells were recorded using the voltage-clamp mode. Typical inward currents were recorded at a holding potential of −100 mV, at which virtually all possible voltage-gated conductances were eliminated. LA was applied focally to the entire taste cell from a pipette positioned near the cell and delivered by a PicoSpritzer III (Parker Hannifin Corp.) controlled by the data acquisition and analysis software. For estimations of ion selectivity, ramp protocols from −100 mV to +100 mV (500 ms duration) were used to generate the instantaneous current–voltage relationship for the LA-induced TRPM5 current in various solutions. Data were analyzed by an unpaired, two-tailed Student's *t* test (see Fig. 3C), or one-way ANOVA followed by Bonferroni's *post hoc* analysis (see Figs. 2D, 4E,J, 5D, 7C). Significance was set at $\alpha = 0.05$ for all the analyses.

*Calcium imaging.* Single taste cells were loaded with Fura-2AM (5 $\mu$M; Invitrogen) for 1 h in HHP with 2% FBS at room temperature in the dark. The coverslips were then mounted onto an imaging chamber (RC-25F, Warner Instruments), placed on an inverted microscope (Nikon, Eclipse TE2000-S), and perfused continuously with Tyrode's. Cells were illuminated with a 100 W xenon lamp and excitation wavelengths (340/380 nm) were delivered by a monochromator (Bentham FSM150, Intracellular Imaging Inc.) at a rate of 20 ratios per minute. Fluorescence was measured by a CCD camera (DVC-340M) coupled to a microscope and controlled by imaging software (Incyt Im2, Intracellular Imaging). The ratio of fluorescence (340 nm/380 nm) was directly converted to calcium concentrations using a standard curve generated for the imaging system using a Fura-2 calcium imaging calibration kit (Invitrogen). LA and other compounds were applied extracellularly with a bath perfusion system over the entire cell surface at a flow rate of 4 ml/min, permitting complete exchange of the extracellular solution in <20 s. For generation of concentration–response curves, some taste cells were treated with LA

in an ascending concentration series, while others were tested in random order. No differences were seen using these two methods. Data were analyzed by a paired (see Figs. 1G, 5F) or unpaired (see Fig. 7E) two-tailed Student's *t* test, and significance was set at $\alpha = 0.05$.

*Behavioral assays.* Procedures for the 48 h, two-bottle preference test have been described in detail previously (Gilbertson et al., 2005; Laugerette et al., 2005; Cartoni et al., 2010). Two groups of 8 mice each (1 group of wild-type male mice and 1 group of TRPM5-null male mice) were tested in this paradigm. Differences between the two groups were analyzed for statistical significance using an unpaired, two-tailed Student's *t* test, and significance was set at $\alpha = 0.05$ (see Fig. 8A). In the conditioned taste aversion (CTA) experiments, two groups of mice (1 group of wild-type male mice and 1 group of TRPM5-null male mice) were used. Each group was further assigned to categories to receive either LiCl (experimental manipulation, CTA) or saline (control) injections during testing in the following sample sizes: wild-type male LiCl, $n = 7$; wild-type male NaCl, $n = 3$; TRPM5-null LiCl, $n = 9$; TRPM5-null male NaCl, $n = 5$. Details of the CTA behavioral tests have been described previously (Pittman et al., 2008). Briefly, mice had *ad libitum* access to water until 24 h before conditioning and testing, at which time the mice were placed on a 23.5 h water restriction schedule for the duration of the experiment. Two hours after the training/conditioning/testing on each of the water restriction days, all mice were given 30 min access to water. All taste stimuli were mixed daily from reagent-grade chemicals and presented at room temperature. In addition to water, there were 10 test stimuli consisting of 0.1, 0.3, 1, 3, 10, 30, and 100 $\mu$M LA, 100 mM sucrose, 3 mM denatonium benzoate, and 100 mM NaCl. Taste aversions were conditioned through 2 consecutive daily pairings of the conditioned stimulus (CS) and the unconditioned stimulus (US). At 9:00 A.M. on each conditioning day, mice first received a 1 ml intraoral application of the CS solution. Immediately after the intraoral application, the US was administered through intraperitoneal injections (20 ml/kg body weight dosage) of 150 mM LiCl to induce gastric distress or 150 mM NaCl (saline) as a control condition. All mice receiving a LiCl injection showed behavioral signs of gastric malaise, the unconditioned response, within 20 min of the injection. Two hours after the conditioning, all mice were given 30 min access to water. All testing was conducted in a MS-160 Davis Rig gustatory behavioral apparatus. A fan was located near the chamber to direct constant airflow along the longitudinal axis of the stimulus delivery tray, serving to reduce olfactory cues for any given stimulus. Mice were trained to lick during water stimulus trials in the Davis Rig apparatus for 3 consecutive days before the initial conditioning day. Following the second conditioning day, 2 consecutive days of testing in the Davis Rig apparatus assessed the formation of conditioned and generalized taste aversions. Each daily test session consisted of 2 blocks of 12 trials with stimulus durations of 5 s, wait times for the first lick of 150 s, and interstimulus intervals of 2 s. Each block included 1 trial of each test stimulus and 2 trials of water stimuli. The stimulus order within each block was randomly assigned. Total number of licks per stimulus was summarized across the 2 trials per test session and normalized using a lick ratio (licks per test stimulus/licks to water) to account for individual variances in the water-restricted motivation across the mice. Differences between LiCl- and saline-injected mice within each group were analyzed for statistical significance using an unpaired, two-tailed Student's *t* test, and significance was set at $\alpha = 0.05$ (see Fig. 8B).

*RT-PCR.* Procedures for RNA isolation and RT-PCR have been described previously (Gilbertson et al., 2005). Primers for transient receptor potential channel type M4 (TRPM4) (sense/antisense; 5′-GAGGATCATGAC-CCGAAAGG-3′/5′-TTCACTTTGGGCGATGTC-3′; 735–754/898–915) were designed using Oligo 6.0 Primer Analysis Software (Molecular Biology Insights).

## Results

### LA depolarizes and elicits an intracellular calcium rise in taste cells

To explore the mechanism that enables the taste system to recognize and respond to dietary fat, we have used cell-based approaches including both functional calcium imaging and patch-clamp record-
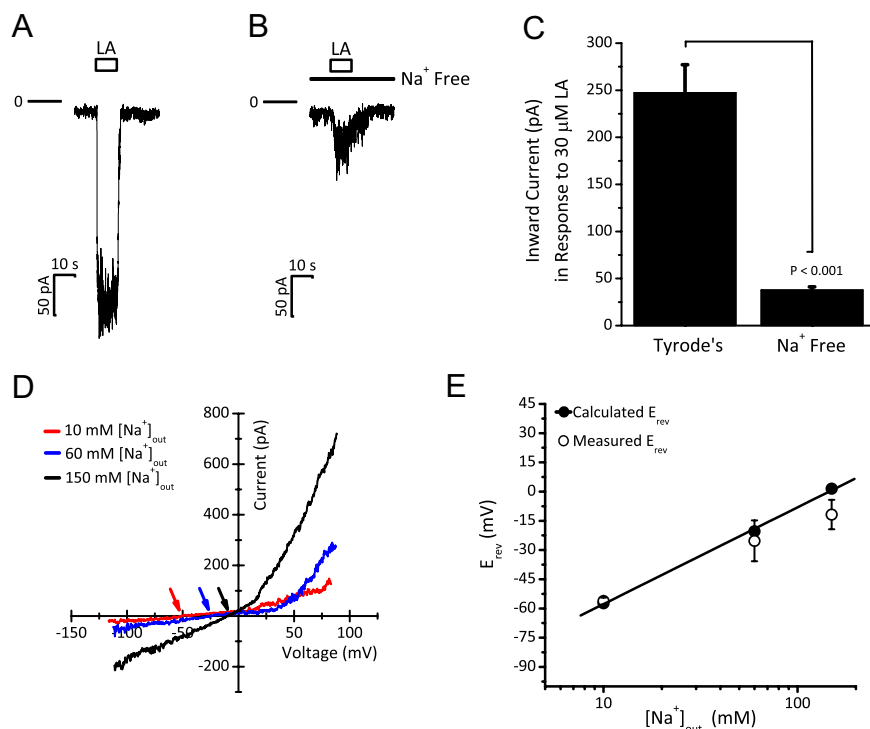
**Figure 3.** Rapid and focal application of 30 $\mu$M LA elicited a monovalent cation-permeable current. **A, B**, LA-induced inward currents (holding potential = −100 mV) in taste cells in Tyrode's (**A**) and Na$^+$-free Tyrode's (**B**). **C**, Mean responses (peak inward current) ± SEM to LA in Tyrode's (control, $n = 6$) and Na$^+$-free Tyrode's ($n = 15$). **D**, Current–voltage relationship of LA-induced current at extracellular Na$^+$ concentrations of 10, 60, and 150 mM. **E**, Comparison between theoretical and measured reversal potentials ± SEM of LA-induced current at extracellular Na$^+$ concentrations of 10 mM ($n = 14$), 60 mM ($n = 11$), and 150 mM ($n = 7$). Reversal potentials (indicated by arrows) were corrected for liquid junction potentials.

ing. We first loaded single taste cells isolated from both fungiform and circumvallate papillae with the ratiometric fluorescent dye Fura-2AM and measured the LA-induced intracellular calcium change. LA elicited a robust and reversible increase in intracellular calcium in taste cells (Fig. 1A,B). The magnitude of the LA-induced intracellular calcium rise was reproducible within cells with no noticeable decrease in the amplitude upon repeated stimulation. We also used a series of concentrations of LA (1, 3, 10, 30, and 100 $\mu$M) and generated the concentration–response curve fit with a Boltzmann function which showed that the EC$_{50}$ = 13.7 $\mu$M (Fig. 1C). In the following experiments, LA was applied at 30 $\mu$M, a concentration that produced significant but not maximum responses. In patch-clamp recording experiments, LA, applied by bath perfusion, elicited a large and reversible depolarization (Fig. 2A) that followed a time course similar to the change in intracellular calcium. Like the LA-induced change in the intracellular calcium concentration, the LA-induced depolarization was reproducible with no significant difference in the amplitude upon repeated applications of LA.

To investigate the dependence of LA-induced intracellular calcium rise on extracellular calcium, intracellular calcium, and extracellular sodium, we performed a series of ion substitution experiments. In the absence of extracellular calcium, LA-induced intracellular calcium rise was significantly reduced (Fig. 1E,G). When intracellular calcium stores were depleted by thapsigargin, an inhibitor of sarco-endoplasmic reticulum calcium ATPase, LA-induced intracellular calcium rise was significantly reduced as well (Fig. 1F,G), consistent with the interpretation that calcium ions from both intracellular stores and extracellular environment contribute to the fatty acid responses in taste cells.

Interestingly, removal of extracellular sodium ions also caused a significant decrease in the LA-induced intracellular calcium rise (Fig. 1D,G).

To determine what cations contributed to the LA-induced membrane depolarization, we manipulated the concentrations of ions extracellularly. Removal of extracellular calcium ions did not have a significant effect upon the LA-induced depolarization (Fig. 2B,D). On the contrary, when extracellular sodium ions were removed, LA-induced depolarization was significantly reduced (Fig. 2C,D). These results suggest that sodium influx is necessary for LA-induced depolarization and that there may be an additional site for calcium entry downstream of sodium entry (depolarization).

**LA activates sodium-dependent inward currents in taste cells**
We found in whole-cell voltage-clamp experiments (holding potential = −100 mV) that rapid and focal application of LA caused a fast inward current in individual taste cells (Fig. 3A). When extracellular sodium ions were removed, this LA-induced inward current was significantly reduced (Fig. 3B,C). To determine the ionic dependence of the LA-induced current, we performed a series of ion substitution experiments to investigate the permeability properties of this conductance. In standard conditions with concentrations of monovalent cations equal on both sides of the membrane, the current–voltage relationship of LA-induced inward current showed a reversal potential ≈ 0 mV, suggesting that LA activated a nonselective monovalent cation-permeable pathway. Consistent with this interpretation, changes in the concentration of extracellular sodium ions led to a corresponding shift in the reversal potential of LA-induced inward current, which can be closely predicted by the Goldman-Hodgkin-Katz equation for sodium-, potassium-, and cesium-permeable conductance with the assumption that all three ions have equal permeability (Fig. 3D,E).

**LA-induced responses depend on the G-protein-PLC pathway**
Recently, several GPCRs have been identified as putative receptors for free fatty acids (Milligan et al., 2006; Rayasam et al., 2007; Hirasawa et al., 2008) and their expression in taste tissues has been verified (Matsumura et al., 2007, 2009; Cartoni et al., 2010). A very recent report showed that GPR120-null and GPR40-null mice exhibited a diminished preference for LA and oleic acid, and diminished taste nerve responses to several fatty acids, strongly suggesting that GPR40 and GPR120 may play a role as the primary receptors for the taste of fat (Cartoni et al., 2010). To further confirm the role of G-proteins in the fatty acid transduction pathway, we used guanosine-5′-$O$-(2-thiodiphosphate) (GDP-$\beta$-S) to reversibly block the activation of G-proteins, and examined its effect on the LA-induced depolarization and inward current. Electrophysiological experiments showed that LA-induced depolarization and inward current were significantly reduced when the activation of G-proteins was inhibited (Fig. 4A,B,E,F,G,J). We ex-
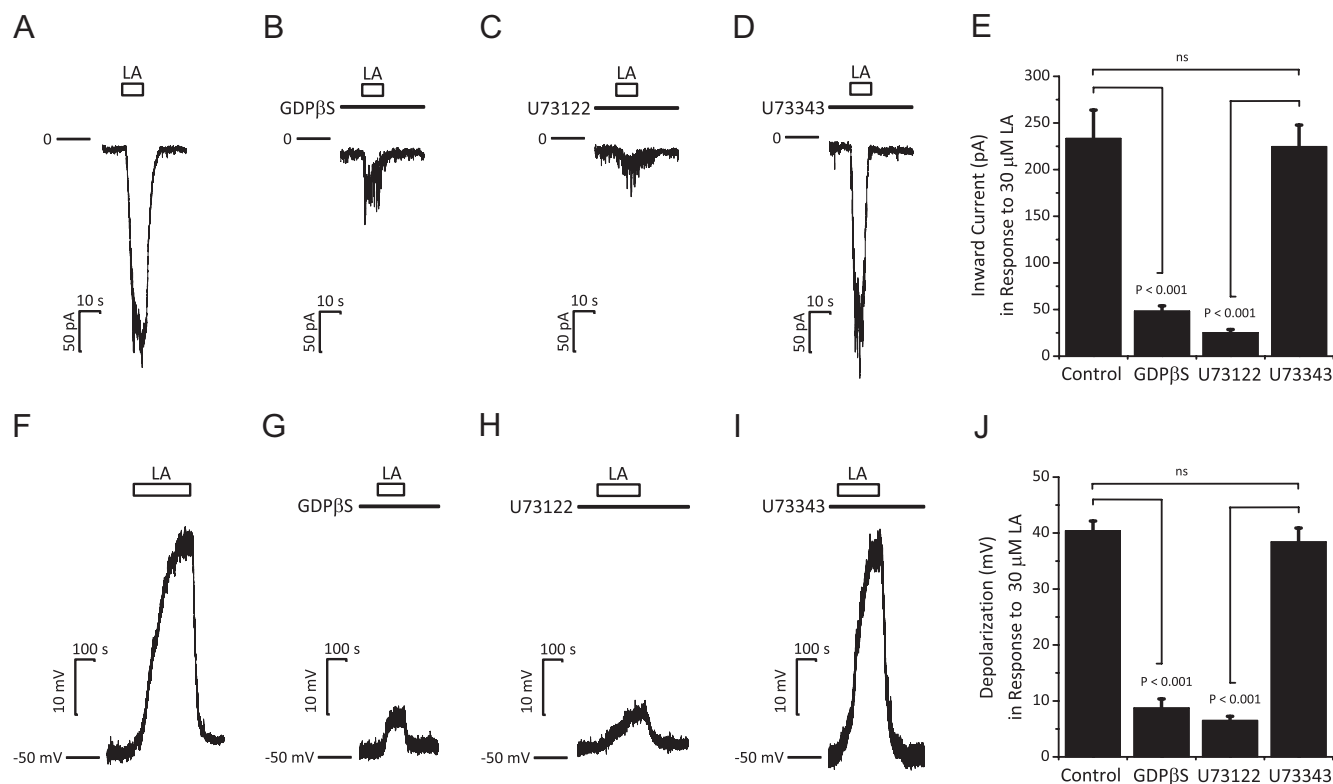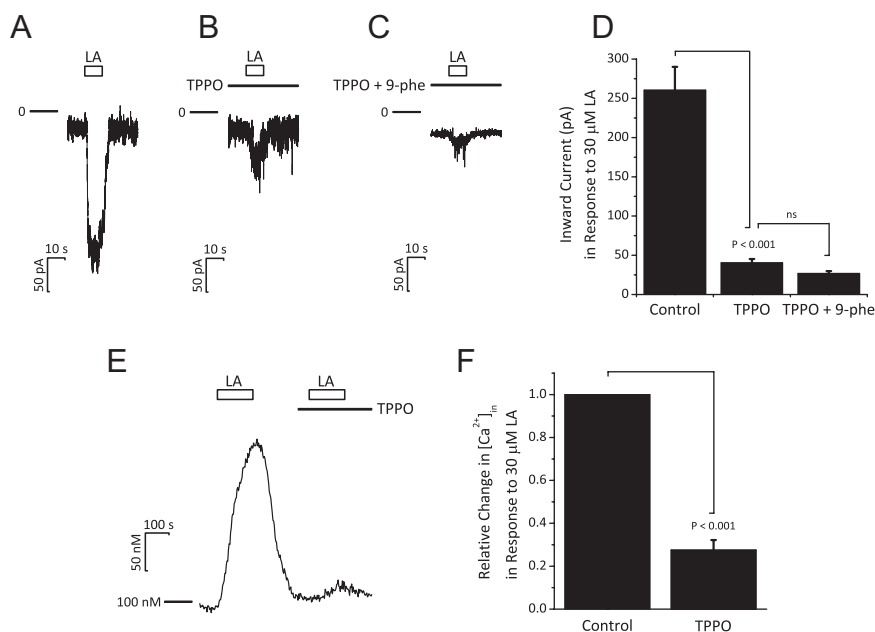
**Figure 4.** LA-induced responses were greatly reduced upon blocking the activity of G-proteins and PLC. **A–D**, LA was applied at a final concentration of 30 $\mu$M. Rapid and focal application of LA induced an inward current (holding potential = $-100$ mV) in control (**A**), GDP-$\beta$-S (1 mM)-treated (**B**), U73122 (3 $\mu$M)-treated (**C**), and U73343 (3 $\mu$M, the inactive analog of U73122)-treated (**D**) taste cells. **E**, Mean responses (inward current) $\pm$ SEM to LA in control conditions ($n = 5$), with GDP-$\beta$-S (1 mM) treatment ($n = 10$), with U73122 (3 $\mu$M) treatment ($n = 11$), and with U73343 (3 $\mu$M) treatment ($n = 10$). **F–I**, Current-clamp studies in taste cells held at their 0 current level showing that LA induced membrane depolarization in control (**F**), GDP-$\beta$-S (1 mM)-treated (**G**), U73122 (3 $\mu$M)-treated (**H**), and U73343 (3 $\mu$M, the inactive analog of U73122)-treated (**I**) taste cells. **J**, Mean responses (depolarization) $\pm$ SEM to LA in control conditions ($n = 14$), with GDP-$\beta$-S (1 mM) treatment ($n = 7$), with U73122 (3 $\mu$M) treatment ($n = 11$), and with U73343 (3 $\mu$M) treatment ($n = 10$).

amined the involvement of PLC in the fatty acid transduction pathway by using the PLC blocker U73122. In the presence of U73122, the LA-induced depolarization and inward current were significantly reduced (Fig. 4C,E,H,J), whereas these responses were not affected when the taste cell was treated with its inactive analog U73343 (Fig. 4D,E,I,J). Our data suggest that LA-induced responses are downstream of the G-protein-PLC pathway and support a pathway involving the activation of fatty acid-activated GPCRs (Cartoni et al., 2010).

## LA-induced currents in taste cells are carried primarily through TRPM5 channels

During the past few years, the calcium-activated, monovalent cation-selective channel TRPM5 has been shown to play an essential role in the transduction of sweet, bitter, and umami tastes (Pérez et al., 2002; Hofmann et al., 2003; Liu and Liman, 2003; Prawitt et al., 2003; Zhang et al., 2003). However, tastant-induced TRPM5 currents have not been successfully recorded in native taste cells electrophysiologically. Since we have shown that the LA-induced inward current is mon-



**Figure 5.** LA-induced responses were significantly reduced by the TRPM5 channel antagonist TPPO. **A–C**, LA (30 $\mu$M) induced inward currents in taste cells in Tyrode's (**A**), in the presence of the TRPM5 antagonist TPPO (100 $\mu$M) (**B**), and in the presence of both the TRPM5 antagonist TPPO (100 $\mu$M) and the TRPM4 antagonist 9-phenanthrol (100 $\mu$M) (**C**). **D**, Mean responses (peak inward current) $\pm$ SEM to LA in Tyrode's (control, $n = 5$), with TPPO (100 $\mu$M) treatment ($n = 12$), and with both TPPO (100 $\mu$M) and 9-phenanthrol (100 $\mu$M) treatments ($n = 10$). **E**, LA (30 $\mu$M)-induced intracellular Ca$^{2+}$ rise in single taste cells in Tyrode's and with TPPO (100 $\mu$M) treatment. **F**, Mean responses (relative change in intracellular Ca$^{2+}$ concentration) $\pm$ SEM to LA in Tyrode's (control) and with TPPO (100 $\mu$M) treatment ($n = 76$).
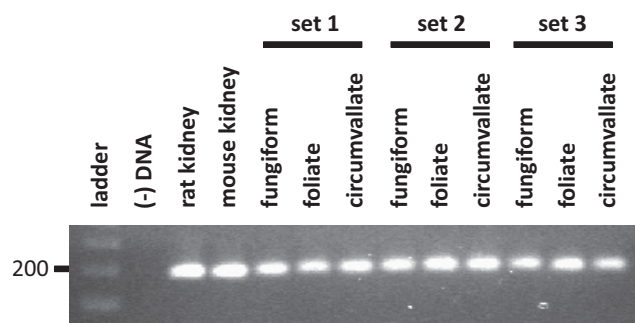
**Figure 6.** Expression of TRPM4 channels in mouse taste cells. Ethidium bromide-stained gel of PCR products showing TRPM4 was expressed in three sets of pooled mouse taste buds from the fungiform, foliate, and circumvallate papillae. Positive controls for TRPM4 using rat kidney cDNA and mouse kidney cDNA are shown. The negative control lane ($-$DNA) represents the omission of cDNA from the PCR.

ovalent cation selective and downstream of PLC activation, we hypothesized that TRPM5 channels may be involved in this process. To test our hypothesis, we used the TRPM5 channel blocker triphenylphosphine oxide (TPPO) (Palmer et al., 2010) to block the activity of TRPM5 channels, and examined its effect in both calcium imaging and patch-clamp recording experiments. TPPO treatment significantly reduced the LA-induced inward current and intracellular calcium rise (Fig. 5$A,B,D,E,F$), consistent with the interpretation that TRPM5 channels are involved in the LA-activated signaling pathway. Interestingly, the LA-induced inward current was not completely blocked by the TPPO treatment, suggesting either that TPPO does not completely block TRPM5 channels, or that there might be other TRPM5-independent pathways involved in the LA-induced inward current. Since TRPM4 is also monovalent cation selective, has properties very similar to TRPM5 channels (Launay et al., 2002), and is apparently expressed in taste cells (Fig. 6), we further tested whether the residual current after TPPO treatment was carried by TRPM4 channels. We used TPPO and 9-phenanthrol (Grand et al., 2008), a TRPM4 channel blocker, together to block both TRPM5 and TRPM4 channels. Patch-clamp recording data showed that the addition of 9-phenanthrol did not significantly reduce LA-induced current any further, suggesting that TRPM4 channels are not responsible for the small, residual LA-induced inward current (Fig. 5$C,D$). Further studies showed that blocking TRPM4 channels using 9-phenanthrol alone did not significantly reduced LA-induced current (data not shown). Based upon its permeability properties, voltage dependence, and pharmacology, our results strongly suggest that LA-induced currents in taste cells are carried primarily through TRPM5 channels.

**Mice lacking TRPM5 channels exhibit no preference for and show reduced sensitivity to LA**

To directly assess the role of TRPM5 channels in the fatty acid signaling pathway, we used a transgenic mouse model with a TRPM5 gene deletion (Damak et al., 2006). Single taste cells were isolated from both fungiform and circumvallate papillae from mice lacking TRPM5. In these cells, LA-induced inward current and intracellular calcium rise were significantly reduced (Fig. 7$A,C,D,E$), validating the involvement of TRPM5 channels in fatty acid transduction. Consistent with the findings in wild-type mice, the residual LA-induced inward current cannot be further reduced by blocking TRPM4 channels with 9-phenanthrol (Fig. 7$B,C$).

Recently, behavioral experiments have shown that wild-type mice preferred a LA emulsion to the vehicle in two-bottle tests
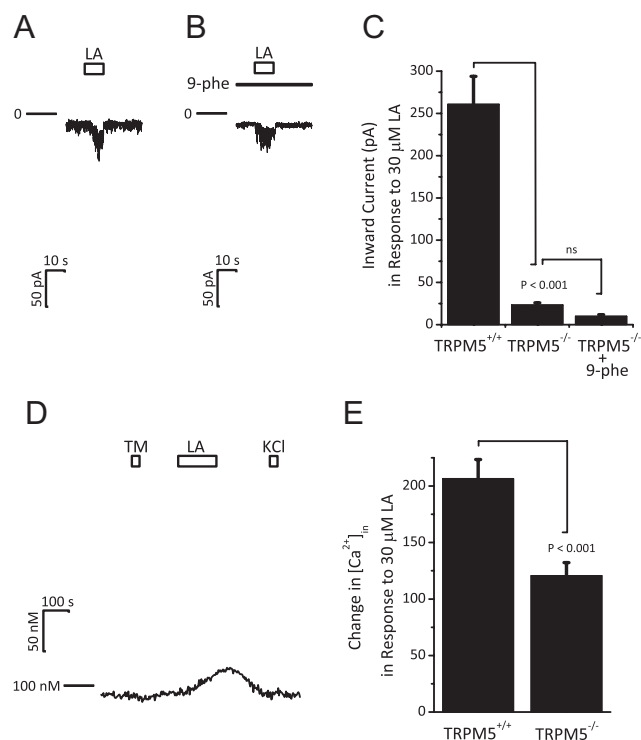


**Figure 7.** LA-induced currents were carried by TRPM5 channels. LA was applied at a final concentration of 30 $\mu$M for **A–D**. **A**, **B**, LA-induced inward current in taste cells isolated from TRPM5$^{-/-}$ mice in Tyrode's (**A**) and in the presence of the TRPM4 antagonist 9-phenanthrol (100 $\mu$M) (**B**). **C**, Mean responses (peak inward current) ± SEM to LA in Tyrode's ($n = 5$) in taste cells from TRPM5$^{+/+}$ mice, and to LA in Tyrode's with ($n = 10$) and without ($n = 12$) 9-phenanthrol (100 $\mu$M) treatment in taste cells from TRPM5$^{-/-}$ mice. **D**, LA-induced intracellular Ca$^{2+}$ rise in single type II taste cells isolated from TRPM5$^{-/-}$ mice. TM, Tastant mixture. **E**, Mean responses (change in intracellular Ca$^{2+}$ concentration) ± SEM to LA in single type II taste cells isolated from TRPM5$^{+/+}$ ($n = 49$) and TRPM5$^{-/-}$ mice ($n = 67$).

(Laugerette et al., 2005). However, there are no reports investigating the preference for fatty acid in TRPM5-null mice. We performed a 48 h two-bottle preference test using both wild-type and TRPM5-null mice. The results showed that TRPM5-null mice were indifferent to a LA emulsion, whereas wild-type mice preferred LA to vehicle as expected (Fig. 8$A$) (Laugerette et al., 2005). Based on this finding, we hypothesized that the TRPM5-null mice lose their ability to respond to fatty acids. To test this hypothesis, we performed a series of brief-access (5 s) behavioral assays, which eliminated post-ingestive cues for fatty acids, following the formation of a CTA to LA (conditioned stimulus, 100 $\mu$M LA) (Pittman et al., 2008). In the CTA assays, wild-type mice showed significant aversions at relatively low LA concentrations (3 $\mu$M), whereas the TRPM5-null mice did not show any significant aversions at LA concentrations up to 100 $\mu$M, suggesting their loss of sensitivity to LA at the concentrations tested (Fig. 8$B$). These results validate the critical role TRPM5 channels play in the fatty acid transduction pathway.

## Discussion

Fatty acids, like LA, have been suggested to fulfill the role of the prototypical taste stimulus for dietary fat. This viewpoint is based upon a number of observations. First of all, free fatty acids have been shown to function as specific extracellular messengers where they activate a variety of systems (e.g., muscle, cardiac cells) (Ordway et al., 1991; Honoré et al., 1994; Petrou et al., 1995). Second, there are molecules in the oral cavity that work to

generate and transport free fatty acids, including lingual lipase and the von Ebner's gland proteins. Lingual lipase, the enzyme responsible for efficient free fatty acid release from dietary triglycerides, is capable of hydrolyzing ~70 $\mu$mol of triglycerides per minute (Kawai and Fushiki, 2003). Von Ebner's gland proteins, structurally similar to lipocalins, a family of proteins that play critical roles in the transport of lipophilic molecules (Flower et al., 2000; Grzyb et al., 2006), bind no other taste molecules than free fatty acids (Schmale et al., 1993; Kock et al., 1994; Creuzenet and Mangroo, 1998). Based on these observations, we began to hypothesize that the gustatory system might be able to detect and respond to fat, especially free fatty acids. Gilbertson et al. (1997) for the first time showed that free fatty acids could activate taste cells through the inhibition of delayed rectifying potassium channels. Recently, several additional fatty acid-responsive proteins have been identified that may be involved in the initiation of fatty acid transduction, and their expression in the taste system has been verified (Baillie et al., 1996; Fukuwatari et al., 1997, Laugerette et al., 2005; Milligan et al., 2006; Rayasam et al., 2007; Sclafani et al., 2007; Matsumura et al., 2007, 2009; Hirasawa et al., 2008; Cartoni et al., 2010).

However, the underlying mechanisms for fatty acid transduction in taste cells remain unclear. In this report, we showed that the free fatty acid, LA, was capable of activating taste cells. Applied extracellularly, LA induced significant membrane depolarization and elicited an intracellular calcium rise in taste cells, which has been shown to be a necessary precursor for the release of neurotransmitters. The concentration–response curve showed the EC$_{50}$ = 13.7 $\mu$M, which fell well within the range of free fatty acid concentrations that can be generated during fat feeding and found in most fat-containing foods. Thus, free fatty acids contained in or produced from fat-containing foods can reach a concentration high enough to stimulate taste cells. While most electrophysiological studies in the taste system were based on taste cells from the circumvallate papillae, our data showed that taste cells from both fungiform and circumvallate papillae responded to fatty acids, suggesting that both the anterior and posterior part of the tongue are involved in fatty acid detection.

Using ion substitution and pharmacological experiments, we also showed that calcium ions from both intracellular stores and extracellular environment contribute to the fatty acid-induced increases in the intracellular calcium concentration. When taste cells were incubated in thapsigargin, there appeared a robust rise in resting calcium levels, indicating the possible activation of store-operated calcium entry (SOCE) triggered by the depletion of endoplasmic reticulum. The application of LA first caused a rapid decrease in the basal calcium concentration, probably due to its ability to inhibit SOCE (Yang et al., 2005), and then increased the intracellular calcium concentration gradually, with the final magnitude of the intracellular calcium rise significantly reduced (Fig. 1F). How the fatty acid responses interact with the
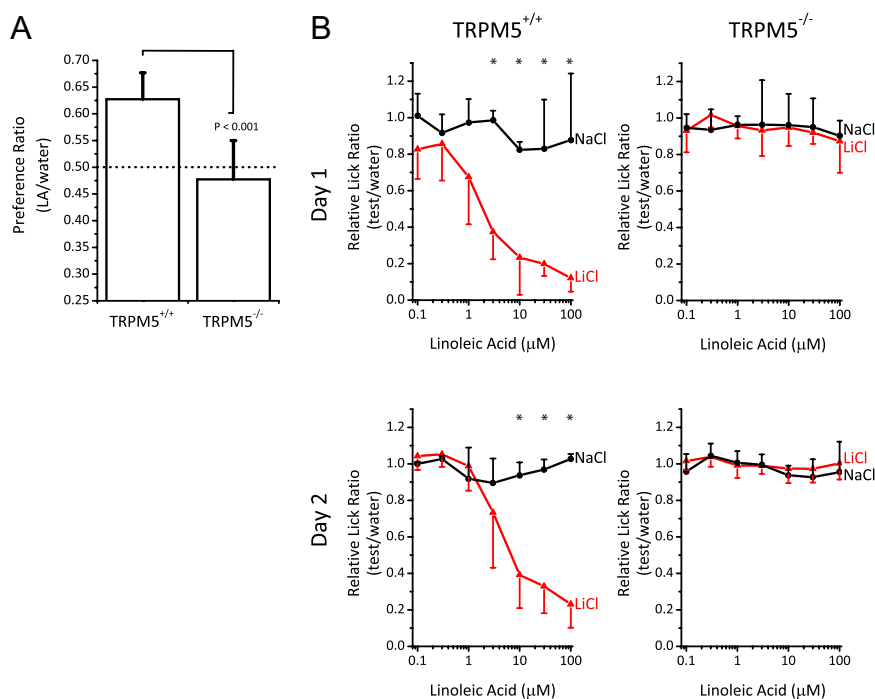


**Figure 8.** Mice lacking TRPM5 channels exhibit no preference for and show reduced sensitivity to LA. ***A***, Preference ratio for LA compared with vehicle in TRPM5 $^{+/+}$ and TRPM5 $^{-/-}$ mice during two-bottle 48 h LA vs vehicle tests. LA was applied at a final concentration of 30 $\mu$M. TRPM5 $^{-/-}$ mice ($n = 8$) were indifferent to a LA emulsion, whereas TRPM5 $^{+/+}$ (wild-type) mice ($n = 8$) preferred LA to vehicle (water). ***B***, Mean lick ratios $\pm$ SD for LA in TRPM5 $^{+/+}$ and TRPM5 $^{-/-}$ male mice on day 1 and day 2 after CTA. TRPM5 $^{+/+}$ LiCl, $n = 7$; TRPM5 $^{+/+}$ NaCl, $n = 3$; TRPM5 $^{-/-}$ LiCl, $n = 9$; TRPM5 $^{-/-}$ NaCl, $n = 5$. Asterisks indicate significant differences between LiCl-injected (triangle, solid red line) and NaCl-injected (dot, solid black line) groups ($p < 0.005$).

SOCE and adjust the regulation of intracellular calcium concentration remains an interesting open question.

We also showed that the LA-induced depolarization and intracellular calcium rise was significantly reduced when the activity of G-proteins or PLC was inhibited, indicating that the LA-induced responses are downstream of the G-protein-PLC pathway. This finding dovetailed with the idea that fatty acid-activated GPCRs may initiate the fatty acid transduction, an idea that has recently been supported empirically (Cartoni et al., 2010). Furthermore, we showed that LA activated a monovalent cation-selective inward current in taste cells. Based on its permeability properties, voltage dependence, and pharmacology, we have demonstrated that this LA-induced current was primarily carried by TRPM5 channels. These results also marked the first time that tastant-induced TRPM5 currents have been successfully recorded in native taste cells electrophysiologically. When TRPM5 channels were inhibited by the antagonist TPPO, or deleted from the genome, the LA-induced responses were significantly reduced, strongly suggesting that TRPM5 channels play a critical role in the fatty acid transduction. Since the G-protein-PLC pathway and TRPM5 channels have been shown to be involved in the transduction of sweet, bitter, and umami, it appears that the signaling pathway the taste system uses to detect fatty acid is qualitatively very similar in many respects to that of sweet, bitter, and umami pathways, at least in those cells that express TRPM5.

Interestingly, the LA-induced inward current was not completely blocked by TPPO, suggesting either that TPPO could not completely block TRPM5 channels, or that there might be other TRPM5-independent pathways involved in this process. Since TRPM4 channel is also monovalent cation selective and shares

similar properties to TRPM5 channel, we also tested the possibility that this residual LA-induced current was carried by TRPM4 channels using the TRPM4 channel blocker 9-phenanthrol. However, 9-phenanthrol did not significantly reduce this residual current any further, suggesting that this small, residual LA-induced inward current was not through TRPM4 channels. Thus, it is very possible that there exist other parallel fatty acid transduction pathways in the taste cells. If, indeed, these additional pathways exist, our behavioral data conducted on mice lacking TRPM5 would argue that they do not contribute significantly to the recognition of LA.

A recent report (Cartoni et al., 2010) suggested that both GPR120, which is primarily expressed in fungiform and circumvallate taste receptor cells, and GPR40, expressed mainly in type I taste cells from the circumvallate papillae, contribute to fatty acid signaling. Our data would argue that GPR120 plays a more critical role for LA taste since GPR120 is expressed primarily in type II (TRPM5-expressing) cells, which do not apparently express GPR40. Loss of TRPM5 resulted in a complete inhibition of preference for LA and the ability to form a conditioned taste aversion against this fatty acid. Together, these results suggest that type II taste cells might be the primary receptor cells for LA.

Our data strongly suggest a gustatory cue exists for free fatty acids. Nonetheless, there appear to be significant species differences in taste reactivity to fatty acids. First, humans do not secrete copious amounts of salivary lingual lipase, a key enzyme that generates free fatty acids from triacylglycerol in rats. However, numerous studies have shown that concentrations of free fatty acids present in foods or liberated during food preparation lie within the effective range for human detection (Chow, 1992; Metzger et al., 1995; Mattes, 2001, 2009a). Recent studies showed that humans can detect free fatty acids varying in their chain length and degree of saturation (Mattes, 2009b). However, evidence for a fatty acid transduction system in human taste cells is still lacking. In addition, free fatty acids seemed to trigger aversive responses in humans rather than the largely attractive responses in rodents. In the present study, despite our best efforts, it is not possible to completely exclude other potential cues for fatty acids, including odor, irritancy, and somatosensory cues that may contribute to the responses we observed in the behavioral studies.

Another concern is due to the hydrophobic properties of fatty acids. Polyunsaturated fatty acids have been found to alter the function of many membrane proteins by affecting membrane fluidity and tension (Bruno et al., 2007). It is certainly possible that this may also contribute to the fatty acid transduction, particularly the small residual responses seen independent of the TRPM5-mediated mechanism. Also, we cannot exclude the possibility that fatty acids may permeate through the cell membrane and elicit signaling pathways inside the cell. Indeed, the binding sites for LA on fatty acid-activated GPCRs, like GPR120, are completely unknown. Further, those fatty acids that permeate the membrane may themselves be activating signaling pathways directly. We cannot rule this out, but we have shown the importance of G-protein activation and PLC pharmacologically in these experiments. So, if intracellular LA is responsible, it is working through a very similar pathway. Also, it is possible that permeation of the acid form of LA might lead to changes in intracellular pH. However, it is unlikely this could account for the responses reported in this study. First, rapid and focal application of LA caused a fast inward current within 1 s in individual taste cells. The speed of the responses offers strong support for the idea that LA activates its receptors extracellularly. Using much stronger (higher concentration) acids, intracellular acidification takes tens of seconds to develop and elicits modest changes in intracellular pH (Lyall et al., 2001). Second, studies suggest that type II (TRPM5-expressing) cells do not respond to sour (acidic) taste even in 100 mM citric acid (Tomchik et al., 2007). Accordingly, even if fatty acids did weakly acidify the cytosol, the modest acidification is not likely to trigger any significant responses due to the lack of transduction machinery for sour taste in type II cells.

In conclusion, our findings specifically support the idea that the key components in dietary fat, the essential fatty acids, are capable of directly activating taste receptor cells at physiologically relevant concentrations and, in that way, may be an initial step in providing support that fat may represent a bona fide sixth basic taste. Moreover, it appears from our data that TRPM5 plays a central role in the generation of the fatty acid-induced receptor potential where it functions in a fashion analogous to its role in the transduction of other complex taste stimuli, like sweet, bitter, and umami compounds. However, unlike the cell specificity found for other G-protein-mediated taste stimuli, most fatty acid-responsive cells also respond to taste mixtures (i.e., type II cells), suggesting that the peripheral gustatory response to fatty acids, at least LA, may be coded quite differently at the periphery than for other taste stimuli. Additional experiments are needed to clarify this issue at both peripheral and central levels.

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