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The Role of GPR84 in Medium-chain Saturated Fatty Acid Taste Transduction

Yan Liu
Utah State University

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THE ROLE OF GPR84 IN MEDIUM-CHAIN SATURATED FATTY ACID TASTE TRANSDUCTION

by

Yan Liu

A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biology

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                                             Dean of the School of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2016
THE ROLE OF GPR84 IN MEDIUM-CHAIN SATURATED FATTY ACID TASTE TRANSDUCTION

by

Yan Liu, Doctor of Philosophy

Utah State University, 2016

Major Professor: Dr. Timothy A. Gilbertson
Department: Biology

Previous research has shown the gustatory recognition of the long-chain unsaturated fatty acids. In this study, I showed for the first time that medium-chain saturated fatty acids (MCFAs) are effective taste stimuli at both the cellular and behavioral levels. The mechanisms of gustatory recognition of MCFAs in mice were also partially elucidated using pharmaceutical approaches. The inward currents induced by capric acid in mouse taste cells were significantly inhibited by the antagonists of G-protein signaling, protein kinase A (PKA), and protein kinase C (PKC), but they were not affected by the phospholipase C (PLC) and phosphodiesterase (PDE) inhibitors, which suggested that MCFA taste transduction involves G-protein, PKA, and PKC activation, but does not involve PLC and PDE. Furthermore, transient receptor potential channel type M5 (TRPM5) knockout mice showed unaffected cellular and behavioral responses, indicating MCFA taste transduction in mice was TRPM5-independent. The receptor of MCFAs on taste cells was also investigated in this study. Previous research has shown that GPR84 is
a novel G protein-coupled receptor (GPCR) activated by MCFAs with 9 to 14 carbons, and the real-time PCR results supported the mRNA expression of GPR84 in mouse taste cells. Therefore, GPR84 is a good candidate for the MCFA receptor in taste cells. By using a cell line that had an inducible GPR84 + Gqi9 (a chimeric G-protein), ligand specificity experiments showed that the ligands of GPR84 included saturated fatty acids with 6 to 12 carbons and other fatty acids (oleic acid, arachidic acid) previously not known to activate this receptor. Compared to wild type cells, taste cells lacking GPR84 showed significantly lower calcium responses and inward currents to capric acid (C_{10}), and GPR84 knockout mice showed decreased taste sensitivities to both lauric (C_{12}) and capric acids behaviorally, suggesting that GPR84 plays an essential role in MCFA taste transduction process.
PUBLIC ABSTRACT

The Role of GPR84 in Medium-Chain Saturated Fatty Acid Taste Transduction

Yan Liu

The prevalence of overweight or obese in adults has nearly doubled during the past two decades, and obesity increases the risk of heart disease, diabetes, and even some types of cancer. One of the factors closely linked with the obesity epidemic is overconsumption of dietary fat. Accumulating evidence has supported the existence of the “taste of fat”, and more and more studies have focused on identifying the mechanisms of fatty acid detection by gustatory system. In this study, I showed for the first time that medium-chain saturated fatty acids (MCFAs) were able to activate taste cells isolated from mice, and they could also be tasted by mice behaviorally. These data suggested that MCFAs were effective taste stimuli. The signaling pathways involved in the taste transduction of MCFAs were also studied here. A novel G protein-coupled receptor (GPCR) GPR84 was suggested to be the main receptor of MCFAs in taste cells, because the mice lacking the GPR84 gene had significantly reduced taste responses to MCFAs both cellurally and behaviorally. My study also suggested that downstream of GPR84 signaling involves G-protein, protein kinase A (PKA), and protein kinase C (PKC) activation, but does not involve phospholipase C (PLC) and phosphodiesterase (PDE). The knowledge on fatty taste may help us reduce fat intake and in turn, prevent obesity in the future.
DEDICATION

To my dear father Deyou Liu, mother Yulan Liu, and husband Xiaoxi Wang for their unconditional love over the years.
ACKNOWLEDGMENTS

Almost six years ago, I arrived in the U.S and started my PhD study. For many times, I thought I was never going to graduate, but I did it and I have so many people to thank.

The first person I want to thank is my major professor Dr. Tim Gilbertson. He is intelligent, knowledgeable, generous, patient, and kind. He gave me countless valuable advices not only in research, but also in career and even life. He has been a mentor and a friend, and his guidance made this journey meaningful and rewarding. I would like to thank our lab manager Dr. Dane Hansen for teaching me the techniques I needed, helping with all the trivial matters in lab, and talking to me as a friend when I needed to vent out. In addition, I would like to thank my former lab mate and also my dear friend Dr. Han Xu. She taught me almost all the techniques I needed to complete my dissertation, and helped solve a lot of tough problems. I also want to thank my committee members, Dr. Brett Adams, Dr. Keith Mott, Dr. Silvana Martini, and Dr. Joan Hevel for their time and effort on helping me complete my study. And I would like to thank all my lab mates and my friends in Logan for their support in both work and life.

For my whole life, my parents has been giving me a lot of encouragement and they respected my decision all the time. I feel so lucky to be their only daughter. Also, I have a little cute boy to thank, my son, Lex. He exhausted me but at the same time gave me a lot of energy to help me finish my PhD study. I want to thank him for being such a joy in my life.

At last, I would like to thank my husband, Xiaoxi Wang. He is my best friend who has been my cheerleader for the last six years. Without his constant encouragement and
support, I would not be as happy as I am. Without his willingness to take care of Lex most of the time, it would take me a lot longer to finish my dissertation. I am grateful that he is always on my side and I know I can depend on him no matter how hard life is.
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<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-Bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium ions</td>
</tr>
<tr>
<td>[Ca&lt;sup&gt;2+&lt;/sup&gt;]&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Intracellular calcium concentration</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>CALHM1</td>
<td>Calcium homeostasis modulator 1</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CD36</td>
<td>Cluster of differentiation 36</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CS</td>
<td>Conditioned stimulus</td>
</tr>
<tr>
<td>CTA</td>
<td>Conditioned taste aversion</td>
</tr>
<tr>
<td>CT</td>
<td>Chorda tympani nerve</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DB</td>
<td>Denatonium benzoate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>dNTP</td>
<td>2-deoxynucleotide 5'-triphosphate</td>
</tr>
<tr>
<td>DRK</td>
<td>Delayed rectifying potassium</td>
</tr>
<tr>
<td>EC50</td>
<td>Half maximal effective concentration</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial sodium channel</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FAT</td>
<td>Fatty acid transporter</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>GAD67</td>
<td>Glutamic acid decarboxylase67</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine-5'-diphosphate</td>
</tr>
<tr>
<td>GDP-ß-S</td>
<td>Guanosine 5′-[ß-thio]diphosphate trilithium salt</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GL</td>
<td>Glossopharyngeal nerve</td>
</tr>
<tr>
<td>GLAST</td>
<td>Glutamate aspartate transporter</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
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<td>GPR120</td>
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<td>GPR40</td>
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<td>GPR84</td>
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GPR84\textsuperscript{+/+}  Wild type
GPR84\textsuperscript{+/-}  Heterozygous GPR84 knockout
GPR84\textsuperscript{+-}  GPR84 knockout
GTP  Guanosine-5'-triphosphate
HBSS  Hank’s buffered salt solution
HCl  Hydrogen chloride
HEK293  Human embryonic kidney 293
HEPES  2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HF  High fat
HHP  Hanks’ buffered salt solution with HEPES buffer and Na pyruvate
IBMX  3-isobutyl-1-methylxanthine
IP\textsubscript{3}  Inositol 1,4,5-triphosphate
IP\textsubscript{3}R3  Inositol 1,4,5-triphosphate receptor type 3
K\textsuperscript{+}  Potassium ions
KCl  Potassium chloride
KO  Knockout
KOH  Potassium hydroxide
LCFA  Long-chain unsaturated fatty acids
LiCl  Lithium chloride
LPS  Lipopolysaccharide
MCFA  Medium-chain saturated fatty acid
MgCl\textsubscript{2}  Magnesium chloride
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium ions</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NST</td>
<td>Nucleus of the solitary tract</td>
</tr>
<tr>
<td>NTPDase2</td>
<td>Nucleoside triphosphate diphosphohydrolase 2</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PLCβ2</td>
<td>Phospholipase C β2 isoform</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKD2L1</td>
<td>Polycystic kidney disease 2-like 1 protein</td>
</tr>
<tr>
<td>PKD1L3</td>
<td>Polycystic kidney disease 1-like 3 protein</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROMK</td>
<td>Renal outer medullary potassium channel</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<td>---------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>SFA</td>
<td>Saturated fatty acid</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>TET</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRC</td>
<td>Taste receptor cell</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
</tr>
<tr>
<td>TRPM5</td>
<td>Transient receptor potential melastatin member 5</td>
</tr>
<tr>
<td>TRPM5+/+</td>
<td>Wild type</td>
</tr>
<tr>
<td>TRPM5−/−</td>
<td>TRPM5 knockout</td>
</tr>
<tr>
<td>US</td>
<td>Unconditioned stimulus</td>
</tr>
<tr>
<td>VGCC</td>
<td>Voltage-gated calcium channel</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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</table>
Rationale for the Proposed Research

Obesity has become one of the most common health problems worldwide and there has been a corresponding increase in cardiovascular disease, diabetes, cancer, and other nutrition-related disorders as a consequence of the obese state. Given that an increase in dietary fat intake is regarded as one of the factors closely linked with the obesity epidemic (Bray et al., 2004), it has become increasingly important to identify the mechanisms the body uses to recognize dietary fat. A review about the detection of fat (Gilbertson, 1998) stated that, besides the texture of the food, rodents and humans also recognize the presence of fat in foods by the gustatory mechanisms in the mouth, this suggests that an orosensory system may be responsible for the detection and preference of fat.

Currently, most of the studies on transduction mechanisms for fat (i.e. fatty acid) taste have focused on long-chain polyunsaturated fatty acids. Interestingly, in the course of our work in the laboratory, we have found that a variety of saturated medium-chain fatty acids (notably, lauric acid) were capable of activating taste cells. There is limited knowledge concerning the possible transduction mechanisms of medium-chain fatty acids (MCFAs) in taste cells. Expression data from our laboratory are consistent with taste cells containing a potential receptor for MCFAs, GPR84. GPR84 is a novel G protein-coupled receptor (GPCR) activated by MCFAs and is expressed predominantly in hematopoietic tissues. It has been suggested that GPR84 activation by MCFAs couples primarily to a pertussis toxin-sensitive Gi/o pathway (Wang et al., 2006).
The basic hypothesis that we will test is that **taste cells can respond to MCFAs, and GPR84 is the primary receptor for MCFAs in the mammalian taste system**. My dissertation research focused on the cellular and behavioral responses to MCFAs in mice, and to determine the role of GPR84 in the detection of medium-chain fatty acid by taste cells, we investigated their expression and function in mouse taste cells and compared the preference to MCFAs in wild-type mice and in mice that lack GPR84 (GPR84 knockout mice).
Overview of Obesity

The worldwide prevalence of obesity has nearly doubled during the past three decades (Finucane et al., 2011). According to the National Health and Nutrition Examination Survey, in 2011–2014, the rate of obesity was over 36% in adults and 17% in children (Ogden et al., 2015), and more than two-thirds of adults were overweight or obese from 2010 to 2011 (Ogden et al., 2014). According to Centers for Disease Control and Prevention, all the states had a prevalence of obesity above 20%, and 22 states had a prevalence of obesity over 30% in 2014 (Figure 1.1, (CDC, 2014)). Obesity alone is not a serious disease, however, it can increase the risk of a number of other chronic diseases among adults, including heart disease (Lavie et al., 2009, Logue et al., 2011), diabetes (Meyer et al., 2011, Sharma and Lau, 2013), high blood pressure (Wang et al., 2010, Finucane et al., 2011), nonalcoholic fatty liver disease (Tiniakos et al., 2010), osteoarthritis (Lementowski and Zelicof, 2008), stroke (Kurth et al., 2002), and even some types of cancer, such as breast, colon, endometrial, and kidney cancer (Hursting et al., 2007, Lavie et al., 2009, Crosbie et al., 2010, Nguyen and El-Serag, 2010, McGuire and Fitzpatrick, 2011, Flores et al., 2012, Chan et al., 2014, Kaaks and Kuhn, 2014, Renehan et al., 2015).

Treating obesity and obesity-related diseases is one of the largest costs in the United States. Currently, an estimate of this cost range from $147 billion to nearly $210 billion per year (Finkelstein et al., 2009, Cawley and Meyerhoefer, 2012). The fundamental cause of obesity is energy imbalance, and the primary factors that contribute to energy imbalance is excessive food energy intake and inadequate energy expenditure (Keith et al., 2006). Overconsumption of dietary fat is regarded as one of the factors closely linked with the
obesity epidemic (Bray et al., 2004).

Saturated Fatty Acids and Health

Saturated fatty acids (SFAs) are those whose carbon atoms are saturated with hydrogen atoms, and they are very common in the daily food supply. Dairy products, fatty meats, palm oil, and coconut oil are primary sources of saturated fatty acids. In addition, they can also be found in processed foods including sweets and snacks, such as cookies, cakes, pastries, crisps, chocolate, and so on (Funaki, 2009). Overconsumption of saturated fats poses a greater health risk than polyunsaturated fats. First, dietary SFAs, such as capric acid, lauric acid, and myristic acid, are capable of causing insulin resistance and inflammatory responses in white adipose tissue (Funaki, 2009, van Dijk et al., 2009), which are closely related to obesity (Pereira-Lancha et al., 2012). Overloading of saturated fat in adipocytes can induce degradation of insulin receptor substrate-1 (Gao et al., 2004), increase reactive oxygen species production (Lin et al., 2005), and activate Toll-like receptor 4 (Song et al., 2006, Wang et al., 2012), all of which are able to impair insulin sensitivity and stimulate adipose tissues to secrete proinflammatory factors. Saturated fat was also able to stimulate more weight gain and hepatic lipid accumulation than unsaturated fat (de Wit et al., 2012). Genetically, several studies showed that SFA intake might also increase the obesity-associated genetic risk in adults (Corella et al., 2011, Phillips et al., 2012, Casas-Agustench et al., 2014). In addition, the processes of obesity-related disease were also influenced by saturated fatty acid intake, such as non-alcoholic fatty liver disease, which could be attenuated by replacing dietary saturated fats with
polyunsaturated fats (Enos et al., 2014, Morrison et al., 2015). Besides obesity, human epidemiological studies showed that higher saturated fat intake led to an increased risk of sudden cardiac death (Jouven et al., 2001, Chiuve et al., 2012). Consumption of high levels of SFAs can also increase total cholesterol concentrations as well as low-density lipoprotein cholesterol levels (Grundy and Denke, 1990), which in turn may contribute to cardiovascular disease (Lloyd-Jones et al., 2010). Some previous studies indicated that a switch from SFAs to polyunsaturated fatty acids significantly decreased the risk of cardiovascular disease (Leren, 1970, Turpenien et al., 1979, Siri-Tarino et al., 2010, Morrison et al., 2015). Therefore, reducing the consumption of saturated fatty acids to a level as low as possible is a European Public Health recommendation to reduce the risk of cardiovascular disease. Taken together, overconsumption of SFA poses a threat to health, and understanding the mechanisms of SFA detection in the oral cavity can help us identify potential strategies to reduce SFA intake, such as designing fat substitutes, and, in turn, help create a healthier diet.

Taste Transduction

How exactly are different taste substances being recognized? To understand these mechanisms, we must first understand a very important mediator – the taste bud. Taste buds are clusters of 50 ~ 100 taste cells distributed throughout the oral cavity. They are onion-shaped structures and primarily located in the three taste papillae on the surface of mammalian tongues, fungiform, foliate, and circumvallate (Figure 1.2, Calvo and Egan, 2015)). The apical side of the taste buds is exposed to saliva and the basal side is innervated
with afferent neuron fibers including chorda tympani (CT) and glossopharyngeal (GL) nerves (cranial nerves VII and IX, respectively, Figure 1.3, (Purves et al., 2001)). The taste information is finally delivered to the gustatory cortex through gustatory nucleus of the solitary tract complex (2001). Based on their respective functions and gene expression profiles, taste cells are normally classified into three cell types, Type I, II, and III cells, and the taste information delivery requires a dialogue among different types of taste cells (Figure 1.4 (Calvo and Egan, 2015)).

Type I cells are glial-like cells and they are the most numerous cells in taste buds. Analogous to the function of glial cells in the nervous system, Type I cells provide support to other cells, and they can also regulate the molecular environment of the taste buds, such as neurotransmitter and ion concentrations, to insure the normal function of the taste transduction process. For example, Type I cells express glutamate/aspartate transporters (GLASTs) (Lawton et al., 2000) and nucleoside triphosphate diphosphohydrolase 2 (NTPDase2) (Bartel et al., 2006), which are able to take up glutamate into the cells and degrade extracellular 5′-triphosphate, respectively. In addition, they also express an inward rectifier channel, renal outer medullary potassium channel (ROMK), which is responsible for removal of accumulating extracellular K+ after action potentials (Dvoryanchikov et al., 2009). Previous studies also showed that Type I taste cells were involved directly in salty taste transduction (Vandenbeuch et al., 2008) via the epithelial sodium channels (ENaCs), which mediated low concentration of NaCl (attractive salt) taste transduction (Chandrashekar et al., 2010).

Type II cells, which are also called taste receptor cells (TRCs), are the primary
sensors for tastants. Type II cells express a variety of receptors in order to recognize different taste qualities, and all of the taste receptors discovered so far are G protein-coupled receptors (GPCRs). The T1R family of taste receptors is responsible for sensing sweet and umami tastants. The heterodimeric combination of T1R2 and T1R3 functions as a sweet receptor, and it can recognize natural sweet substances such as sucrose, fructose, glucose, and some artificial sweeteners including saccharin and sucralose (Max et al., 2001, Nelson et al., 2001, Xu et al., 2004). Heterodimeric complex of T1R1 and T1R3 subunits is an important umami receptor and it can sense L-glutamate and other L-amino acids. Behavioral and electrophysiological studies showed that mice lacking T1R1 or T1R2 lose their response to umami or sweet tastants, respectively, and T1R3 knock out mice diminished responses for both sweet and umami stimuli (Damak et al., 2003, Zhao et al., 2003). The T2R family functions as sensing bitter tastants, such as quinine, denatonium benzoate, and caffeine (Chandrashekar et al., 2000, Bufe et al., 2002, Meyerhof et al., 2010). TRCs also express voltage-gated Na⁺ and K⁺ channels in order to fire action potentials and allow release of neurotransmitters. One of the most important neurotransmitters, ATP, is released from Type II taste cells, and interacts with ionotropic ATP receptors (P₂X₂ and P₂X₃) on sensory nerve fibers or neighboring Type III taste cells. The mice lacking P₂X₂ and P₂X₃ showed eliminated taste nerve responses and exhibited greatly reduced taste sensitivities to sweet, umami and bitter tastants (Finger et al., 2005). A voltage-gated ATP channel, calcium homeostasis modulator 1 (CALHM1), was found recently that is essential for taste transduction. CALHM1 is expressed exclusively in Type II taste cells, and knocking out of CALHM1 eliminated ATP release from Type II taste cells. Also, mice
lacking CALHM1 have significantly reduced taste sensitivities and gustatory nerve responses to sweet, bitter, and umami tastants (Taruno et al., 2013a, Taruno et al., 2013b).

Type III cells are believed to be presynaptic cells because they can synapse with afferent nerve terminals (Yee et al., 2001) and express many synapse-related proteins, including synaptosomal-associated protein 25, voltage-gated Ca$^{2+}$ channel, synapsin-II, γ-aminobutyric acid (GABA), and the neurotransmitter-synthesizing enzymes (DeFazio et al., 2006, Dvoryanchikov et al., 2011). The main function of Type III cells is to integrate the input signals generated by TRCs, and this is also the reason why Type III cells can respond to all taste qualities although they don’t express any taste-specific receptors. In fact, Type III taste cells are not absolutely necessary for taste transduction. Mice lacking Type III cells can still respond to sweet, bitter, and umami tastes (Huang et al., 2006). An important neurotransmitter produced by Type III taste cells, serotonin (5-HT), is produced when the ATP released from TRCs interact with the ATP receptors on Type III cells (Huang et al., 2007, Huang et al., 2009). Recent studies have showed that instead of functioning in neurotransmission, 5-HT was crucial for regulating the ATP release from 5-HT receptor-expressed Type II taste cells (Jaber et al., 2014). In addition, Type III cells can respond to sour taste directly partly because they express polycystic kidney disease 2-like 1 protein (PKD2L1) and polycystic kidney disease 1-like 3 protein (PKD1L3) channels (LopezJimenez et al., 2006, Huang et al., 2008), and mice lacking PKD2L1 showed reduced taste sensitivities to sour tastants (Horio et al., 2011).

Type IV (basal) cells are precursor cells that contribute to taste cell renewal, and they are able to differentiate into the mature Type I-III taste cells (Barlow and Klein, 2015).
In general, there is a well-described pathway for the transduction of most taste stimuli. Initially, taste compounds have to diffuse and interact with their corresponding receptors on the TRCs. This interaction then leads to a series of downstream signaling events, which allow $\text{Ca}^{2+}$ release into cytosol of TRCs. $\text{Ca}^{2+}$ can then interact with and open some $\text{Ca}^{2+}$-dependent ion channels, which in turn depolarizes the taste cell membrane and leads to the generation of action potentials. Membrane depolarization activates voltage-gated $\text{Ca}^{2+}$ channels on the cell membrane, and allows extracellular $\text{Ca}^{2+}$ influx into the cytosol, which then binds to synaptic proteins. Eventually, neurotransmitters are released from the synaptic vehicles in TRCs and bind to gustatory afferent nerve fibers directly or indirectly through adjacent Type III cells.

There are two models of peripheral coding of taste: labeled-line model and across-fiber model. In the labeled-line models, one distinct taste quality is encoded by one population of taste receptor cell expressing unique taste receptors, and then only one set of dedicated afferent nerve fibers (Barretto et al., 2015). While in the across-fiber model, taste receptor cells and gustatory afferent nerves are broadly tuned to respond to more than one taste quality (Carleton et al., 2010, Liman et al., 2014).

Taste of Fat

Sweet, sour, bitter, salty, and umami are five basic taste qualities that are well known and have been studied for decades. But how was “fatty” first considered as a candidate taste quality? This was actually based on the function of fat because one of the most important functions of “taste” is that it should be able to detect nutritional or
physiological requirements. For example, sweet represents sensing of carbohydrates that serve as an energy source. Similar with sweet, fat is also a very important energy source and it can be found abundantly in human diet. Moreover, fat can contribute to the palatability of foods as evidenced by the attraction to foods containing high levels of fat, such as ice cream, donuts, cheese and so on. Therefore, over a decade ago, scientists started to hypothesize that fatty may represent a sixth taste quality.

In order to effectively investigate if the dietary fat can be detected by the gustatory system, we must first identify the substance being recognized within the oral cavity after eating fat. Ingested fat is typically in the form of triglycerides, three fatty acids bound to a glycerol backbone, and it can be degraded into free fatty acids within 1-5 s in the mouth of rat by lingual lipase, which is secreted from von Ebner’s glands around circumvallate and foliate papillae in the tongue. The role of lingual lipase has been shown by experiments that have investigated the action of lingual lipase inhibitors, which can inhibit free fatty acid production in mouth. Inhibition of lingual lipase significantly reduced the preference for triglycerides but not for free fatty acids in rat (Kawai and Fushiki, 2003). Similar results were found in humans, but with different types of lipases in human oral cavity (Voigt et al., 2014). A recent study investigated the salivary nonesterified fatty acids after the subjects chewed high fat food for 1 min, and the concentrations of free fatty acids (palmitic, oleic, linoleic, and stearic acids) increased significantly, and these concentrations were sufficient to stimulate taste cell response in mice (Kulkarni and Mattes, 2013). Taken together, free fatty acid appears to be the primary stimulus contributing to the fat taste.

Numerous reports have demonstrated the hedonic preference for fat in humans and
rodents. Several studies proved that human preference for foods strongly depended on the relative proportions of fat in the samples tasted (Drewnowski and Greenwood, 1983, Mela and Sacchetti, 1991, Drewnowski, 1997), and it has been proven that mice and rats showed a robust preference for high-fat foods (Greenberg and Smith, 1996, Imaizumi et al., 2000, Takeda et al., 2000). Until quite recently, researchers believed post-ingestive cues (Lucas et al., 1998), aroma (Ramirez, 1993, Kinney and Antill, 1996), and texture (Rolls et al., 2003, Kadohisa et al., 2005) rather than taste, were the only factors contributing to sensory perception of fat. During the last decade, however, accumulating behavioral research has shown that humans and rodents were still able to recognize the presence of fat in foods after eliminating these three factors.

Sham feeding paradigms allow the elimination of post-ingestive cues, and by using this method, Mindell et al. found that sham-fed rats showed a strong preference for corn oil over mineral oil in 2-bottle preference tests, which suggests the orosensory effects of fat detection (Mindell et al., 1990). Post-ingestive effects can also be excluded by short-term (5 min) preference tests because these occur only after ~13 min following ingestion of fat (Ackroff et al., 1990). Olfactory effects can be removed by intranasal application of zinc sulfate to generate anosmic animals. Takeda and colleague showed that although the threshold for detection of fatty acids was higher, anosmic mice can still recognize and prefer corn oil (Takeda et al., 2001). The texture of fatty acids can also be masked by suspending all the test stimuli in xanthan gum solution. Tsuruta and colleagues showed that rats prefer long-chain fatty acid fluids to vehicles in short-term two-bottle preference test after the texture was masked with xanthan gum solution. Additionally, rats had different
preferences for different fatty acids, which suggested that rats can discriminate different kinds of fatty acids (Tsuruta et al., 1999). Further, the texture of most free fatty acids at their physiologically relevant concentrations is very similar to water. McCormack and colleagues measured the viscosity of 88 μM solution of free fatty acids and showed that there was a negligible difference in viscosity between fatty acids and water (1.5% difference) (McCormack et al., 2006). One of the first studies to prove the role of gustation for fatty acids after minimizing all the effects of olfaction, texture and postingestive was conducted by Fukuwatari and colleagues. They used anosmic rats to eliminate olfaction, suspended all the test substances in 0.3% xanthan gum to mask the texture, and performed 5 min preference tests to eliminate the postingestive influences. The results showed that rats still preferred oleic acid to the control, which showed convincingly the gustatory recognition of fatty acids (Fukuwatari et al., 2003). Besides preference tests, conditioned taste aversion is another method to test the ability of rodents to taste the fatty acids (Garcia et al., 1985). Several studies showed that both rats and mice could be conditioned to avoid free fatty acids in the low micromolar range (McCormack et al., 2006, Gaillard et al., 2008).

Accumulating evidence from human studies also indicates the gustatory perception of fatty acids. The taste of fatty acids was first identified in humans by Schiffman and Dackis in 1975, which demonstrated that people could describe the taste of fatty acid solutions (Schiffman and Dackis, 1975). However, in this test, olfaction and texture were not excluded. About 20 years later, human behavioral tests were performed again. Detection thresholds for fatty acids were typically determined using an 3-alternative, forced-choice, ascending (3-AFC), sip and spit method (Mattes, 2009). The participants are asked
to discriminate three samples including two control samples and one stimulus sample starting from the lowest concentration. If a correct identification is made, the same concentration will be given again, and if an incorrect identification is made, the next higher concentration will be given. Threshold is the lowest concentration that is correctly identified for 3 consecutive times. One study using young and elderly subjects with nose clips showed that there were measurable thresholds for fatty acid detection and young subjects had lower thresholds (Schiffman et al., 1998), which suggested orosensory system was responsible for the detection of fat. Nasser and colleagues showed that 6-N-2-propylthiouracil (PROP) tasters could discriminate linoleic acid added to high-fat ice cream (Nasser et al., 2001). Similarly, linoleic acid tasters and non-tasters, who were grouped by correct identification of a 10 µM linoleic acid solution, were used to discriminate fatty acids with different carbon lengths, and linoleic acid tasters were able to discriminate between linoleic acid and oleic acid added ice cream (Kamphuis et al., 2003). The contribution of taste for fatty acids on humans was first isolated by Chale-Rush and colleagues, where the texture was masked by gum acacia solution and the odor cue was eliminated by blocking the nares (Chale-Rush et al., 2007a, b). After other sensory systems were minimized, the detection threshold for linoleic, oleic, and stearic acids were still measurable, suggesting fatty acids can be detected by gustatory system. Recent studies on humans showed further that lauric and caproic acids had detectable oral detection thresholds (Mattes, 2009), and humans had different oral sensitivities and sensations for fatty acids with different carbon lengths (Running and Mattes, 2014, Running et al., 2015).

Taken together, the studies mentioned above suggested rodents and humans are
capable of detecting free fatty acids independently even when olfaction, texture and postingestive factors were eliminated, which supports the conclusion that free fatty acids are effective taste stimuli.

Fatty acid taste transduction

Evidence that “fatty” constitutes a basic taste quality has been supported recently by cell-based assays, and more and more studies have focused on identifying the mechanisms of fatty acid taste transduction. Here are the main components proven to be involved in these mechanisms.

DRK channels

Delayed rectifying $\text{K}^+$ (DRK) channels are the first discovered ion channels that are expressed in taste cells and involved in fat taste transduction. Gilbertson and colleagues were the first to show that extracellular application of $\text{cis}$-polyunsaturated fatty acids was able to activate native taste cells from rats by inhibiting DRK channels, but no significant effect on $\text{K}^+$ channels were found when saturated, monounsaturated, and trans-polyunsaturated fatty acids were applied (Gilbertson et al., 1997). Because DRK channels are responsible for repolarization during an action potential, inhibiting DRK channels by fatty acids can prolong depolarization phase of an action potential and, in turn, alter (i.e. enhance) taste cell activity (Gilbertson et al., 1997). Among the major DRK channels expressed on taste buds, including $\text{Kv1.3}$, $\text{Kv1.5}$, $\text{Kv2.2}$, $\text{Kv3.1}$, and $\text{Kv3.2}$, $\text{Kv1.5}$ is the most highly expressed in rat taste cells. It is very sensitive to polyunsaturated fatty acids and electrophysiological and pharmacological assays have indicated that $\text{Kv1.5}$ is the
major DRK channel that contributes to the DRK, and, hence fatty acid-sensitive, current (Liu et al., 2005).

**CD36**

Another protein responsible for taste detection of fatty acids is CD36 (cluster of differentiation 36), which is a transmembrane protein that can bind a variety of lipids including long-chain fatty acids (Baillie et al., 1996). It is able to transport fatty acids across the cell membrane, which is the reason that CD36 is also called fatty acid translocase (FAT) (Zhang et al., 2003). CD36 is expressed in lingual gustatory papillae in mouse, rat, and human, especially in the circumvallate papillae (Fukuwatari et al., 1997, Laugerette et al., 2005, Simons et al., 2011). The role of CD36 in fatty acid taste transduction has been actively studied in recent years. Laugerette *et al.* compared the preference for the diet containing long-chain fatty acids (LCFAs) in wild type and CD36 knockout mice, and the results showed that the CD36 knockout mice lost their spontaneous preference for a LCFA-enriched diet (Laugerette et al., 2005). Similar results were found in some later studies (Sclafani *et al.*, 2007, Chen *et al.*, 2013), suggesting that CD36 plays an important role in fatty acid taste transduction. However, CD36 is not essential in this process. Previous research showed that although the preference of CD36 knockout mice for high fat (HF) diet was abolished when the HF diet contained low concentrations of fat, but it came back at high concentration of fat (2.5%-20%) (Chen *et al.*, 2013). Also, Ca$^{2+}$ responses were eliminated in CD36-null human and mouse taste cells with low concentrations of linoleic acid, but not with high concentration of the same stimulus (Ozdener *et al.*, 2014). With more and more studies on the primary LCFA receptor GPR120, there is a theory proposed
that CD36 may function as a chaperone protein that facilitates free fatty acids to bind GPR120, which then activates downstream taste transduction. The data in our laboratory showed that knocking down of CD36 reduced but not abolished the Ca\(^{2+}\) responses to linoleic acid in human taste cell line, and CD36 knockout mice showed lower but not eliminated taste sensitivities to linoleic acid, suggesting that CD36 is not required but may facilitate the taste transduction of LCFAs.

**G Protein-Coupled Receptors**

Another type of protein that is involved in fatty acid taste transduction is a subclass of G protein-coupled receptors (GPCR). Several GPCRs have been suggested to be activated by free fatty acids, including GPR120, GPR40, GPR41, GPR43, and GPR84. The ligands for these receptors are fatty acids with different carbon lengths, which are generally classified as long-, medium-, and short-chain fatty acids (Gilbertson et al., 2010). For long chain, polyunsaturated fatty acids, the common point for initiation of the fatty acid-induced receptor potential is transient receptor potential channel type M5 (TRPM5). TRPM5 is another important ion channel involved in fatty acid taste transduction. In a recent study of our laboratory, knocking out of TRPM5 reduced the taste cell response to linoleic acid, and TRPM5 knockout mice showed no preference for and diminished taste sensitivities to linoleic acid (Liu et al., 2011). Our laboratory and others have already confirmed the expression of GPR120, GPR40, GPR41/43 and GPR84 in taste cells by real time PCR, which suggested the possible roles of these receptors in taste transduction of fatty acids.

**GPR120**

GPR120 is the receptor of saturated and unsaturated long-chain fatty acids
(Hirasawa et al., 2005) and it is expressed in a variety of tissues. Hirasawa et al. initially identified GPR120 in the intestinal tract and also mouse intestinal endocrine cell line STC-1. They suggested that GPR120 could promote unsaturated long-chain fatty acid-induced glucagon-like peptide-1 (GLP-1) secretion (Hirasawa et al., 2005). Later, Tanaka et al. proved that GPR120 in the intestine could also mediate fatty acid-induced gut peptide hormone cholecystokinin (CCK) production (Tanaka et al., 2008). Our laboratory confirmed that GPR120 was involved in CCK release from line STC-1 cells (Shah et al., 2012), and both of GLP-1 and CCK can elicit satiety and inhibits food intake (Smith and Gibbs, 1975, Gutzwiller et al., 1999). Besides intestinal cells, GPR120 is also highly expressed in adipose tissue and proinflammatory macrophages, and Oh et al. suggested that GPR120 could be activated by omega-3 fatty acids and, in turn, caused anti-inflammatory effects (Oh et al., 2010). The role of GPR120 in taste transduction has been studied recently. Matsumura et al. showed that GPR120 was mainly expressed in the Type II taste cells (Matsumura et al., 2009), which was further confirmed by a later study (Cartoni et al., 2010). The GPR120 protein was also detected in human gustatory epithelia by immunohistochemical experiments (Galindo et al., 2012). Two bottle taste preference test showed that knocking out of GPR120 significantly reduced the preference for linoleic acid in mice (Cartoni et al., 2010), suggesting a role of GPR120 in the taste transduction of long-chain fatty acid. The downstream pathway after GPR120 activation in taste system is still not entirely clear. A recent study showed that a GPR120 agonist induced a significant rise of GLP-1 in mouse taste cells, which indicated that similar to the intestine, GPR120 activation by LCFA can also induce the release of GLP-1 in gustatory system (Martin et
GPR40

GPR40 belongs to the GPR40 family, which also includes GPR41 and GPR43. By using a ligand fishing strategy, the ligands for GPR40 were determined to be medium- and long-chain saturated and unsaturated free fatty acids (Briscoe et al., 2003, Itoh et al., 2003, Kotarsky et al., 2003). GPR40 is enriched in pancreatic β-cells as well as some pancreatic β-cell lines, and long-chain fatty acids can stimulate and modulate insulin secretion in a GPR40-dependent manner (Itoh et al., 2003). Additionally, GPR40 is also expressed in endocrine cells in the gastrointestinal tract where it can modulate incretin secretion and therefore regulates insulin secretion stimulated by free fatty acids (Edfalk et al., 2008). The expression of GPR40 in taste cells was also reported recently. It has been suggested that GPR40 is expressed mainly in Type I taste cells, and knocking out GPR40 decreased the taste nerve responses to several medium- and long-chain fatty acids. GPR40 knockout (KO) mice were also used in taste preference test, and it turned out that GPR40 KO mice showed lower, but not absent, preference for linoleic acid and oleic acid than wild type mice, indicating that GPR40 contributes to the taste of fatty acids (Cartoni et al., 2010). The downstream signaling pathway of GPR40 remains to be determined, but it may couple with G\textsubscript{aq} and G\textsubscript{ai} family.

GPR41 and GPR43

GPR41 and GPR43 are another two family members in the GPR40 family and they are closely related to each other. Also by adopting a ligand fishing strategy, GPR41 and GPR43 are believed to be activated by short-chain fatty acids, although their ligands have
different carbon chain lengths (Brown et al., 2003, Le Poul et al., 2003). GPR41 is expressed mainly in adipose tissue and knocking down of GPR41 significantly reduced the leptin gene expression (Brown et al., 2003, Xiong et al., 2004). GPR41 is also found in enteroendocrine cells in the gut epithelium and ascending colon, and may function as the sensor of short-chain fatty acid and eventually regulates host energy balance (Samuel et al., 2008, Tazoe et al., 2008, Tazoe et al., 2009). Another function of GPR41 was discovered recently, that is GPR41 mediates regulation of sympathetic nervous system by short-chain fatty acids (Kimura et al., 2011).

GPR43 is highly expressed in leukocytes, and it may play a role in leukocyte differentiation because GPR43 expression was greatly induced in leukemia cells during differentiation to monocytes (Senga et al., 2003). GPR43 is also expressed in intestine and colon and may be involved in intestine and colon function (Karaki et al., 2006, Tazoe et al., 2008, Tazoe et al., 2009). Furthermore, GPR43 knockout mice had lower body fat composition, improved glucose control, and lower plasma lipid under high-fat diet, suggesting that GPR43 play a very important role in energy and lipid metabolism (Bjursell et al., 2011). A recently published article showed that GPR43 could mediate short-chain fatty acid induced GLP-1 secretion (Tolhurst et al., 2012). The downstream signaling pathways of both GPR41 and GPR43 remain to be determined. As far as we know, GPR41 coupled with G_{i/o}, whereas GPR43 coupled through G_{q} and G_{i/o}. Their activation can then increase intracellular Ca^{2+} concentration and decrease intracellular cyclic adenosine monophosphate (Brown et al., 2003, Le Poul et al., 2003). The data from my laboratory showed that GPR41 and GPR43 were both expressed in taste cells (unpublished data).
GPR84

GPR84 is a member of the rhodopsin superfamily of GPCRs (Joost and Methner, 2002), and was first discovered by using an expressed sequence tag data mining strategy in 2001 (Wittenberger et al., 2001). In the same year, GPR84 was also identified from human peripheral blood neutrophils by a degenerate-primer RT-PCR method and was suggested to be expressed mainly in bone marrow, lung, and peripheral blood leukocytes (Yousefi et al., 2001). The human and murine gpr84 genes, which have an open reading frame of 1191 bp, both encode GPR84 protein of 396 amino acids and share 85% identity (Wittenberger et al., 2001).

The nature of the ligands that act on GPR84 was never reported until 2006, when Wang and his coworkers investigated the potency of various fatty acids at GPR84 using [^35S]GTPγS binding assays and finally demonstrated that GPR84 was activated by medium-chain saturated fatty acids (MCFAs) of C₉ to C₁₄ in length, and the most potent ligands for GPR84 are capric (C₁₀), undecanoic (C₁₁), and lauric acids (C₁₂) (Wang et al., 2006). A later study showed that 2- and 3-hydroxy MCFAs were more effective agonists for GPR84 than nonhydroxylated MCFAs (Suzuki et al., 2013). The signaling transduction pathway following GPR84 activation still remains to be determined. It has been suggested that GPR84 activation by MCFAs coupled primarily to a pertussis toxin-sensitive Gᵢ/o pathway, in which MCFAs stimulation led to calcium mobilization, 3’,5’-cyclic AMP inhibition, and [^35S]GTPγS binding (Wang et al., 2006).

The function of GPR84 is not well understood. A previous study showed that in GPR84 knockout mice, activated T cells expressed higher level of interleukin-4 (IL-4) than
wild type mice, suggesting a role for GPR84 in the immune system (Venkataraman and Kuo, 2005). Additionally, GPR84 activation can induce the secretion of interleukin-12 (IL-12) p40 in the lipopolysaccharide (LPS)-stimulated monocyte/macrophage cell line, and in the same cell line, the mRNA level of GPR84 was strongly up-regulated under LPS activation, which indicated the possible role of GPR84 in monocyte/macrophage activation (Wang et al., 2006, Lattin et al., 2008). GPR84 is also a proinflammatory receptor. One recent study showed that GPR84 was up-regulated in adipocytes co-cultured with macrophage cells or TNFα, and MCFAs suppressed adiponectin mRNA expression via GPR84 in TNFα-primed adipocytes (Nagasaki et al., 2012). Another study showed that GPR84 activation increased the release of IL-8 from polymorphonuclear leukocytes and TNFα from macrophages (Suzuki et al., 2013). GPR84 may also play an important role in neuroimmunological processes. In the central nervous system, the expression of GPR84 is up-regulated in microglia during endotoxemia, and also in monocytic cells upon LPS stimulation (Bouchard et al., 2007).

Putative Transduction Pathway for Long-Chain Unsaturated Fatty Acids in Taste Cells

We have elucidated putative the taste transduction pathway for long-chain unsaturated fatty acids (LCFAs) (Figure 1.5), and this pathway remains an important frame of reference for the possible GPR84-mediated MCFA taste transduction pathway. LCFAs are transported by CD36 to its cognate GPCR (e.g. GPR120) or interact with the GPCR directly to initiate the signaling pathway. The G-protein that coupled with the GPCR is then activated, and the βγ subunits of the activated G-protein dissociate and stimulate
phospholipase C β2 (PLCβ2). PLCβ2 cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), both of which are second messengers. IP3 diffuses and binds to IP3 receptor 3 (IP3R3) located on the endoplasmic reticulum (ER) membrane. IP3R3 is also an IP3-dependent calcium channel, and IP3R3 activation leads to a release of Ca2+ from the ER calcium store to cytosol. Ca2+ then binds to a cation channel, TRPM5, on the cell membrane, causing extracellular Na+ influx into the cytosol and, in turn, depolarization of the taste cells. DRK channels are activated and opened by the depolarized membrane potential, but fatty acid-sensitive DRK channels are inhibited by free polyunsaturated fatty acids, which are able to prolong the depolarization phase of an action potential. A large enough depolarization opens voltage-gated Ca2+ channels (VGCCs) on the cell membrane and allows extracellular Ca2+ influx into the cytosol. The combination of cell depolarization and elevated intracellular Ca2+ level then triggers the release of neurotransmitters.

Hypothesized GPR84-Mediated MCFA Taste Transduction Pathway

As discussed above, in leukocytes, GPR84 activation by MCFAs couples to a pertussis toxin-sensitive G\textsubscript{i/o} pathway, where it caused calcium mobilization and 3′,5′-cyclic AMP (cAMP) inhibition. Since α-gustducin (McLaughlin et al., 1992), which is abundantly expressed in taste cells, is the α-subunit of a G-protein that belongs to the G\textsubscript{i/o} family (McLaughlin et al., 1992), it’s reasonable to hypothesize that α-gustducin is responsible for this GPR84-mediated MCFA signaling pathway in taste cells. The function of α-gustducin in taste transduction is not entirely clear. However, it has been proposed
that α-gustducin was coexpressed with T1R and T2R receptors (Kim et al., 2003, Stone et al., 2007), which are receptors for sweet, umami, and bitter taste. Together with the fact that knocking out of α-gustducin significantly compromised these three tastes (Ruiz et al., 2003, Glendinning et al., 2005), it is believed that α-gustducin might play a key role in sweet, umami, and bitter taste transduction pathway. α-Gustducin is able to activate phosphodiesterase (PDE) and in turn decrease intracellular concentration of cAMP. In taste cells, bitter and umami stimuli were both able to induce a reduction of cAMP level, and for bitter, it was α-gustducin dependent (Yan et al., 2001, Abaffy et al., 2003). The role of the α-gustducin-induced decrease in cAMP during taste transduction is still not quite clear, and limited research has been done in this field. However, it has been proposed that cAMP was able to suppress an inward whole-cell current in taste cells, indicating that there is a kind of cAMP suppressible cation channel in taste cells, and decrease of cAMP level can activate these channels and depolarize the taste cells (Kolesnikov and Margolskee, 1995). Another function of cAMP is to activate protein kinase A (PKA) (Meinkoth et al., 1993), which is able to inhibit PLCβ2 (Liu and Simon, 1996) and IP3R3 (Soulsby and Wojcikiewicz, 2007). Therefore, decreased cAMP concentration can cause the decreased activity of PKA, which will relieve its inhibition to PLCβ2 and IP3R3. Then, similar with the polyunsaturated fatty acids taste transduction pathway, IP3 and DAG can be generated and Ca\(^{2+}\) can be released from ER store. Ca\(^{2+}\) and DAG together have been shown to activate some isoforms of protein kinase C (PKC) (Nishizuka, 1995), which is able to phosphorylate and close K\(^{+}\) channels on the membrane (Varkevisser and Kinnamon, 2000), and, in turn, depolarize the taste cells, open VGCCs, and increase intracellular Ca\(^{2+}\) levels (Figure 1.6). The partner of
α-gustducin, β3γ13 (Huang et al., 1999), may function as the βγ subunit involved in the LCFAs taste transduction pathway discussed above. Alternatively, it remains possible that the GPR84 pathway is similar to that seen with LCFAs.

References


Casas-Agustench P, Arnett DK, Smith CE, Lai CQ, Parnell LD, Borecki IB, Frazier-Wood


Chen CSY, Bench EM, Allerton TD, Schreiber AL, Arceneaux KP, Primeaux SD (2013)
Preference for linoleic acid in obesity-prone and obesity-resistant rats is attenuated by the reduction of CD36 on the tongue. Am J Physiol-Reg I 305:R1346-R1355.


DeFazio RA, Dvoryanchikov G, Maruyama Y, Kim JW, Pereira E, Roper SD, Chaudhari N (2006) Separate populations of receptor cells and presynaptic cells in mouse taste


regional, and global trends in body-mass index since 1980: systematic analysis of
health examination surveys and epidemiological studies with 960 country-years
Flores MB, Rocha GZ, Damas-Souza DM, Osorio-Costa F, Dias MM, Ropelle ER,
Camargo JA, de Carvalho RB, Carvalho HF, Saad MJ, Carvalheira JB (2012)
Obesity-induced increase in tumor necrosis factor-alpha leads to development of
colon cancer in mice. Gastroenterology 143:741-753 e741-e744.
Expression of the putative membrane fatty acid transporter (FAT) in taste buds of
Role of gustation in the recognition of oleate and triolein in anosmic rats. Physiol
Behav 78:579-583.
NA, Montmayeur JP, Besnard P (2008) The gustatory pathway is involved in CD36-
mediated orosensory perception of long-chain fatty acids in the mouse. Faseb J
22:1458-1468.
Galindo MM, Voigt N, Stein J, van Lengerich J, Raguse JD, Hofmann T, Meyerhof W,
Senses 37:123-139.


Keith SW, Redden DT, Katzmarzyk PT, Boggiano MM, Hanlon EC, Benca RM, Ruden D,


FASEB J 9:484-496.


Ramirez I (1993) Role of olfaction in starch and oil preference. American Journal of
Renehan AG, MacKintosh ML, Crosbie EJ (2015) Obesity and endometrial cancer: unanswered epidemiological questions. BJOG.


Soulsby MD, Wojcikiewicz RJ (2007) Calcium mobilization via type III inositol 1,4,5-trisphosphate receptors is not altered by PKA-mediated phosphorylation of serines
916, 934, and 1832. Cell Calcium 42:261-270.


Figure 1.1. Prevalence of self-reported obesity among U.S. adults by state and territory, Behavioral Risk Factor Surveillance System, 2014.
Figure 1.2. Localization of taste papillae on human tongue. Fungiform are spread on the middle and front of tongue; circumvallate are located on the back of tongue; foliate are on the sides of tongue (Calvo and Egan, 2015).
Figure 1.3. Ascending gustatory pathway. Chorda tympani (cranial nerves VII) and glossopharyngeal (cranial nerves IX) nerves innervate the taste buds from fungiform and circumvallate, respectively. They carry the taste signals to the gustatory nucleus of the solitary tract in medulla, the thalamus, and finally to the primary gustatory cortex (Purves et al., 2001).
Figure 1.4. Schematic representation of a taste bud and four types of taste cells. Type I cells are glial-like cells providing support to other cells and regulating molecular environment of the taste buds. They were also involved in salty taste transduction. Type II cells are primary sensors for tastants and express a variety of receptors to recognize different taste qualities. Type III cells are presynaptic cells that synapse with gustatory afferent nerves, and they also contribute to sour taste. Taste cell precursors (also known as Type IV cells) are responsible for taste cell renewal (Calvo and Egan, 2015).
Figure 1.5. Putative transduction pathway for long-chain unsaturated fatty acids (LCFAs) in taste cells. LCFAs are transported by CD36 or directly bind to their cognate GPCRs and activated the G-proteins. The $\beta\gamma$ subunits of the activated G-protein dissociate and stimulate PLC$\beta$2, which then cleave PIP$_2$ into DAG and IP$_3$. IP$_3$ then interact with IP$_3$R3 on the ER membrane and release Ca$^{2+}$ from the ER calcium store to cytosol. Ca$^{2+}$ activate TRPM5 and allow extracellular Na$^+$ influx into the cytosol, which leads to taste cell depolarization and followed by opening of DRK channels. The fatty acid-sensitive DRK channels are inhibited by free LCFAs, which prolongs the depolarization phase of an action potential and opens VGCCs. The combination of cell depolarization and elevated intracellular Ca$^{2+}$ level then triggers the release of neurotransmitters.
Figure 1.6. Hypothesized GPR84-mediated MCFA taste transduction pathway. MCFAs bind to GPR84 and activate gustducin. The $\beta_3\gamma_{13}$ subunits work the same as shown in the LCFA taste transduction pathway (Figure 1.5), however, the inherent activity of PKA can inhibit PLC$\beta_2$ and IP$_3$R3. $\alpha$-gustducin activates PDE and in turn cleaves the cAMP to AMP. Decreased cAMP then inhibits the activity of PKA, which relieves its inhibition to PLC$\beta_2$ and IP$_3$R3, and finally the Ca$^{2+}$ are released from ER store. Ca$^{2+}$ and DAG work together to activate PKC, which can phosphorylate and close DRK channels, and, in turn, depolarize the taste cells and trigger neurotransmitter release. Decreased cAMP can also release its inhibition to the cAMP suppressible cation channel and depolarize the taste cells.
CHAPTER II

GUSTATORY MECHANISMS FOR MEDIUM-CHAIN FATTY ACID DETECTION IN MICE

Abstract

It has been almost twenty years since investigators started to study the gustatory recognition of fatty acids, and in the interim most of the studies in this area have focused on the long-chain unsaturated fatty acids. However, medium-chain saturated fatty acids (MCFA) are also an important form of dietary fatty acids, and there is limited knowledge concerning the gustatory responsiveness to and possible transduction mechanisms of MCFAs in taste cells. In this study, I showed that MCFAs are effective taste stimuli at both the cellular and behavioral levels. To elucidate the mechanisms of gustatory recognition of MCFAs in mice, antagonists of several important players that might be involved in this pathway were used in electrophysiological experiments. Capric acid-induced currents were significantly inhibited by GDP-β-S and the inhibitors of protein kinase A (PKA) and protein kinase C (PKC), which suggested that the currents were activated downstream of G-protein, PKA and PKC activation. However, these currents were not affected by the phospholipase C (PLC) and phosphodiesterase (PDE) inhibitors, suggesting they were not involved in the MCFA taste transduction pathway. Further, loss of transient receptor potential channel type M5 (TRPM5) did not affect either the lauric acid-induced intracellular Ca\textsuperscript{2+} change or taste sensitivity to lauric acid, indicating that MCFA transduction in mouse taste cells is TRPM5-independent. My results indicated that MCFAs
are sapid compounds that work through a pathway that is independent of the well-documented pathway for long-chain unsaturated fatty acids (Liu et al., 2011) and suggested increasing complexity to mechanisms underlying the ‘taste of fat’.

**Introduction**

Obesity has become one of the most common health problems worldwide and there has been a corresponding increase in cardiovascular disease, diabetes, cancer, and other nutrition-related disorders following obesity. Given that an increase in dietary fat intake is regarded as one of the factors closely linked with the obesity epidemic (Bray et al., 2004), it has become increasingly important to identify the mechanisms the body uses to recognize dietary fat. A review about the detection of fat (Gilbertson, 1998) stated that, besides the texture of the food, rodents and humans also recognize the presence of fat in foods by the gustatory mechanisms in the mouth, this suggests that an orosensory system may be responsible for the detection and preference of fat.

Currently, most of the research on transduction mechanisms for fat (i.e. fatty acid) taste has focused on long-chain polyunsaturated fatty acids (LCFAs). The Gilbertson laboratory completed a series of studies that have elucidated the pathway for the taste transduction of linoleic acid, which is a typical long-chain fatty acid. Linoleic acid interacts with a GPCR in a subset of Type II taste cells, which then activates G proteins-PLC pathway and produces second messengers, which finally activate the taste cells through TRPM5 channels (Liu et al., 2011). This pathway is similar to the sweet, bitter, and umami taste transduction pathways. Besides LCFAs, saturated fatty acids are very common in our food supply, and include items such as dairy products, fatty meats, palm oil, coconut oil,
and many processed foods (Funaki, 2009). Compared with LCFAs, saturated fatty acids actually pose a greater health risk. However, there is limited knowledge concerning their ability to activate the gustatory system and even less about their possible transduction mechanisms. In the course of our work in the laboratory, we have found that a variety of MCFAs were capable of activating taste cells. And some human studies suggested that MCFAs had detectable oral detection thresholds (Mattes, 2009, Running and Mattes, 2014, Running et al., 2015).

In this study, I explored the ability of the gustatory system to respond to MCFAs in mice by using both cellular and behavioral assays, and showed for the first time that they were effective taste stimuli. I also identified both Type II and Type III taste cells were able to respond to MCFAs. Moreover, I characterized the transduction pathway by using pharmacological approaches and a knockout mice model. Together, this study greatly expands our knowledge for gustatory detection of dietary fat.

Materials and Methods

Animals

The production of TRPM5 knockout strain has been described in detail previously (Damak et al., 2006), and the GFP-PLCβ2 and GFP-GAD67 strains have also been described in previous studies (Chattopadhyaya et al., 2004, Kim et al., 2006). All experiments were performed on adult (2-6 months) male C57BL/6J, TRPM5 knockout, GFP-PLCβ2, or GFP-GAD67 mice, and they were raised with water and normal mouse chow provided ad libitum in a room with 12-h:12-h day/night cycle. 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.

**Solutions**

Tyrode’s solution contained (in mM) 140 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose, and 10 Na pyruvate; adjusted pH to 7.40 with NaOH and osmolarity to 300 ~ 330 mOsm/L with NaCl. Calcium-magnesium free Tyrode’s solution (Ca²⁺-Mg²⁺ free Tyrode’s) contained (in mM): 140 NaCl, 5 KCl, 2 BAPTA, 10 HEPES, 10 glucose, and 10 Na pyruvate; pH was adjusted to 7.40 with NaOH and osmolarity to 300 ~ 330 mOsm/L with NaCl. Hanks’ buffered salt solution (HBSS) with HEPES, Na pyruvate, and fetal bovine serum (HHP with 2% FBS) contained: 98% 1× HBSS, 1% HEPES, 1% Na pyruvate, and 2% heat-inactivated fetal bovine serum (FBS). Intracellular solution used in whole-cell voltage clamp recording contained (in mM): 140 K gluconate, 1 CaCl₂, 2 MgCl₂, 10 HEPES, 11 EGTA, 3 ATP, and 0.5 GTP; pH adjusted to 7.20 with KOH and osmolarity to 305 ~ 315 mOsm/L with K gluconate as necessary. U73122, an inhibitor of PLC, Guanosine 5’-[β-thio]diphosphate trilithium salt (GDP-β-S), an inhibitor of G-protein activation, and H-89 dihydrochloride hydrate, an inhibitor of PKA were purchased from Sigma-Aldrich (St. Louis, MO). U73122 was dissolved in chloroform, aliquoted to 0.6 µmol per tube, and then evaporated to film under a nitrogen stream. Immediately before each experiment, an aliquot was dissolved in ethanol to 0.6 mM, and then diluted in Tyrode’s solution to a final concentration of 3 µM. GDP-β-S was dissolved in water to a stock solution of 50 mg/mL, and diluted in the intracellular solution to a final concentration of 1 mM. H-89 was also dissolved in water to a 10 mg/mL stock, and diluted in Tyrode’s
solution to a final concentration of 3 µM. IBMX (3-isobutyl-1-methylxanthine (Enzo Life Science, Farmingdale, NY), an inhibitor of PDE, was dissolved in DMSO to a 50 mg/mL stock solution, and then diluted in Tyrode’s solution to a final concentration of 0.1 mM. Bisindolylmaleimide II, an inhibitor of PKC was purchased from VWR Scientific (Radnor, PA). It was dissolved in DMSO to a stock solution of 100 mM, and then diluted in Tyrode’s solution to a final concentration of 0.2 µM. All the stock solutions was stored in -20 °C, and diluted right before experiments. The sodium salt forms of all the fatty acids were bought from Sigma-Aldrich (St. Louis, MO), and the fatty acid solutions were prepared fresh by dissolving in Tyrode’s solution (cellular assays) or water (behavioral assay). Fatty acid perfusion in calcium imaging was followed by 0.1% fatty acid free bovine serum albumin (BSA, Sigma) in Tyrode’s solution.

**Taste Cell Isolation**

Tongues were removed, then rinsed and immediately immersed in ice-cold Tyrode’s solution. A mixed enzyme solution of collagenase I (1 mg/ml), dispase II (2.45 mg/ml), and trypsin inhibitor (1 mg/ml) was injected between the muscle layer and the lingual epithelium of the tongues. The injected tongue was incubated in a Ca²⁺-Mg²⁺ free Tyrode’s solution and bubbled with O₂ for 45 min at room temperature. Following incubation, the lingual epithelium was removed from the underlying muscle layer with forceps, pinned out in a Sylgard-lined petri dish and incubated in the same enzyme solution for appropriately 7 minutes. Then the epithelium was incubated in the Ca²⁺-Mg²⁺ free Tyrode’s solution for another 10 minutes. Taste buds were moved from the epithelium under low magnification microscope by gentle suction from a fire-polished pipette and
plated immediately onto a coverslip coated with Corning® Cell-Tak™ Cell and Tissue Adhesive (Corning, NY) for calcium imaging and patch clamp recording.

**Calcium Imaging**

The procedure of calcium imaging has been described in detail in previous studies (Liu et al., 2011). Single taste cells were loaded with intracellular calcium indicator Fura-2, AM (5 µM, Invitrogen) in HHP buffer with 2% FBS for 1 hour at room temperature in a dark room. The coverslip was then mounted onto an imaging chamber (RC-25F or RC-26Z, Warner Instruments), placed on the stage of an inverted microscope (Nikon TE-100), and perfused continuously with Tyrode’s or tastant solutions dissolved in Tyrode’s. A monochromator (Bentham FSM150, Intracellular Imaging Inc.) was used to deliver excitation wavelengths (340 and 380 nm) with a 100-watt xenon lamp to illuminate the Fura-2 AM loaded taste cells. The cell fluorescence was recorded through a CCD camera linked to the inverted microscope, and controlled by an imaging software (Incyt Im2™). The monochromator was calibrated beforehand to construct a standard calibration curve of the ratio of fluorescence at 340 and 380 nm vs. calcium concentration using the Calcium Calibration Buffer Kit (Life Technologies, CA) with pentapotassium salt of Fura-2 as the indicator. During the calcium imaging experiment, fluorescence ratio of 340 nm/380 nm was directly converted to calcium concentration using this calibration curve. Different MCFA solutions were applied extracellularly with a bath perfusion system at a flow rate of 4 ml/min for 2.5 min, following with 1 min of 0.1% fatty acid free BSA solution, and then the solution was switched to regular Tyrode’s until the calcium signal returned to near baseline level. Data analyses were based on the area under the curve (AUC) of each calcium
response calculated with Gaussian multi-peak function by the analytical software Origin 7 (OriginLab, Northampton MA). The AUC between the taste cells from wild type and TRPM5 knockout mice were compared by an unpaired two-tailed Student’s t-test and significance was set at $\alpha = 0.05$.

**Whole Cell Voltage Clamp Recording**

Capric acid-induced currents were recorded in voltage clamp mode with an Axopatch-200B amplifier (Molecular Devices, Sunnyvale, CA). Borosilicate patch pipettes were pulled on a Sutter P-97 puller (Sutter Instruments, Novato CA) and then fire polished on a microforge (model MF-9; Narishige, East Meadow NY) to a resistance of 4-11 MΩ. Series resistance and cell capacitance were compensated optimally before recording. pCLAMP software (version 10, Molecular Devices, Sunnyvale, CA), which interfaced to the amplifier with a Digidata 1322 A A/D board (Axon Instruments, Union City, CA), was used to deliver commands and collect data. Data were collected at 2-5 kHz and filtered on-line at 1 kHz. A patch pipette was sealed onto the cell membrane to obtain resistance of at least 1 GΩ, and then more suction was applied to rupture the membrane patch (whole cell mode). Typical input resistance of cells recorded in this fashion was 1 to several GΩ. Capric acid-induced currents were recorded at a holding potential of -100 mV. Our prototypical MCFA, capric acid (200 µM), was applied focally from a pipette positioned near the cell and delivered for a period of 10 s by a PicoSpritzer III (Parker Hannifin Corp, Cleveland, OH) controlled by pCLAMP software. Different inhibitors except GDP-β-S, which was dissolved in the intracellular solution, were diluted in Tyrode’s buffer and applied in bath 30 secs before and during capric acid application. The
magnitude of the currents induced by capric acid before and after inhibitor application were compared by a paired, two-tailed Student’s t-test. Significance was set at $\alpha = 0.05$ for all the analysis.

**Conditioned Taste Aversion Assay**

Details of the conditioned taste aversion (CTA) assay have been described previously (Pittman et al., 2008, Liu et al., 2011). Before the tests, mice were given *ad libitum* access to water until 24 hours prior to water training when the mice were placed on a 23.5-hour water restriction schedule for the duration of the CTA assay. Right after the training/conditioning/testing on each of the water restriction days, all mice were given 30 minutes access to water. Mice were first water trained in a MS-160 Davis Rig gustatory behavioral apparatus for 3 ~ 4 days until they have similar lick numbers for each of 24 presentations of water. Then taste aversions were conditioned through 3 consecutive days of pairing the conditioned stimulus (CS) and the unconditioned stimulus (US). During conditioning, mice were given 5 minutes access to 600 µM of lauric or capric acid as the CS. Once the mice stopped licking, they were given the same fatty acid orally with syringes. The US was immediately administered through intraperitoneal injections (20 ml/kg body weight dosage) of 150 mM of LiCl to induce gastric distress or 150 mM of NaCl as a control condition. Signs of gastric malaise include lying in an extended prone position, immobility, and a lack of rearing or cage exploration compared with the controls. 11 test taste stimuli were used on testing days besides water, including 10, 30, 100, 300, and 600 µM of lauric or capric acid (depending on the CS used), 100 µM of linoleic acid, 300 µM of undecanoic acid, 300 µM of capric or lauric acid (depending on the CS used), 100 mM
sucrose, 2 mM denatonium benzoate, and 100 mM NaCl. All testing was conducted in the same MS-160 Davis Rig as water training. In order to reduce olfactory cues for the fatty acids, a fan was placed near the animal chamber to provide constant airflow along the longitudinal axis of the stimulus delivery tray. The test session included 2 blocks of 12 presentations with shutter opening for 5 s, and the wait times for the first lick were 150 s. The sequences of the 12 stimuli were arranged randomly. In order to compensate individual variances in the water-restricted motivation across the mice, the total number of licks per stimulus was summarized between the 2 trials and normalized by a ratio of licks to test stimulus/licks to water. Trials with zero lick number were excluded from the subsequent analysis. The unpaired, two-tailed Student’s t-test were used to analyze the differences between LiCl and NaCl-injected groups within each tastant and significance was set at $\alpha = 0.05$.

Results

**MCFAs Activated Mouse Taste Cells.**

To explore the ability of mice taste cells to respond to MCFAs, I used two different kinds of cellular assays with capric acid (C10:0) and lauric acid (C12:0) as representative MCFAs stimuli since they were suggested to be the most potent ligands for the receptor of MCFAs based on my preliminary data and that from a previous study (Wang et al., 2006). Intracellular calcium concentration change is a good indicator for cell activity, so functional calcium imaging was conducted on single taste cells stimulated with capric acid or lauric acid from wild type mice. Taste cells were isolated using our established methods (Liu et al., 2011). In functional calcium imaging, the taste cells were loaded with Fura-2
AM and stimulated with a series of concentrations (10 µM, 30 µM, 100 µM, and 300 µM) of lauric acid in Tyrode’s solution for 2.5 min. Bath application of lauric acid at concentrations greater than 30 µM induced a robust intracellular Ca\(^{2+}\) rise in single taste cells, and the responses were concentration dependent (Figure 2.1). I also used 100 µM of capric acid as a stimulus in the same calcium imaging procedure, and about 8% of the taste cells showed capric acid-induced calcium responses (AUC = 16777 ± 2728, n = 26, Figure 2.2A).

To verify the results, I also performed whole-cell voltage clamp recording on mouse taste cells. 200 µM capric acid was applied by rapid perfusion with a stimulus pipette positioned near the isolated taste cells, and induced a quick inward current (-68.6 ± 5.2 pA, n = 46, Figure 2.2B) on a slightly higher percentage of taste cells than the proportion that responded using calcium imaging. The inward current reached a sharp peak about 1 s after applying the capric acid, and then gradually decreased during the rest of the stimulus. The taste cells also received repetitive stimulations of capric acid on different time intervals. The results showed that the response to capric acid adapted very quickly, and one second was already enough for the taste cells to recover from the previous stimulation. Both calcium imaging and patch clamp recording experiments suggested that MCFAs were able to activate native mouse taste cells.

**Both Type II and Type III Taste Cells Were Activated by MCFAs.**

After confirming that taste cells were able to respond to MCFAs, I conducted additional experiments to determine the subtypes of taste cells that respond to MCFAs. We have in the laboratory transgenic mice expressing enhanced green fluorescent protein (GFP)
under control of the PLCβ2 (GFP-PLCβ2) (Kim et al., 2006) or GAD67 (GFP-GAD67) (Chattopadhyaya et al., 2004, Tomchik et al., 2007) promoter. PLCβ2 is the beta-2 isoform of PLC, which is a key player in Ca^{2+} release after taste receptors are stimulated. Many taste cells express PLCβ2 and it is considered to be a marker for Type II tastes cells because it is essential for the taste transduction of sweet, bitter, and umami (Zhang et al., 2003, Dotson et al., 2005). GAD67 is an isoform of glutamic acid decarboxylase (GAD), which is an enzyme that responsible for GABA synthesis, and it is suggested to be the marker for Type III taste cells (Dvoryanchikov et al., 2011). I performed both functional calcium imaging and patch clamp recording using the isolated taste cells from GFP-PLCβ2 and GFP-GAD67 mice. In calcium imaging, the criterion for calcium responses is its reversibility and having a peak amplitude that was at least ten times the standard deviation of baseline fluctuation. 62 out of 166 (37.3%) GFP positive taste cells from GFP-PLCβ2 mice had calcium responses to MCFAs (100 µM of lauric and capric acids mixture, Figure 2.3 A), and 29 out of 52 (55.8%) GFP positive taste cells from GFP-GAD67 mice were activated by MCFAs in calcium imaging (Figure 2.3 B), which suggested that MCFAs could activate both Type II and Type III taste cells in mice.

In order to verify the calcium imaging data, I performed the patch clamp recording on the taste cells from both GPF labeled strains. Consistent with the calcium imaging data, capric acid induced robust inward currents on the GPF labeled taste cells from both strains of mice (Figure 2.3 E, F), including 66% (8 out of 12) Type II taste cells and 75% (6 out of 8) type III taste cells. The time course of the currents induced in both type of taste cells were similar with each other, they all reached a sharp peak about 1s after capric acid
application, and then decreased slowly. All the results indicated that both Type II and Type III taste cells are MCFA sensitive.

**Wild Type Mice Were Able to Detect MCFAs Behaviorally.**

To determine the ability of MCFAs to be detected *in vivo*, I performed CTA paradigm on wild type mice (Liu et al., 2011), where the mice were conditioned to avoid lauric acid by using 600 µM of lauric acid as the conditioned stimulus. The results of the CTA assays showed that LiCl-injected wild type mice significantly avoided lauric acid at concentrations higher than 30 µM (Figure 2.4 A), which suggested that lauric acid was an effective taste stimulus *in vivo*. Control mice (injected with NaCl) showed no such aversion. And the taste aversion to lauric acid had generalized to both capric and undecanoic acid (Figure 2.4 B), as expected since they all belong to the same MCFA category and were reported as ligands for GPR84 (Wang et al., 2006). There was no generalization with other GPCR-mediated taste stimuli, such as sweet, bitter, or linoleic acid (Figure 2.4 B). Overall, the results were consistent with the cellular assays mentioned above.

**TRPM5 Is Not Essential for MCFAs Taste Transduction**

Previous studies in our laboratory concluded that transient receptor potential channel type M5 (TRPM5) was essential in long-chain fatty acid transduction in mouse taste cells (Liu et al., 2011). Cellular responses to linoleic acid in Type II taste cells, for example, occurred in a qualitatively similar fashion to sweet, bitter and umami stimuli. Therefore, I wanted to test if TRPM5 was also essential for MCFA transduction pathway, and a transgenic mouse model lacking TRPM5 (TRPM5−/−) was used in cell-based and behavioral assays to do so. MCFA-induced changes in intracellular calcium in the taste
cells isolated from TRPM5⁻/⁻ mice were measured using functional calcium imaging as described above. Lauric acid (100 µM) elicited qualitatively similar changes in intracellular Ca²⁺ rise in taste cells from TRPM5⁻/⁻ mice (AUC = 26130 ± 10560, n = 7, Figure 2.5 A) compared to taste cells from wild type mice (AUC = 28710 ± 6388, n = 11, Figure 2.5 B). The responses of the taste cells were calculated by area under the calcium response curves, and there was no significant difference between wild type and TRPM5⁻/⁻ groups (p = 0.8267, Figure 2.5 C), consistent with the interpretation that TRPM5 channels are not an essential component in MCFA transduction signaling pathway.

CTA assays were also conducted to verify the role of TRPM5 in MCFA taste transduction. In a manner similar to that already described (see above), mice (TRPM5⁻/⁻ or wild type) were injected with either LiCl or NaCl (controls) immediately following a single exposure to 600 µM of lauric acid. Similar to wild type mice, LiCl-injected TRPM5⁻/⁻ mice showed significant avoidance to lauric acid in the same low micromolar range (Figure 2.5 D), suggesting TRPM5⁻/⁻ mice have similar taste sensitivity to lauric acid as wild type mice. These results further confirmed that TRPM5 channel is not required in MCFA transduction in the mouse taste system.

**Downstream Signaling Pathway for MCFA Taste Transduction**

In order to begin to elucidate the transduction pathway for MCFAs, I used pharmacological approaches to determine the involvement of potential second messenger pathways, where I inhibited suspected second messengers that might be involved in the downstream signaling as hypothesized earlier (Chapter I).

The Gilbertson laboratory has elucidated the taste transduction pathway for LCFAs,
which couples to G-protein and PLCβ2 (Liu et al., 2011), and this pathway remains an important frame of reference for the possible MCFA taste transduction pathway (see Figure 1.5 in Chapter I). Therefore, I first investigated the involvement of G-proteins and PLCβ2. GDP-β-S (1 mM) was used to block the activation of G-proteins by mixing into the intracellular solution during whole cell voltage clamp recording, and the inward currents induced by capric acid were recorded both with and without GDP-β-S. The results showed that the inward currents produced by capric acid (Figure 2.6 A) stimulation were almost completely abolished when the activation of G-proteins was blocked (n = 14, Figure 2.6 B), suggesting that G-protein was required in the taste transduction of capric acid.

The blocker of PLCβ2, U73122 (3 µM), was also used during voltage clamp recording as stated above. But instead of being applied intracellularly, U73122 was mixed in the extracellular solution and applied 30 seconds before and during the focal release of capric acid. The results showed that U73122 had no significant effect on the capric acid-induced inward current (control: -55.3 ± 14.0 pA; U73122: -59.8 ± 20.0 pA; p = 0.7241, n = 5, Figure 2.6 C, D), which indicated that in contrast to the long-chain fatty acid taste transduction pathway, taste cells activation by MCFAs does not depend on PLCβ2.

Previous studies showed that cell activation by MCFAs caused cAMP inhibition (Wang et al., 2006), therefore, the next component under investigation was phosphodiesterase (PDE), because it catalyzes the breakdown of cAMP into AMP. The inhibitor of PDE, IBMX (0.1 mM), was used in the same manner as U73122 described above (Figure 2.7). The current induced by capric acid (-57.2 ± 9.8 pA, n = 10) was not significantly reduced by IBMX (-58.8 ± 9.4 pA, n = 10, p = 0.8062), which indicated that
PDE was not activated downstream after taste cells were activated by MCFAs.

The involvement of PKA and PKC was also investigated based on my initial hypothesis (see Figure 1.6 in Chapter I). The antagonists of PKA, H-89 (3 µM), was applied extracellularly during voltage clamp recording (Figure 2.8), and it significantly inhibited the capric acid-induced current (control: -72.9 ± 19.0 pA; H89: -32.4 ± 5.6 pA, n = 6, p < 0.05), suggesting PKA played an important role in MCFA transduction. By using the same method, the inhibitor of PKC, bisindolylmaleimide II (0.2 µM), was also applied on taste cells. Similar with the PKA inhibitor, the inward current produced by capric acid (-66.2 ± 10.7 pA, n = 8) was also significantly reduced by PKC inhibitor (-53.1 ± 9.6 pA, n = 8, p < 0.05). However, the inhibition effect of PKC inhibitor was smaller compared with the PKA inhibitor (Figure 2.9), suggesting that although PKC was involved in MCFA taste transduction, it likely plays a less important role in this process.

Discussion

In the current study, I have shown that MCFAs are able to elicit calcium response and inward current in native taste cells of mice, including both Type II and Type III taste cells. MCFAs are also effective taste stimuli in vivo, proved by the conditioned taste aversion assays. In addition, capric acid-induced currents were significantly inhibited by GDP-β-S and inhibitors of PKA and PKC, which suggested that the currents were activated downstream of G-protein, PKA, and PKC activation. However, these currents were not affected by the PLCβ2 and PDE inhibitors, suggesting PLCβ2 and PDE were not involved in the MCFA taste transduction pathway.

It has been almost twenty years since we started to study the gustatory recognition
of fatty acids, and most of the studies focused on the long-chain unsaturated fatty acids, especially linoleic acid, since it is a qualitatively more effective fatty acid taste stimulus. However, dietary fatty acids also include other forms of fatty acids, such as MCFAs, and there is limited knowledge concerning the possible transduction mechanisms of MCFAs in taste cells. So far, only one group showed that MCFAs were detectable in humans (Mattes, 2009, Running and Mattes, 2014), no other studies have reported the taste of MCFAs or on the ability of MCFA to activate taste cells. This study is the first that supported the notion that MCFAs are effective taste stimuli at both the cellular and behavioral levels.

Intracellular calcium concentration change is a good indicator for cell activity, so functional calcium imaging was conducted on single taste cells from wild type mice while stimulated with MCFAs. The calcium responses induced by MCFAs were similar with those produced by linoleic acid in respect to amplitude and time course (Liu et al., 2011). The inward current elicited by MCFAs, however, were generally smaller than those attributed to linoleic acid (Liu et al., 2011). This may reflect the fact that LCFAs activate TRPM5 downstream, while MCFAs, like lauric or caproic acid, do not. The nature of the channel carrying the MCFA-induced current has not been identified in the present study. Despite best efforts to provide a more complete characterization of the MCFA-induced current, ion substitution experiments produced equivocal results due to the relatively small amplitude of these currents and their adapting nature. This difficulty may be exacerbated by our finding that both Type II and Type III cells respond to MCFAs and these different cell types may not necessarily be utilizing a common transduction pathway. Future experiments performed on identifiable cell types will be required to determine the
molecular nature of this channel in the same way the involvement of TRPM5 was assessed in LCFA transduction.

Different types of taste cells express unique receptors and channels and they have different functions, the taste recognition requires cross talk among Type I, II, and III taste cells (Besnard et al., 2016). Therefore, the knowledge of which type of taste cells respond to a certain taste stimulus can help us predict the possible signaling pathway being activated. Previous studies in our lab showed that long-chain unsaturated fatty acids activated primarily Type II cells but also a small fraction of Type III taste cells. Similar to this, MCFAs could also elicit responses in a similar fraction of both Type II and Type III taste cells. This finding provided the first clue to investigate if a similar signaling pathway was involved both in MCFA and LCFA transduction.

To date, GPCRs, like GPR120, are believed to be the cognate receptors for fatty acids on taste cells (Matsumura et al., 2007, Cartoni et al., 2010). I hypothesized that GPR84 is the primary receptor of MCFAs in taste transduction based on the current knowledge on MCFAs signaling pathways (Wang et al., 2006) and their expression in the taste system, which will be more fully described in the next chapter. Previous studies concluded that MCFAs activated GPR84, and then coupled to a pertussis toxin-sensitive pathway (Wang et al., 2006). In addition, research in our lab showed that the linoleic acid induced responses coupled with activation of G-proteins (Liu et al., 2011). Consistent with previous studies, the capric acid-induced inward current was virtually eliminated by GDP-β-S, suggesting that MCFAs-induced responses couple to G-proteins. This result further confirmed the possible involvement of GPCRs in initiating the MCFA-activated signaling
pathway in the peripheral taste system.

Previous studies concluded that TRPM5 played a critical role in long-chain fatty acid transduction in mouse taste cells. The taste cells from TRPM5 knockout mice showed significantly decreased responses to linoleic acid, and behaviorally, mice lacking TRPM5 showed no preference and reduced sensitivity to linoleic acid (Liu et al., 2011). Similar experiments were done on TRMP5 knockout mice, but instead of using linoleic acid as the tastant, I used lauric acid. However, knocking out of TRPM5 didn’t have significant effects on either taste cell responses in vitro nor taste sensitivity in vivo, suggesting that TRPM5 was not involved in the MCFA signaling pathway in mouse taste cells. Another important component involves in long-chain fatty acid taste transduction is PLC (Liu et al., 2011), but applying the antagonist of PLCβ2 did not reduce the taste cell responses to capric acid. The differences in the involvement of TRPM5 and PLC indicated that MCFA transduction pathway in mouse taste cells is significantly different with for the long-chain unsaturated fatty acids.

In leukocytes, GPR84 activation by MCFAs couples to a pertussis toxin-sensitive G\textsubscript{i/o} pathway, where it caused calcium mobilization and 3',5'-cyclic AMP (cAMP) inhibition (Wang et al., 2006). α-gustducin, which belongs to the G\textsubscript{i/o} family, is abundantly expressed in taste cells (McLaughlin et al., 1992) and might play a key role in sweet, umami, and bitter taste transduction pathway (Ruiz et al., 2003, Glendinning et al., 2005). Therefore, I hypothesized that α-gustducin is the G-protein that responsible for MCFA signaling pathway in taste cells, and α-gustducin is able to activate phosphodiesterase (PDE) and, in turn, decrease intracellular cAMP in taste cells (Kolesnikov and Margolskee, 1995,
Kinnamon, 2009). I didn’t have an α-gustducin knockout model to test its involvement directly, but I found that by applying the antagonist of PDE during the whole cell voltage clamp recording, the inward current induced by capric acid was not reduced significantly, which suggested that PDE was not involved in the MCFA signaling pathway. Therefore, the MCFA activated G-protein might not be α-gustducin. My data showed that the antagonist of PKA significantly reduced the inward current induced by capric acid, suggesting MCFA signaling pathway involves the activation of PKA, which is also an indirect evidence of the cAMP involvement because PKA activation requires cAMP (Meinkoth et al., 1993). How exactly cAMP was produced is still under investigation, but one known signaling pathway for sugar transduction can provide some insight into this process. Sucrose and other sugars activate Gs by interacting with GPCRs, and the activated Gαs then activate adenylyl cyclase (AC) to produce cAMP. cAMP is able to activate PKA, which in turn close the basolateral K+ channels by phosphorylation (Avenet et al., 1988a, b, Naim et al., 1991, Margolskee, 2002, Abaffy et al., 2003). Similar with this sugar transduction pathway, MCFA taste transduction also involves G-protein and PKA activation, and my next step will be testing the involvement of AC in this process by using AC blockers.

I also used PKC inhibitor during whole cell voltage clamp and the results showed that the inward current produced by capric acid was slightly but significantly decreased by PKC inhibitor, which indicated that PKC activation was followed with taste recognition of capric acid, although it may be not as important as the role of PKA. The involvement of PKC in taste transduction is not a new concept. It has been demonstrated that the
transduction of synthetic sweeteners required the activation of PKC (Varkevisser and Kinnamon, 2000). How exactly PKC was activated is unclear. A well-known PKC activation pathway is PLC dependent, where PLC hydrolyses phosphatidylinositol 4,5 bisphosphate and releases inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). IP₃ and DAG can be generated and Ca²⁺ can be released from ER store. Ca²⁺ and DAG together then work together to activate protein kinase C (PKC) (Nishizuka, 1995). However, PLC is not involved in MCFA transduction based on my studies, so it likely relies on a PLC-independent PKC activation pathway, which is poorly understood but has been studied in several studies (Villalba et al., 2002, Yang et al., 2006, Hao et al., 2015).

References

Cartoni C, Yasumatsu K, Ohkuri T, Shigemura N, Yoshida R, Godinot N, le Coutre J,


Damak S, Rong M, Yasumatsu K, Kokrashvili Z, Perez CA, Shigemura N, Yoshida R, Mosinger B, Jr., Glendinning JI, Ninomiya Y, Margolskee RF (2006) Trpm5 null mice respond to bitter, sweet, and umami compounds. Chem Senses 31:253-264.


to sucrose stimulation in membranes of pig circumvallate taste papillae. Comp Biochem Physiol B 100:455-458.


Figure 2.1. Calcium response induced by lauric acid was concentration dependent. Intracellular calcium changes in taste cells were measured using functional calcium imaging when applying a series of concentrations of lauric acid in Tyrode’s solution for 2.5 min. Then the cells were perfused with 1 mg/ml BSA in Tyrode’s solution to wash out lauric acid. Bath application of lauric acid that is higher than 30 µM induced a robust intracellular Ca\(^{2+}\) rise in taste cells, and the response was concentration dependent.
Figure 2.2. Taste cell responses induced by capric acid (CA). (A). Bath application of capric acid (100 µM) elicited an intracellular calcium rise in mouse taste cell. (B). Focal application of capric acid (200 µM) induced a rapid inward current in mouse taste cell. (C). Capric acid (200 µM) was applied on mouse taste cells (n=6) repetitively on different time intervals, and 1 s interval was already enough for the cells to recover and generate similar current response again.
Figure 2.3. Both Type II and Type III taste cells were activated by MCFAs. In calcium imaging, lauric (LA) and capric (CA) acids mixture (100 μM) induced robust calcium increases in GFP positive taste cell from both GFP-PLCβ2 (A) and GFP-GAD67 mice (C). And among all the cells collected, 37.3% (62 out of 166) of GFP-PLCβ2 and 55.8% (29 out of 52) of GFP-GAD67 taste cells responded to MCFAs. Voltage clamp recording data showed that capric acid (200 μM) elicited rapid inward currents in both GFP-PLCβ2 (B) and GFP-GAD67 (D) taste cells.
Figure 2.4. Wild type mice were able to detect MCFAs. (A) Relative lick ratios (mean ± SEM) for different concentrations of lauric acid (10, 30, 100, 300, 600 μM) in wild type mice on day two after CTA. n = 7 for LiCl group, and n = 5 for NaCl group. Asterisks indicate significant differences between two groups (p < 0.05). (B) Relative lick ratios (mean ± SEM) for other MCFAs and GPCR-mediated taste stimuli.
Figure 2.5. TRPM5 is not essential for MCFAs taste transduction. Lauric acid (LA, 100 μM) induced similar intracellular calcium rise in both TRPM5+/+ (A) and TRPM5-/- taste cells (B). The area under the curve (mean ± SEM) to of these two groups were compared using an unpaired two-tailed Student’s t-test (α = 0.05) with the sample size as n = 7 for TRPM5+/+ taste cells and n = 9 for TRPM5-/- taste cells (C). (D) On day two after CTA test on TRPM5-/- mice, relative lick ratios (mean ± SEM) were recorded and calculated for different concentrations of lauric acid (10, 30, 100, 300, 600 μM). n = 4 for LiCl group, and n = 6 for NaCl group. Asterisks indicate significant differences two groups (p < 0.05).
Figure 2.6. MCFA taste transduction involves G-protein activation but does not involve PLCβ2. Focal application of capric acid (CA, 200 μM) induced an inward current in wild type taste cells (A), and this current was almost completely abolished by GDP-β-S (B). However, capric acid (200 μM) induced similar inward currents with (C) and without (D) U73122 (PLCβ2 inhibitor). The amplitudes of the currents (mean ± SEM) in both groups were not significantly different (E, n = 5, p = 0.7241). Data were analyzed by a paired two-tailed Student’s t-test and significance was set at α = 0.05.
Figure 2.7. MCFA taste transduction does not involve PDE activation. Capric acid (CA, 200 µM) induced an inward current in the wild type taste cell (A), and this current was not reduced significantly by the PDE inhibition, IBMX (B, \( p = 0.8062 \)). (C) Mean response ± SEM to capric acid with \( n = 10 \) or without IBMX \( (n = 10) \) inhibition. Data were analyzed by a paired two-tailed Student’s t-test and significance was set at \( \alpha = 0.05 \).
Figure 2.8. MCFA taste transduction involves PKA activation. The rapid inward current (A) induced by capric acid (CA, 200 µM) was significantly inhibited by the antagonist of PKA (H89, n = 6, p < 0.05, B, C). The amplitudes of the currents were expressed as mean ± SEM. Data were analyzed by a paired two-tailed Student’s t-test and significance was set at α = 0.05.
Figure 2.9. MCFA taste transduction involves PKC activation. Capric acid (CA, 200 µM) induced a fast inward current in mouse taste cells (A), and this current was slightly but significantly reduced by the antagonist of PKC (Bisindolylmaleimide II, n = 8, p < 0.05, B, C). The amplitudes of the currents were expressed as mean ± SEM. Data were analyzed by a paired two-tailed Student’s t-test and significance was set at α = 0.05.
CHAPTER III

GPR84 IS ESSENTIAL FOR THE TASTE OF MEDIUM-CHAIN SATURATED FATTY ACIDS

Abstract

Previous research has shown that GPR84 is a novel G protein-coupled receptor (GPCR) activated by medium-chain saturated fatty acids (MCFAs) of C9 to C14 in length (Venkataraman and Kuo, 2005, Wang et al., 2006), and the real-time PCR results showed that the mRNA of GPR84 was expressed in taste cells. Fura-2 based ratiometric calcium imaging was used to characterize GPR84 in a cell line that had an inducible GPR84 + Gqi9 (a chimeric G-protein; (Wang et al., 2006)). I found that GPR84 functioned as a receptor for saturated fatty acids from C6 to C12 and other fatty acids (oleic acid, arachidic acid) previously not thought to activate this receptor. Using whole cell patch clamp recording, I have shown that capric acid (C10) induced a quick inward current in wild type taste cells at resting potentials, and this current was significantly reduced in taste cells from GPR84 knockout mice. The calcium response to capric acid in GPR84 knockout taste cells was also significantly reduced compared to the wild type taste cells. In behavioral studies, the mice lacking GPR84 showed reduced taste sensitivities to both lauric (C12) and capric acid (C10). All the data above supported the idea that GPR84 plays a very important role in MCFA taste transduction process.
Introduction

The prevalence of individuals who are overweight or obese has nearly doubled during the past two decades, and obesity increases the risk of heart disease, diabetes, and even some types of cancer (Hursting et al., 2007, Wang et al., 2010, Logue et al., 2011, Meyer et al., 2011). Overconsumption of dietary fat is regarded as one of the factors closely linked with the obesity epidemic (Bray et al., 2004). Therefore, understanding the mechanisms of fatty acid detection in the oral cavity can help the identification of potential strategies to reduce fat intake, such as designing fat substitutes, and, in turn, help create a healthier diet.

Accumulating evidence has established the existence of the “taste of fat”, and more and more studies have focused on identifying the mechanisms of fatty acid taste transduction. Gilbertson and colleagues were the first to show that extracellular application of cis-polyunsaturated fatty acids was able to activate native taste cells from rats by inhibiting delayed rectifying potassium channels (Gilbertson et al., 1997). Subsequently, it became clear that there were additional receptors upstream of delayed rectifying potassium channel, and support for both cluster of differentiation 36 (CD36) and fatty acid-activated GPCRs emerged (Gilbertson and Khan, 2014). Support for the involvement of fatty acid receptors in taste system, two bottle taste preference tests showed that knocking out of GPR120 or GPR40 significantly reduced the preference for linoleic acid in mice (Cartoni et al., 2010b). Furthermore, downstream of these receptors, G-protein, phopholipase beta-2 (PLCβ2), and transient receptor potential channel type M5 (TRPM5) are important second massagers involved in fatty acid taste transduction based on recent studies in our
All the studies above, however, were focusing on the gustatory mechanism of long-chain unsaturated fatty acids, little research has been done on unveiling the taste transduction of saturated fatty acids, although they pose greater threats to human health. We have found that a variety of medium-chain saturated fatty acids (MCFAs) were capable of activating taste cells, and some studies in humans suggested that MCFAs had detectable oral detection thresholds (Mattes, 2009, Running and Mattes, 2014, Running et al., 2015). GPR84 is a novel G protein-coupled receptor (GPCR) expressed predominantly in hematopoietic tissues, and can be activated by MCFAs of C9 to C14 in length (Wang et al., 2006). It has been suggested that GPR84 activation by MCFAs couples primarily to a pertussis toxin-sensitive Gi/o pathway, in which MCFAs caused calcium mobilization, 3’,5’-cyclic AMP inhibition, and [35S]GTPγS binding (Wang et al., 2006).

In this study, I investigated the expression of GPR84 in native taste cells, and the ligand specificity of GPR84 on a cell line expressing inducible GPR84 receptors. GPR84 knockout mice model was used to study the involvement of GPR84 receptor in MCFA taste transduction in both cellular and behavioral assays.

**Materials and Methods**

**GPR84-Expressing Cell Line**

The special HEK293 cell line was designed to express GPR84 in an inducible fashion under control by the tetracycline (TET) promoter. It was constructed by and a generous gift of International Flavor and Fragrances Inc. (International Flavors & Fragrances, Inc.). The specific constructs I utilized was an inducible GPR84 + Gqi9 (a
chimeric G-protein; (Wang et al., 2006)). This cell line has been cloned, validated by PCR/qPCR and verified for function in FLIPR®-based calcium assays. All cells were cultured in the cell culture medium DMEM with GlutaMax (Invitrogen) supplemented with 10% tetracycline-free fetal bovine serum (FBS, Fisher). 10 μg/ml Blasticidin S HCl (Invitrogen) and 100 μg/ml Hygromycin B (Invitrogen) were added to the medium to select the antibiotic-resistant cells. To induce the expression of transfected GPR84 receptor genes, 0.5 μg/ml doxycycline were added to the culture medium 24 hours before experiments. Non-induced cells served as controls.

**Animals**

The GPR84 knockout model (GPR84−/−) was created by Taconic (Hudson, NY) on a pure C57BL/6N background. It was made by incorporating a knockout gene, in which the single exon was replaced by a LacZ/Neo cassette by homologous recombination (Figure 3.1). GPR84 knockout mice were bred with two heterozygote (GPR84 +/-) parents, and GPR84+/− and GPR84−/− mice were identified by genotyping. I checked their general health condition by measuring their body weight and body composition every week for 20 weeks. All experiments were performed on adult (2-5 months) male C57BL/6J or GPR84 knockout mice, and they were raised with water and normal mouse chow provided *ad libitum* in a room with 12-h:12-h day/night cycle. 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.
Genotyping

DNAs were extracted from ear tissues by DirectPCR lysis Reagent (Viagen Biotech, CA) according to manufacturer’s protocol. Then GPR84 genotyping was carried out by two separate polymerase chain reaction (PCR) to specifically amplify either the wild type or the knockout allele. The primers used are shown in Table 3.1. Final reaction cocktail (20 μL) contains 1× GoTaq® Reaction Buffer (Promega, WI), 1.75 mM Mg²⁺, 0.2 mM dNTPs, 0.3 µM forward and reverse primers, 1 µL template DNA extracted by DirectPCR® Lysis Reagent, and 0.025 U/µl GoTaq® DNA Polymerase (Promega, WI). The PCR was performed in Eppendorf® Mastercycler (Sigma-Aldrich, MO) with the programming as follows: 10 cycles of 94 °C for 15 s, 65 °C for 30 s (decrease 1 °C per cycle), and 72 °C for 40 s. Then 30 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 40 s. After PCR, the samples were electrophoresed in 2% agarose in 1×TAE (Tris-Acetate-EDTA) buffer (Fisher BioReagents, NJ) with 1×SYBR® Safe DNA Gel Stain (Invitrogen, CA). Then the gel was imaged in a Gel Doc XR+ system (Bio-Rad, CA).

Solutions

Tyrode’s buffer contained (in mM) 140 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose, and 10 Na pyruvate; pH was adjusted to 7.40 with NaOH and osmolarity to 300 ~ 330 mOsm/L with NaCl, if necessary. Calcium-magnesium free Tyrode’s (Ca²⁺-Mg²⁺ free Tyrode’s) solution contained (in mM): 140 NaCl, 5 KCl, 2 BAPTA, 10 HEPES, 10 glucose, and 10 Na pyruvate; adjusted pH to 7.40 with NaOH and, as needed, osmolarity to 300 ~ 330 mOsm/L with NaCl. Solution used to dilute Fura-2 AM for calcium imaging on taste cells (HHP with 2% FBS) contained: 98% 1× Hanks' Balanced Salt Solution, 1%
HEPES, 1% Na pyruvate, and 2% heat-inactivated fetal bovine serum (FBS). Intracellular solution used in whole-cell voltage clamp recording contained (in mM): 140 K gluconate, 1 CaCl₂, 2 MgCl₂, 10 HEPES, 11 EGTA, 3 ATP, and 0.5 GTP; pH was adjusted to 7.20 with KOH and osmolarity was adjusted to 305 ~ 315 mOsm/L with K gluconate. All the fatty acids (sodium salt) were bought from Sigma-Aldrich (St. Louis, MO), and they were dissolved in Tyrode’s solution (cellular assays) or water (behavioral assay) to make fatty acid solutions.

**Taste Cell Isolation**

Details of taste cell isolation procedure were described before (Liu et al., 2011). Briefly, tongues of the mice were removed, rinsed, and then immersed immediately in the ice-cold Tyrode’s solution. An enzyme mixture including collagenase I (1 mg/ml), dispase II (2.45 mg/ml), and trypsin inhibitor (1 mg/ml) was injected under the lingual epithelium of the tongue, which was then incubated at room temperature in Ca²⁺-Mg²⁺ free Tyrode’s solution with O₂ bubbled for 45 minutes. The lingual epithelium was separated from the underlying muscle layer and pinned out in a Sylgard-lined petri dish, followed by incubation in the same enzyme mixture for 7 minutes, and subsequently in the Ca²⁺-Mg²⁺ free Tyrode’s solution for another 10 minutes. Taste bud isolation was performed under low magnification microscope with a fire-polished pipette, and the isolated taste buds were placed on a coverslip coated with Corning® Cell-Tak™ Cell and Tissue Adhesive (Corning, NJ) for calcium imaging and patch clamp recording.

**Real-Time PCR**

RNA was extracted from taste cells isolated from mouse by RNeasy Plus Micro Kit
(Qiagen, Valencia, CA) and analyzed with ExperionTM RNA HighSens Analysis Kit (BIO-RAD, Hercules, California). Then cDNA was synthesized by the iScript cDNA Synthesis Kit (BIO-RAD, Hercules, California). All the procedures above were performed according to the manufacturers’ protocols. Commercially available TaqMan® Gene Expression Assays (Thermo Fisher Scientific, Waltham, MA) were then used to detect the expression of GPR84. Final reaction cocktail (20 μL) contains: 1× reaction buffer, 0.2 mM dNTPs, 2.5 mM Mg2+, 0.06 μM forward and reverse primers for GAPDH, 0.2 μM GAPDH probe, TaqMan® gene expression mix for GPR84 (1.25 μL in 25 μL reaction, Thermo Fisher Scientific, MA), 2 μL template cDNA, and 0.05 U/μl HotMaster Taq (5 Prime, MD). The GAPDH probe was Texas Red-labeled and the GPR84 probe was FAM-labeled. The expression levels of GPR84 from different types of papillae was normalized to the expression level of the housekeeping gene GAPDH within the same sample by the equation: 

\[ \Delta C_t^{GPR84} = C_t^{GPR84} - C_t^{GAPDH} \]

Ct is the number of cycles required for the fluorescence signal to cross the threshold. Then GPR84 expression was compared to GPR120 in circumvallate (calibrator) by the equation 

\[ \Delta \Delta C_t = \Delta C_t^{GPR84} - \Delta C_t^{GPR120} \]

At last, the \( \Delta \Delta C_t \) was exponentially transformed to represent the relative expression (R) to the calibrator: 

\[ R = 2^{\Delta \Delta C_t} \]

**Calcium Imaging**

The procedure of calcium imaging has been described in detail in a previous study (Liu et al., 2011). For cell lines, suspended cells in culture medium were plated onto 12 mm coverslips with cell medium and incubated in the incubator for about 2 hours until most of the cells attached stably to the coverslips. Then the plated cells were rinsed with
Tyrode’s solution, loaded with 4 μM of the ratiometric intracellular calcium indicator Fura-2, AM (Invitrogen) in Tyrode’s with 0.25% pluronic acid solution, and incubated at 37°C for 45 min in the cell incubator. For taste cells, single taste cells on coverslips were loaded with the Fura-2, AM in HHP buffer with 2% FBS for 1 hour at room temperature in dark. The coverslips were then mounted onto an imaging chamber (RC-25F or RC-26Z, Warner Instruments) and imaged under an inverted microscope (Nikon TE-100). The excitation wavelengths of 340 and 380 nm were generated by a monochromator (Bentham FSM150, Intracellular Imaging Inc.) to excite Fura-2. Each cell was outlined and the fluorescence in the outlined area was recorded by an imaging software coupled to the monochromator (Incyt Im2™) through a CCD camera linked to the microscope. A standard curve of the ratio of fluorescence (340 nm/380 nm) vs. calcium concentration was calibrated beforehand using Calcium Calibration Buffer Kit (Life Technologies, CA) with pentapotassium salt of Fura-2 as the indicator. During calcium imaging, the calcium concentration was directly calculated by the calibration curve from the ratio of fluorescence. MCFA solutions were applied by a bath perfusion system at a flow rate of 4 ml/min for 2.5 minutes, followed by 1 min of 0.1% fatty acid free bovine serum albumin (BSA) in Tyrode’s solution and then regular Tyrode’s solution to wash away the fatty acids until the calcium signal returned to the baseline. The calcium response was quantified by the area under the curve (AUC), which was calculated with Gaussian multi-peak function by Origin 7 (OriginLab, Northampton MA). The calcium response between the taste cells from wild type and GPR84<sup>−/−</sup> mice were compared by an unpaired two-tailed Student’s t test and significance was set at α = 0.05. To generate the does-response curves for lauric and capric
acids on GPR84-HEK cells, a series of lauric or capric acid at concentrations of 1, 3, 10, 30, and 100 μM were applied to the cells, and AUC was calculated as the calcium response. Does-response curves were fitted using GraphPad software (Graphpad Software Inc., CA) to the following equation:

\[ Y = Min + \frac{(Max-Min)}{(1+10^{((LogEC50-X))})} \]

Where Max and Min are plateaus in the units of the Y axis.

**Whole Cell Voltage Clamp Recording**

Voltage clamp mode with an Axopatch-200B amplifier (Molecular Devices, Sunnyvale, CA) was used to record the capric acid-induced currents in taste cells. Borosilicate patching pipettes were pulled by a Sutter P-97 puller (Sutter Instruments, Novato CA) and fire polished by a microforge (model MF-9; Narishige, East Meadow NY). The resistances of the patching pipettes were 4-11 MΩ. pCLAMP software (version 10, Molecular Devices, Sunnyvale, CA) was used to deliver commands and collect data, and it was interfaced to the amplifier with a Digidata 1322 A A/D board (Axon Instruments, Union City, CA). Data were collected at 2-5 kHz and filtered on-line at 1 kHz. In order to record a whole cell current, a seal with the resistance higher than 1 GΩ was needed between the patching pipette and the cell membrane, and then more suction was applied to rupture the membrane patch to reach a whole cell mode. Series resistance and cell capacitance were compensated optimally before recording, and the whole cell currents were recorded with a holding potential of -100 mV. Focal application of capric acid was delivered by a pipette positioned near the cell and controlled by a PicoSpritzer III (Parker Hannifin Corp, Cleveland, OH). The currents recorded in wild type and GPR84 knockout taste cells were
compared by an unpaired, two-tailed Student’s t-test, and significance was set at $\alpha = 0.05$.

**Conditioned Taste Aversion assay**

Details of the conditioned taste aversion (CTA) assay have been described previously (Pittman et al., 2008, Liu et al., 2011). Mice were placed on a 23.5-hour water restriction schedule for the duration of the CTA assay starting at 24 hours before water training, where they were given 30 min access to water right after training/conditioning/testing experiment on each day. Water training and testing were both performed in a MS-160 Davis Rig gustatory behavioral apparatus. Firstly, mice were trained with water for 3 ~ 4 days until they have stable numbers of licks for each of at least 20 presentations. The next 3 consecutive days, taste aversions were constructed by pairing the conditioned stimulus (CS) and the unconditioned stimulus (US). Mice were given 5 minute access to 600 $\mu$M of lauric or capric acid as the CS, and then the same CS orally with syringes right before injecting the US. 150 mM of LiCl was injected intraperitoneally (20 ml/kg body weight dosage) as the US to induce gastric distress, the signs of which included moving slowly, lying in an extended prone position, and stopping exploration in the cage. 150 mM of NaCl was injected to the control group. On testing days, a fan was placed near the animal chamber to provide constant airflow along the longitudinal axis of the stimulus delivery tray to reduce olfactory effect of the fatty acids. Besides water, the test solutions included 10, 30, 100, 300, and 600 $\mu$M of lauric or capric acid, 100 $\mu$M of linoleic acid, 300 $\mu$M of undecenoic acid, 300 $\mu$M of capric or lauric acid, 100 mM sucrose, 2 mM denatonium benzoate, and 100 mM NaCl. The sequences of the tastants were arranged randomly, and the test session included 2 blocks of 12 presentations with shutter
opening for 5 s, and the wait times for the first lick were 150 s. The total number of licks per stimulus was averaged between the 2 trials and normalized relative to the licks to water presentations. Trials with zero licks were removed from the analysis. The differences between LiCl and saline-injected groups within each tastant were compared by an unpaired, two-tailed Student’s t-test and significance was set at $\alpha = 0.05$.

Results

**Evaluation of GPR84 Knockout Mice.**

Weights of GPR84$^{+/+}$, GPR84$^{+/-}$, and GPR84$^{-/-}$ mice were monitored every week from age 4 to 20 weeks (Figure 3.2 A). The results showed that mice with different genotypes all grew steadily, and GPR84$^{+/-}$ and GPR84$^{-/-}$ mice had significantly higher body weights than GPR84$^{+/+}$ mice for every testing week ($p < 0.05$, $n = 4$–10). Body fat compositions were also measured every 4 weeks from age 8 to 20 week (Figure 3.2 B). GPR84$^{+/-}$ mice showed higher body fat composition than GPR84$^{+/+}$ and GPR84$^{-/-}$ mice for all the testing ages. At 20 week, GPR84$^{+/+}$ mice showed significantly lower body fat composition than both GPR84$^{+/-}$ and GPR84$^{-/-}$ mice ($p < 0.05$, $n = 4$–10). Although the deletion of GPR84 caused the differences among three genotype group on growth and body fat composition, GPR84$^{-/-}$ mice were in a healthy state by all indices within the age of my experiments.

**GPR84 Receptor mRNA Is Expressed in Taste Cells.**

To determine if GPR84 receptors present in native taste cells, I examined the expression of its mRNA in taste buds from wild type mice by quantitative real-time PCR
(qPCR). Taste buds from fungiform, foliate, and circumvallate papillae were isolated and analyzed separately. The mRNAs from the taste buds were exacted and then the cDNAs were synthesized and used in qPCR for quantitative evaluation. The mRNA expression of GPR120 was used to compare the GPR84 expression levels. The qPCR results showed that the mRNA of GPR84 expressed in the taste cells from fungiform ($\Delta Ct = 10.2 \pm 0.9$, $n = 4$), foliate ($\Delta Ct = 7.8 \pm 1.2$, $n = 9$), and circumvallate papillae ($\Delta Ct = 7.2 \pm 0.9$, $n = 9$), and the expression in circumvallate was the highest. The expression level of GPR84 is 30% ~ 40% of GPR120 within each papillae (Figure 3.3). Previous studies have shown that GPR120 was highly expressed in taste cells (Cartoni et al., 2010a, Montmayeur et al., 2011), although the GPR84 expression level was not as high as GPR120, it was still significant.

**Ligand Specificity of GPR84.**

The ligand specificity of GPR84 was tested on a heterologous expression cell line which was designed to express GPR84 in an inducible fashion under control by the tetracycline promoter, and the gene expression of this cell line was validated by qPCR. By using functional calcium imaging, we recorded the intracellular calcium rise stimulated by a variety of fatty acids in HEK293 cells with induced GPR84 receptors and Gqi9. The results showed that caproic ($C_{6:0}$), caprylic ($C_{8:0}$), capric ($C_{10:0}$), undecanoic ($C_{11:0}$), lauric ($C_{12:0}$), oleic ($C_{18:1}$), and arachidic acids ($C_{20:2}$) all elicited a robust and reversible calcium increase in the cells induced to express GPR84 receptors, while in the non-induced control cells, they could not induce any calcium responses (Figure 3.4).

To characterize the ligand sensitivity of GPR84, the concentration-response functions for activation of GPR84 by lauric and capric acids were investigated using
ratiometric calcium imaging. Figure 3.5 shows the concentration-response plots measured from the cells with induced GPR84 receptors in response to a series of lauric or capric acids at concentrations ranging from 1 to 100 µM. The EC₅₀ value for lauric acid and capric acid were 27.4 ± 1.1 µM (n = 53 ~ 81) and 4.4 ± 1.3 µM (n = 17 ~ 55), respectively.

**Cellular Responses to MCFAs in GPR84⁻/⁻ Mice**

To study the functional role of GPR84 in MCFA taste transduction, we utilized a transgenic mouse model that is GPR84 deficient (GPR84⁻/⁻). Taste cells were isolated from GPR84⁻/⁻ mice, and both functional calcium imaging and voltage clamp recording were used to characterize the taste cell responses to capric acid. In voltage clamp recording experiments, capric acid was applied near the recorded cells and it induced a rapid inward current in wild type taste cells (Figure 3.6 A, -78.77 ± 9.104 pA, n = 17). This current was significantly reduced in GPR84 knockout taste cells (-15.98 ± 4.985 pA, n = 11, p < 0.01). Consistent with the electrophysiology data, calcium imaging experiments showed that the calcium increases to capric acid in GPR84⁻/⁻ taste cells (Figure 3.7, AUC: 2542 ± 604.5, n = 5) were much smaller compared to the responses in wild type taste cells (AUC: 16777 ± 2728, n = 26, p < 0.05).

**Behavioral Assays on GPR84⁻/⁻ Mice.**

To determine the role of GPR84 in MCFA taste transduction in vivo, I performed CTA assays on GPR84⁻/⁻ mice, and then compared the taste sensitivity between wild type and GPR84 knockout mice. Mice were injected with either LiCl or NaCl (controls) immediately following a single exposure to 600 µM of lauric or capric acid. When lauric acid was used as the conditioned stimulus, GPR84 knockout mice did not show any
significant aversions for lauric acid with concentrations up to 600 μM, suggesting the lack of GPR84 receptors significantly reduced the taste sensitivity of mice to lauric acid (Figure 3.8 A). However, when capric acid was served as conditioned stimulus in another set of CTA assays, the GPR84 knockout mice showed significant aversion to capric acid with concentration higher than 300 μM ((Figure 3.8 B), suggesting that knocking out of GPR84 reduced but not totally abolished the taste sensitivity of mice to capric acid. The underlying nature of the differences between using lauric acid and capric acid as the conditioned stimulus was not clear.

Discussion

In the current study, I have shown that the mRNA of GPR84 is expressed on taste cells from mice, and GPR84 functioned as a receptor for saturated fatty acids from C6 to C12, and, surprisingly, oleic acid and arachidic acids. A GPR84 knockout mice model was used to investigate the role of GPR84 in MCFA taste transduction, and both cellular and behavioral assays proved that GPR84 was critical in gustatory recognition of MCFAs.

Several GPCRs have been suggested to be activated by free fatty acids, including GPR120, GPR40, GPR41, GPR43, and GPR84. The ligands for these receptors are fatty acids with different lengths, which are generally classified as long-, medium-, and short-chain fatty acids. GPR120 and GPR40 were the only GPCRs that have been studied in the gustatory systems so far, and past research showed that mice lacking GPR120 or GPR40 had reduced preferences for long chain unsaturated fatty acids, which suggested that GPR120 and GPR40 mediated taste transduction of long-chain unsaturated fatty acids.
We have found that taste cells responded not only to LCFAs, but also to MCFAs, which suggested the existence of a MCFA receptor(s) on taste cells. The real-time PCR data from our laboratory was consistent with taste cells containing a potential receptor for MCFAs, GPR84. GPR84 is a novel GPCR activated by MCFAs which is expressed predominantly in hematopoietic tissues, it has been proven that GPR84 was activated by saturated fatty acid with carbon chain lengths of 9-14 in CHO (Chinese Hamster Ovary) cells transfected with human GPR84. For the first time, I did a careful and systematic analysis of the ligand specificity of this receptor using ratiometric calcium imaging. In contrast with previous studies, I found that the ligands of GPR84 included not only the MCFAs from C₆ to C₁₂ but also oleic acid and arachidic acid, which were previously not thought to activate this receptor. The EC₅₀ value for lauric acid and capric acid were also in the same molar order of magnitude range seen in earlier work (Wang et al., 2006).

To determine directly the involvement of GPR84 in the initial recognition of MCFAs by taste cells, the changes in intracellular calcium and induced current responses to capric acid were recorded from the taste cells isolated from GPR84 knockout mice. As expected, knocking out of GPR84 significantly diminished the taste cell responses activated by capric acid. However, there were still small residual responses in both calcium rise and inward current, which suggested that there might be another receptor(s) responsible for MCFA taste transduction. Besides GPR84, GPR40 is another GPCR that has been shown to be activated by some MCFAs (Briscoe et al., 2003), however, it has never been studied extensively in taste cells. It is very possible that GPR40 can also contribute to MCFA responses in the gustatory system.
In the CTA assay with lauric acid as the conditioned stimulus, GPR84 knockout mice showed no avoidance to lauric acid with concentrations up to 600 µM. However, in the CTA test with capric acid as the conditioned stimulus, mice lacking GPR84 could not taste capric acid at concentrations up to 100 µM, but still avoided the capric acid with the concentration higher than 300 µM. The study in Chapter II of my dissertation showed that wild type mice could taste the lauric acid with the concentration as low as 30 µM (Figure 2.4). Compared to wild type mice, GPR84 deficient mice showed much lower taste sensitivity to MCFAs, which further confirmed the important role of GPR84 in MCFA taste transduction. The reason that GPR84 knockout mice could still taste high concentrations of capric acid may be because of the same aforementioned reason, which may be due to expression of GPR40 receptors. Different results in CTA assays between lauric and capric acid also suggested differences among various MCFAs. While the reason for this remains unclear at present, they may have different binding kinetics with multiple MCFA receptors.

It has been suggested that the signaling pathways downstream of GPR84 involved activation of a pertussis toxin-sensitive G_{i/o}, inhibition of cAMP production, and mobilization of calcium in CHO cells with transfected GPR84 receptors (Wang et al., 2006). The taste transduction signaling pathway after GPR84 activation has been investigated and discussed in Chapter II. The mechanism of MCFA taste transduction appears to be different from long-chain unsaturated fatty acid taste, where the downstream pathway involves TRPM5 and PLCβ2 activation. Blocking the activation of G-protein, protein kinase A, and protein kinase C significantly reduced the taste cell responses to MCFAs, which suggested their important roles in the downstream pathway of MCFA taste transduction (data shown
In conclusion, I have shown that GPR84 was expressed in taste cells from three taste papillae, and I identified MCFAs with carbon length from 6 to 12 and two long-chain unsaturated fatty acids (oleic acid and arachidic acid) as the ligands for GPR84 receptors. Based on the results of calcium imaging, patch clamp recording, and CTA assays, I found that GPR84 is an important receptor that mediates to a large degree the taste transduction of MCFAs. This new knowledge on the "fatty" taste receptors provided more information concerning how different types of fatty acids are detected in the gustatory system, and it makes us closer to solving the problems of overconsumption of fat, which is one of the main causes to dietary-induced obesity.

References


of K+ channels in taste receptor cells: gustatory cues for dietary fat. Am J Physiol
272:C1203-C1210.

Gilbertson TA, Khan NA (2014) Cell signaling mechanisms of oro-gustatory detection of


type M5 is essential for fat taste. J Neurosci 31:8634-8642.

Sattar N (2011) Obesity is associated with fatal coronary heart disease

Mattes RD (2009) Oral detection of short-, medium-, and long-chain free fatty acids in
humans. Chem Senses 34:145-150.

Meyer MR, Clegg DJ, Prossnitz ER, Barton M (2011) Obesity, insulin resistance and
diabetes: sex differences and role of oestrogen receptors. Acta Physiol (Oxf)
203:259-269


Pittman DW, Smith KR, Crawley ME, Corbin CH, Hansen DR, Watson KJ, Gilbertson TA
(2008) Orosensory detection of fatty acids by obesity-prone and obesity-resistant


Table 3.1. Sequence of PCR primers used to genotype the wild-type and knockout alleles of GPR84.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Sense</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Forward</td>
<td>ATTAGAAAGGCAGCTCAACAGATCG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCTCTATACCACTGAGGTGG</td>
</tr>
<tr>
<td>KO</td>
<td>Forward</td>
<td>GCAGCGCATCGCCTTCTATC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCTCTATACCACTGAGGTGG</td>
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</tbody>
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Figure 3.1. Gene targeting strategy to generate GPR84−/− mice.
Figure 3.2. Evaluation of GPR84 knockout mice. GPR84<sup>+/+</sup>, GPR84<sup>+-</sup>, and GPR84<sup>-/-</sup> mice were evaluated by their growth curves (A) and body fat composition (B). Data points were represented by mean ± SEM (n = 4-10). Asterisks indicate significant differences between two groups (p < 0.05).
Figure 3.3. GPR84 receptors are expressed in mouse taste cells. The mRNA expression of GPR84 and GPR120 in the taste cells from fungiform, foliate, and circumvallate papillae. Data are expressed relative to the expression of GPR120 in the circumvallate papilla (calibrator).
Figure 3.4. Ligand specificity of GPR84 receptor. Ligand specificity was investigated by functional calcium imaging in HEK293 cell line with inducible GPR84 receptors. Bath application of caproic (C$_{6}$:0), caprylic acid (C$_{8}$:0), capric acid (C$_{10}$:0), undecanoic acid (C$_{11}$:0), lauric acid (C$_{12}$:0), oleic acid (C$_{18}$:1), and arachidic acid (C$_{20}$:2) all elicited a robust and reversible calcium in the cells with induced GPR84 receptors, while they did not induce any calcium increase in the non-induced control cells.
Intracellular calcium increases in the cells with induced GPR84 receptor were measured using functional calcium imaging when applying a series of concentrations of lauric and capric acids. The area under response curve was calculated and fitted with a dose-response equation. The EC₅₀ value for lauric and capric acids were 27.4 ± 1.1 µM (n = 53 ~ 81) and 4.4 ± 1.3 µM (n = 17 ~ 55), respectively.
Figure 3.6. The inward currents induced by MCFAs in GPR84$^{-/-}$ taste cells were significantly lower than wild type taste cells. Focal application of capric acid (CA, 200 µM) induced an inward current in wild type taste cells (A), and this current was significantly reduced in GPR84$^{-/-}$ taste cells ($p < 0.01$). (C) Mean response ± SEM to capric acid for wild type taste cells (n = 17) and GPR84 knockout taste cells (n = 11).
Figure 3.7. The intracellular calcium rises elicited by MCFAs in GPR84\(^{-/-}\) taste cells were significantly lower than wild type taste cells. Capric acid (100 µM) induced an intracellular calcium increase in wild type (A) and GPR84\(^{-/-}\) taste cells (B). The area under the curve (mean ± SEM) of these two groups were calculated and compared via an unpaired two-tailed Student’s t-test, and the calcium responses in GPR84\(^{-/-}\) taste cells (n = 5) were significantly reduced compared to the responses in wild type taste cells (n = 26, \(p < 0.05\)).
Figure 3.8. GPR84<sup>−−</sup> mice showed lower taste sensitivities to MCFAs than wild type mice. Relative lick ratios (mean ± SEM) for different concentrations of lauric or capric acid when using lauric acid (A, n = 7 for LiCl group, and n = 3 for NaCl group) or capric acid (B, n = 10 for LiCl group, and n = 9 for NaCl group) as the conditioned stimulus in GPR84 knockout male mice on day two after CTA. Asterisks indicate significant differences between two groups (p < 0.05).
Are Medium-Chain Fatty Acids Effective Taste Stimuli?

Rodents and humans have a hedonic preference for foods rich in fatty acids. Over the last twenty years, it has been proven in numerous behavioral studies that rodents and humans are capable of detecting fatty acids independently even when olfaction, texture, and postingestive factors were eliminated, and moreover, free fatty acids are capable of eliciting cellular responses in native taste cells isolated from rodents. However, most of the studies have been focusing on the long-chain unsaturated fatty acids (LCFAs), and limited research has been done on the taste of saturated fatty acids, even though they exist in dietary food and pose more serious threats to human health. There is only one published paper that studied the gustatory response for medium-chain saturated fatty acids (MCFAs), which showed that human had a detectable oral taste threshold for one MCFA (lauric acid, C\textsubscript{12}) (Mattes, 2009). My research is the first study ever that used both cellular and behavioral assays to determine if one class of fatty acids, MCFAs, was an effective gustatory stimulus.

In Chapter II of my dissertation, I have shown that MCFAs induced both an intracellular free calcium rise and inward currents in the taste cells isolated from wild type mice. Increase of intracellular calcium concentration is a good indicator for cell activation, and calcium is a key ion involved in the neurotransmitter release. Inward currents can lead
to cell membrane depolarization, which is another hallmark for increased cell activity. Moreover, wild type mice showed aversion to MCFAs with low concentrations during conditioned taste aversion (CTA) test, which further confirmed that MCFAs were effective stimuli for rodents. In order to link between the cellular/molecular and behavioral events and provide a whole picture for the gustatory response to MCFAs, whole nerve recording on the gustatory afferent nerves (chorda tympani or glossopharyngeal nerves) while applying MCFAs over the tongue would be a nice complementary experiment. Chorda tympani (CT) and glossopharyngeal (GL) innervate the fungiform and circumvallate papillae, respectively, and the neurotransmitters release from the taste cells could activate both of these nerves. Since the taste cells from circumvallate express more GPR84 than fungiform, I expect that the GL nerve would elicit a more robust response.

Different types of taste cells have unique signaling pathways and functions during taste transduction. Therefore, the studies on which type of taste cells responds to MCFAs could provide clues for the MCFA taste transduction pathways. Somewhat surprisingly, I identified that both Type II and Type III taste cells could be activated by MCFAs, which suggested complicated signaling pathways might be involved in gustatory recognition of MCFAs.

Is GPR84 the Receptor for MCFAs?

During the past couple of decades, several fatty acid activated G protein-coupled receptors (GPCRs) have been deorphanized and they are able to bind fatty acids with different carbon lengths (Briscoe et al., 2003, Brown et al., 2003, Hirasawa et al., 2005, Wang et al., 2006). Among all the fatty acid activated GPCRs, GPR120 and GPR40 were
the only ones that had been studied in taste system and it has been showed that they played important roles in taste perception of LCFAs (Cartoni et al., 2010a). The reason that we started to study GPR84 in taste is because we wanted to investigate how saturated fatty acids were detected through taste pathways, and GPR84 has been proven to be activated by saturated fatty acids, although the ligands of GPR84 only include MCFAs (Wang et al., 2006). Another reason is that we found the mRNA of GPR84 was expressed in taste cells, which indicated that GPR84 is very possibly the main receptor that mediates the taste of MCFAs.

In Chapter III of my dissertation, I provided evidence regarding the expression of GPR84 mRNA in taste cells, however, I didn’t show the expression of GPR84 protein, which would be an additional support of GPR84’s involvement in taste. I tried immunohistochemical assays on the frozen tongue tissue sections from the wild type mice, but didn’t find specific binding of the anti-GPR84 antibody on the taste papillae. There are several reasons that may have contributed to this result. First, the antibodies I tried were anti-human GPR84 antibodies because there was no commercially available anti-mouse GPR84 antibody. The lack of a specific mouse antibody could reduce the binding ability of the antibody to the GPR84 receptors on mice, despite the fact the human and the murine GPR84 proteins are 85% identical in amino acid residues (Wittenberger et al., 2001). Another reason might be the expression of GPR84 protein on taste cells is not high enough to be detected in immunohistochemical assays. Based on the data from our lab, the mRNA expression of GPR84 is about 30% to 40% the expression of GPR120 on each papillae. Previous research validated the protein expression of GPR120 in taste cells (Cartoni et al.,
2010a, Montmayeur et al., 2011), so GPR84 protein is very likely expressed in taste cells, too. Moreover, my experiments on GPR84 knockout model proved its functional expression.

Which type of taste cells expresses GPR84? This can be elucidated by real-time PCR on GFP positive taste cells isolated from GFP-PLCβ2 or GFP-GAD67 mice. I hypothesize that GPR84 is expressed in both Type II and Type III taste cells because both types responded to MCFAs in calcium imaging and patch clamp recording assays.

A GPR84 knockout mouse model was utilized to investigate its involvement in MCFA taste transduction at both cellular and behavioral levels, and loss of GPR84 significantly decreased taste cell responses and taste sensitivities to MCFAs. All the results supported my hypothesis that GPR84 plays an essential role in MCFA transduction. Again, whole nerve recording on the gustatory afferent nerves of GPR84 knockout mice would be additional supporting evidence to provide the link between the taste cell and ultimate behavior.

In previous studies from our laboratory, RNA interference has been used to knock down GPR120 expression on STC-1 cell line, which is a mouse enteroendocrine cell line that expresses many taste signaling elements (Dyer et al., 2005). And the cellular response induced by linoleic acid was significantly inhibited when the expression of GPR120 was reduced (Shah et al., 2012). The same strategy can be used to directly measure the involvement of GPR84 receptors in MCFA transduction, but STC-1 cell line cannot be used as the study model because it does not express GPR84 receptors according to the data of our laboratory. Instead, the taste cell line derived from p53 deficient mice (Sako et al.,
2011) can be utilized in this fashion, and it is a better model to mimic the native taste cells. Although GPR84 knockout model showed significantly decreased cellular and behavioral responses, there were still residual signals left in calcium rise, inward current, and taste sensitivity to capric acid. Therefore, it is possible that there are other GPCRs or other signaling pathways that are involved in MCFA transduction. GPR40 can be activated by medium- and long-chain fatty acid, and it is expressed in taste cells. A previous study suggested that GPR40 can mediate the taste of long-chain fatty acids (Cartoni et al., 2010b). Therefore, GPR40 would be a good candidate as a potential additional MCFA receptor in the taste system. The involvement of GPR40 in MCFA transduction could be investigated using similar methods as used in my studies of GPR84.

What Is the Downstream Transduction Pathway for MCFAs?

The taste transduction pathways for LCFAs have been elucidated during the past decades. It is initiated by the interaction between LCFAs and their corresponding GPCRs. The G-proteins that coupled with the GPCRs are then activated, and the βγ subunits are released and stimulate the PLCβ2 activity, which cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). Then IP3 stimulates the release of intracellular calcium, which eventually activates the transient receptor potential cation channel 5 (TRPM5) (Liu et al., 2011). This pathway is an important reference for the possible GPR84-mediated MCFA taste transduction pathway. Therefore, I first investigated the involvement of the important components in this pathway. However, by using TRPM5 knockout model in both cellular and behavioral assays, I demonstrated that taste activation by MCFAs was not affected by knocking out of TRPM5.
Moreover, applying the antagonist of PLCβ2 did not inhibit the inward current induced by capric acid. All the data suggested that the MCFA taste transduction pathway is different than that for LCFA, and it may involve (a) more complicated signaling pathway(s).

So far, only a few published papers attempted to elucidate the signaling pathways following GPR84 activation. In leukocytes, GPR84 activation by MCFAs coupled with a pertussis toxin-sensitive Gi/o pathway, and followed by calcium mobilization and cAMP inhibition. α-Gustducin is a well-known G-protein highly expressed in taste cells, and it belongs to the Gi/o family (McLaughlin et al., 1992). It plays a key role in sweet, umami, and bitter taste transduction pathway. So I hypothesized that GPR84 coupled to α-gustducin during MCFA transduction. My studies showed that blocking G-protein activation using GDP-β-S abolished the inward current induced by capric acid, which suggested G-protein activation was coupled with MCFA transduction. However, it could not prove directly the involvement of α-gustducin in this process, unless we use a α-gustducin knockout model. So I studied a second messenger activated downstream of α-gustducin, phosphodiesterase (PDE), to investigate the role of α-gustducin indirectly. My results showed that inhibiting PDE did not affect the inward current induced by capric acid, suggesting that PDE was not involved in MCFA taste transduction, and in turn indicating that α-gustducin may not be involved, either.

My study also showed that inhibiting the activity of protein kinase A (PKA) significantly reduced the response of taste cell to MCFAs. Previous studies have shown that in taste cells, sucrose and other sugars activated Gs and released the Ga subunit, which then stimulated adenylyl cyclase (AC) to produce cAMP and eventually activated PKA.
PKA could phosphorylate and close the basolateral potassium channels to elongate the depolarization phase of taste cells (Avenet et al., 1988a, b, Naim et al., 1991, Margolskee, 2002, Abaffy et al., 2003). It is possible that the GPR84-mediated MCFA taste transduction pathway is similar with sugar. To prove this hypothesis, AC inhibitor can be used the same way as other antagonists in my study. The cAMP levels in the epithelial sheets from taste papillae can be measured with or without MCFA stimulation by using methods described before (Abaffy et al., 2003).

Blocking protein kinase C (PKC) slightly but significantly reduced the inward current produced by capric acid, which means a portion of the MCFA transduction depends on PKC activation, and it probably acts in a separate pathway from the one involving PKA. It has been suggested that PKC was required in the taste transduction of synthetic sweeteners (Varkevisser and Kinnamon, 2000), but no published research has ever studied how PKC was activated in taste cells. A well-known PKC activation pathway is as follows: PLC hydrolyses phosphatidylinositol 4,5 bisphosphate to produce IP3 and DAG, and then Ca²⁺ is released from ER store. Ca²⁺ and DAG together then work together to activate PKC (Nishizuka, 1995). However, my study showed that PLC was not involved in MCFA transduction, so it likely relies on a PLC-independent PKC activation pathway that remains to be elucidated.

Capric acid induced a rapidly activating inward current in wild type taste cells, but what is the nature of the ion channel that mediates this current? To characterize this current, a series of ion substitution experiments were performed to determine which ions this channel could conduct. Changing the extracellular concentration of sodium by ion
substitution did not shift the reversal potential to a predictable level (data not show), suggesting this channel was not exclusively sodium permeable, like TRPM5. Therefore, I suspected that this channel may be a calcium channel. Many transient receptor potential (TRP) channels are expressed in sensory organs, including taste, and most of them are calcium permeable (Gees et al., 2010). In order to check if this channel is calcium channel, the sodium, potassium, and chloride ions in the extracellular solution can be substituted with cesium during patch clamp recording, and using current ramps in the absence and presence of the MCFAs. The nature of the MCFA current was difficult to identify during the course of my experiments because I got equivocal results. One possible cause of this may lie in the fact that MCFAs induce different currents in Type II and Type III taste cells, and my patch clamp studies were not conducted on identifiable (i.e. GFP-labeled) cell types. In the future, one could perform ion substitution experiments during voltage clamp recording as stated above only on GFP positive taste cells from GFP-PLCß2 or GFP-GAD mice to elucidate the currents from Type II and Type III taste cells.

Cluster of differentiation 36 (CD36) is a protein that has been actively studied on the taste of fat. It is a transmembrane protein that is able to transport or LCFAs (Baillie et al., 1996, Zhang et al., 2003). The exact role of CD36 on taste transduction remains to be elucidated, however, a theory proposed that CD36 might work as a chaperone protein to facilitate free fatty acids binding their receptors, which then activated downstream taste transduction. The data in our laboratory showed that reduced expression of CD36 inhibited but not abolished the Ca^{2+} response to linoleic acid in human taste cell line. Moreover, CD36 knockout mice showed lower but not eliminated taste sensitivity to linoleic acid,
suggesting that CD36 is not essential but might facilitate the LCFA taste transduction. It would be interesting to check the involvement of CD36 in MCFA transduction by using CD36 deficient mice. Since CD36 is generally believed to only transport or bind LCFAs (Degrace-Passilly and Besnard, 2012), I anticipate the result that knocking out of CD36 will not affect the taste responsiveness to MCFAs.

In conclusion, my dissertation has shown that MCFAs are effective taste stimuli and at least part of the MCFA transduction pathway has been elucidated. So far, the research on fat taste has been limited to the LCFAs, and my research of the taste of MCFAs has added significantly to the understanding of fat taste transduction. These findings will help us identify potential strategies to reduce fatty acid intake, such as designing fat substitutes, and in turn, possibly helping to prevent dietary-induced obesity.

References


Briscoe CP, Tadayyon M, Andrews JL, Benson WG, Chambers JK, Eilert MM, Ellis C,


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PUBLICATIONS

Radioimmunotherapy of Human Colon Cancer Xenografts with $^{131}$I-labeled Anti-CEA Monoclonal Antibody.

Comparison of in vitro and in vivo Characteristics of $^{99m}$Tc-labeled Cyclic RGD Dimers with PEG4/2PEG4 linkers
Journal of Nuclear and Radiochemistry. 2010.32(5):

$^{125}$I/$^{111}$In Labeled Anti-EGFR Monoclonal Antibody Panitumumab: Preparation and Biodistribution Evaluation in Normal Mice


CONFERENCE ABSTRACTS

Radioimmunotherapy of EGFR-positive tumors with 90Y-labeled Panitumumab
2. Liu Y, Gilbertson TA
Medium-chain fatty acid transduction in mouse taste cells is TRPM5-independent
AChemS 34th Annual Meeting, CA, 2012

3. Liu Y, Gilbertson TA
Ligand specificity of orphan G protein-coupled receptor 84
AChemS 35th Annual Meeting, CA, 2013

4. Liu Y, Gilbertson TA
Taste cell responses to medium chain saturated fatty acids are mediated by GPR84

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