DISSERTATION

CHARACTERIZATION OF IP_3 RECEPTORS IN BITTER TASTE TRANSDUCTION

Submitted by

Tod R. Clapp

Department of Biomedical Sciences

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY TOD R. CLAPP ENTITLED *CHARACTERIZATION OF IP_3 RECEPTORS IN BITTER TASTE TRANSDUCTION* BE ACCEPTED AS FULLFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTORATE OF PHILOSOPHY.

Committee on Graduate Work

(Please print name under signature)

Dr. Kurt G. Beam

Dr. Kathryn M. Partin

Dr. Diego Restrepo

Dr. Michael M. Tamkun

Dr. Sue C. Kinnamon

Department Head

ABSTRACT

CHARACTERIZATION OF IP_3 RECEPTORS IN BITTER TASTE TRANSDUCTION

An important second messenger in taste transduction is inositol trisphosphate (IP_3), which has been implicated in the transduction of bitter, sweet and umami compounds. Although the importance of IP_3 is known its target has not been described previously. Here we provide evidence that the downstream target of IP_3 is the type III IP_3 receptor (IP_3R3) in taste cells. This receptor is located on the endoplasmic reticulum and allows Ca^{2+} to pass from the ER into the cytosol.

Rat taste buds contain several types of taste cells distinguishable by morphological characteristics, however, the physiological roles of these cell types is not clear. Knowing that IP_3 is involved in bitter, sweet, and umami taste and that its target is IP_3R3, we have utilized DAB immunoelectron microscopy to determine which cell types express IP_3R3. Our results indicate that a large subset of Type II and small subset of Type III cells display IP_3R3

immunoreactivity, suggesting these cell types are responsible for bitter, sweet, and umami transduction. Interestingly, many immunoreactive Type II cells lacked conventional synapses with nerve fibers, but many were found with subsurface cisternae (SSC) of smooth endoplasmic reticulum at close appositions with nerve fibers.

Of the taste transduction pathways, the role of IP_3 in bitter taste is best understood. Bitter compounds activate a heterotrimeric G-protein consisting of $G\alpha_{gustducin}$, $G\beta3$, and $G\gamma_{13}$. The $G\beta3\gamma_{13}$ subunits raise cytosolic IP_3 by activation of $PLC\beta_2$, while $G\alpha_{gustducin}$ decreases cyclic adenosine monophosphate (cAMP) via activation of phosphodiesterase. $G\alpha_{gustducin}$ null mice show a marked decrease in their ability to detect bitter substances, although the precise role of $G\alpha_{gustducin}$ in the transduction process is not understood. The finding that IP_3R3 is the dominant and perhaps only IP_3 receptor isoform in taste cells is intriguing because work in other systems has shown that IP_3R3 is inhibited by cAMP dependent phosphorylation, suggesting a possible role for $G\alpha_{gustducin}$ in taste transduction. Further, phosphorylation has been shown to inhibit $PLC\beta_2$, another essential component in bitter transduction. In the last chapter we show

preliminary data investigating a possible role of $G\alpha_{gustducin}$ in modulation of $PLC\beta_2$ and IP_3R3.

Tod R. Clapp

Department of Biomedical Sciences

Colorado State University

Fort Collins, CO 80523

Spring 2004

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CHAPTER 1

INTRODUCTION

The sense of taste, or gustation has evolved allowing organisms to detect chemical stimuli in their environment. Taste plays an important role in an organism's survival, helping it select nutritionally advantageous compounds such as sugars and salts and avoid potentially harmful substances such as toxic or acidic compounds. Most animals can distinguish five known taste modalities including salty, sour, bitter, sweet, and umami, which is the taste of amino acids such as monosodium glutamate.

1.1 General anatomy of gustation

In mammals, the sensation of taste begins when sapid stimuli interact with taste receptor cells housed in the epithelium of the oral cavity (for reviews see (Witt and Reutter, 1996; Finger, 2000). These taste receptor cells are organized into discrete end organs called taste buds. Each taste bud contains 50-150 polarized neuroepithelial cells that extend from the basal lamina to the surface of the tongue, where apical microvilli protrude into the oral cavity (Kinnamon, 1987). In vertebrates, lingual taste buds are housed in connective tissue structures called papillae. Murine fungiform papillae contain 1-2 taste buds, are located on the anterior surface of the tongue, and are innervated by the chorda tympani branch of cranial nerve VII. The circumvallate and foliate papillae typically contain several hundred taste buds and are located in the posterior medial and lateral regions of the tongue, respectively (for reviews see (Murray, 1973; Witt and Reutter, 1996; Finger, 2000). Foliate papillae are innervated by both the chorda tympani branch of the facial nerve (CN VII) and the glossopharyngeal nerve (CN IX), while innervation to circumavallate papillae is provided solely by the glossopharyngeal nerve.

1.2 Distinct cell types found in taste epithelium

Taste receptor cells can be classified into four distinct types based on ultrastructural characteristics and immunocytochemical markers. Basal cells are round proliferative cells found in the basolateral region of a taste bud (Farbman,

1965; Murray, 1973; Delay et al., 1986; Roper, 1989). The other three cell types, all candidates for taste transduction are elongate cells that extend from the basal lamina to the taste pore where they extend apical microvilli into the oral cavity. Type I, also known as dark cells, possess an electron-dense cytoplasm. These are slender in shape with several long microvilli of various lengths extending into the oral cavity. Electron micrographs of Type I cells often show them wrapping around other cell types much like glia (Murray, 1973; Pumplin et al., 1997). The nuclei of these cells may be invaginated and typically contains prominent heterochromatin adherent to the inner leaflet of the nuclear lamellae. Perhaps the most distinguishing feature of a Type I cell is the presence of many 100-400 nm electron-dense granules in the apical cytoplasm of the cell. Type II, or light cells are more electron-lucent, spindle- or pyriform-shaped cells with large, round nuclei that lack the heterochromatin and invaginations characteristic of Type I cells. The apical cytoplasm of Type II cells typically contains electron-lucent, swollen cisternae of smooth endoplasmic reticulum and lacks the dense granules found in Type I cells. Several short microvilli of uniform length protrude from the apical region of Type II cells into the taste pore. Type III, sometimes referred to as intermediate cells have a slender, spindle shape and have an electron lucent cytoplasm similar to type II cells. These cells can be distinguished from Type II cells by an elongate nucleus with prominent invaginations and the presence of large, dense-cored vesicles around the nuclear region of the cytoplasm. One very distinguishable feature of Type III cells is the single, large, blunt microvillus that extends into the taste pore. Type III cells also have conventional synapses

onto nerve processes that have been observed in both mouse (Takeda, 1976) and rat (Takeda and Hoshino, 1975; Yee et al., 2001).

Several immunocytochemical markers have been employed to help differentiate between Type I, Type II, and Type III cells at the light level. Antigen H is believed to be a surface marker present on all Type I cells (Pumplin et al., 1997; Pumplin et al., 1999), which also express the glial glutamate transporter GLAST (Lawton et al., 2000). Currently there is no single immunocytochemical marker that describes the complete population of Type II cells, however, $G\alpha_{gustducin}$ is present in a subset of Type II cells (Boughter et al., 1997; Cho et al., 1998; Yang et al., 2000a; Yang et al., 2000b) and the blood group antigen A is a cell surface marker on a separate subset of Type II cells. Several markers label Type III cells. Serotonin (5-HT) immunoreactivity is present in a subset of rat Type III taste cells (Kim and Roper, 1995) and ubiquitin carboxyl terminase (protein gene product 9.5, PGP9.5) is found in a separate subset, as well as a small subset of Type II cells (Yee et al., 2001). Neural cell adhesion molecule (NCAM) is present in most or all intermediate cells (Nelson and Finger, 1993), which we now believe are Type III cells. Brain derived neurotrophic factor (BDNF) immunoreactivity is also present in the majority, if not all Type III cells encompassing those that express NCAM but can also be seen in a small population of Type II cells (Yee et al., 2003). Although these immunocytochemical markers lend insight into cell type they do not completely encompass all cells of a particular type and thus are only used as a guide.

1.3 Taste transduction

In general, the transduction of tastants is initiated when sapid stimuli interact with apical microvillar membranes of taste receptor cells, causing depolarization and/or increases in intracellular Ca^{2+} from both extracellular and intracellular sources (Ogura, 2002). This increase in intracellular Ca^{2+} is believed to result in release of neurotransmitter onto gustatory afferent fibers (for review see (Glendinning et al., 2002). Simple stimuli such as salts and acids interact directly with ion channels to cause depolarization and Ca^{2+} influx, whereas complex stimuli such as sugars, amino acids, and bitter compounds act via G protein-coupled receptors and a variety of second messengers (Kinnamon, 2000).

Although bitter, sweet, and umami stimuli all activate G-protein signaling cascades the downstream events of the bitter signaling path are best understood (Lindemann, 1996; Gilbertson et al., 2000; Glendinning, 2000). Bitter transduction is initiated when bitter substances bind G-protein coupled receptors of the T2R (also called TRB) class located on the apical membrane of a subset of taste receptor cells (Adler et al., 2000; Chandrashekar et al., 2000; Matsunami et al., 2000). These receptors couple to heterotrimeric G-proteins usually consisting of $G\alpha_{gustducin}$ (Ruiz-Avila et al., 1995; Ming et al., 1998) and its $\beta\gamma$ partners, $\beta3\gamma_{13}$ (Rossler et al., 1998; Huang et al., 1999), although other alpha subunits have been implicated (Caicedo et al., 2003). Upon activation, $\beta3\gamma_{13}$ activates phospholipase $C\beta_2$ ($PLC\beta_2$), which then cleaves phosphotydil inositol bisphosphate (PIP_2) into diacyl glycerol (DAG) and inositol trisphopsphate (IP_3)

(Rossler et al., 1998). IP_3 activates inositol trisphosphate receptors (IP_3R's) usually located on the membrane of smooth endoplasmic reticulum (ER)(Otsu et al., 1990), causing release of Ca^{2+} into the cytosol. $G\alpha_{gustducin}$ activates phosphodiesterase (PDE), which converts cAMP to 5'AMP (Yan et al., 2001). The role of the decreased intracellular cAMP is not yet known, however in pancreatic acinar cells cAMP- dependent phosphorylation modulates the effectiveness of the IP_3 receptor (Giovannucci et al., 2000a; Giovannucci et al., 2000b), and in COS-7 cells it modulates the efficacy of $PLC\beta_2$ (Liu and Simon, 1996).

1.4 Transient receptor potential receptors

Calcium entry into the cytosol can occur either from intracellular sources or from the extracellular milieu. In taste cells bitter compounds such as denatonium cause release of intracellular Ca^{2+} via IP_3 and IP_3R's, while an unknown store operated channel mediates influx (Ogura et al., 1997; Ogura et al., 2002). Recently a transient receptor potential (TRP) channel (TRPM5) was identified in taste receptor cells (Perez et al., 2002; Perez et al., 2003) and knockout studies suggest it is essential for bitter, sweet, and umami taste transduction (Zhang et al., 2003). This is of particular interest because some TRP channels function in store operated Ca^{2+} entry (Putney and McKay, 1999; Clapham, 2002; Minke and Cook, 2002; Voets et al., 2002). Although it has been suggested that TRPM5 is the store operated channel in taste receptor cells (Ogura et al., 2002; Perez et al., 2002; Perez et al., 2003), recent expression

studies with heterologous cells have shown that TRPM5 is impermeable to Ca^{2+}, suggesting these channels may serve a function other than store operated Ca^{2+} entry (Hofmann et al., 2003; Liu and Liman, 2003; Prawitt et al., 2003). Further studies will be required to determine if TRPM5, or a heterotetramere that includes TRPM5, operates as a store operated channel in taste cells.

1.5 IP_3 receptors

IP_3 is an important second messenger in taste receptor cells and has been implicated in bitter, sweet (for review see (Lindemann, 2001), and umami (Ninomiya et al., 2000) taste transduction. We know that activation of $PLC\beta_2$ in bitter sensitive cells cleaves PIP_2 into both IP_3 and DAG. While it has been well established in other systems that DAG activates PKC's this has not yet been directly demonstrated in taste cells. Although production of IP_3 in taste receptor cells has been demonstrated no one has previously identified its targets, although they are presumed to be IP_3 receptors found on the endoplasmic reticulum membrane.

Upon binding, IP_3 receptors open allowing Ca^{2+} flux into the cytosol, where Ca^{2+} can mediate a variety of effects including expression of transcription factors, apoptosis (Berridge et al., 2000), and transmitter release (Tse et al., 1997; Fomina and Nowycky, 1999). Upon binding IP_3 these receptors flux Ca^{2+} from the internal stores of the ER lumen to the cytoplasm. IP_3R's are predominantly located within the membrane of ER but some have also been reported in the plasma membrane (PM) and may play a role in Ca^{2+} flux from the extracellular

milieu (for review see (Putney, 1997). IP_3R's are integral membrane proteins encoded by genes approximately 2700 amino acids in length (Furuichi et al., 1994) that assemble as tetrameres (Supattapone et al., 1988; Maeda et al., 1991). Each subunit traverses the membrane six times leaving both the N and C termini in the cytosol. The N-terminus houses the IP_3 binding domain while the C-terminus anchors the protein to the membrane, forms the pore region, and is involved in formation of the tetramere. The large central region of the receptor protein contains the regulatory domain with several binding sites for Ca^{2+}, PKA, tyrosine kinase (TK), ATP, and accessory proteins (Patel et al., 1999).

There are at least three known isoforms of IP_3R's encoded by three different genes with approximately 60-80% homology (Mignery et al., 1989; Sudhof et al., 1991; Blondel et al., 1993; Furuichi et al., 1994; Maranto, 1994; Joseph et al., 1995; De Smedt et al., 1997). Isoforms are differentially expressed in tissues with different expression ratios and exist as both homo and heterotetrameres (Maranto, 1994; Fujino et al., 1995; Furuichi and Mikoshiba, 1995). The three isoforms have different affinities for IP_3 with a relative order of affinity of $IP_3R2>IP_3R1>IP_3R3$ (Sudhof et al., 1991; Maranto, 1994; Newton et al., 1994; De Smet et al., 1999) and also show markedly different regulation by Ca^{2+}. IP_3R1 has a biphasic relationship with Ca^{2+} in that at low Ca^{2+} concentrations potentiate Ca^{2+} release and high concentrations inhibit release (Taylor and Marshall, 1992; Iino and Tsukioka, 1994; Mak et al., 1998) IP_3R2 and IP_3R3 on the other hand appear to only be potentiated by Ca^{2+} (Hagar et al., 1998; Ramos-Franco et al., 1998), however this is controversial, as others have reported that

high Ca^{2+} concentrations also inhibit IP_3R3 (Mak et al., 2001). Phosphorylation also affects IP_3R's ability to permeate Ca^{2+} and affects each isoform differently. Both IP_3R1 and IP_3R2 show potentiation in response to phosphorylation (Bruce et al., 2002; Dyer et al., 2003) whereas IP_3R3 is inhibited by phosphorylation (Giovannucci et al., 2000a; Straub et al., 2002). There also appears to be variation among the isoforms in how they respond to cytosolic ATP levels. IP_3R1's display enhanced gating when cytosolic ATP levels increase whereas IP_3R3's show an increase in open probability to low cytosolic ATP levels (Hagar et al., 1998; Maes et al., 2000; Mak et al., 2001). The widely expressed and studied Ca^{2+} binding protein calmodulin (CAM) appears to have a binding site on all three isoforms (Putney, 1997) and has been suggested to exert tonic inhibition of IP_3R's under physiological conditions (Patel et al., 1999).

1.6 Hypotheses and specific aims

The general hypothesis for this dissertation is: IP_3, generated in response to bitter, sweet, and umami stimuli, binds the Type III IP_3 receptor, which is co-expressed with other signaling components in Type II taste cells. The role of $G\alpha_{gustducin}$ is to modulate this pathway. The specific aims are: (1) To identify the target IP_3 receptor subtype(s) expressed in taste cells; (2) To determine the taste cell type that expresses IP_3R's; (3) To determine if activation of $G\alpha_{gustducin}$ modulates the IP_3 pathway.

1.7 Organization of dissertation

This dissertation is divided into four chapters. Chapter one provides an introduction to taste. Chapter two is a paper entitled "Immunocytochemical evidence for co-expression of Type III IP_3 receptor with signaling components of bitter taste transduction", which has been published in BMC Neuroscience 2001, 2:6. This paper addresses specific aim 1; authorship is T.R. Clapp, L.M. Stone, R.F. Margolskee, and S.C. Kinnamon. Chapter three is a paper entitled "Morphological characterization of rat taste receptor cells that express components of the phospholipase C signaling pathway", which has been published in The Journal of Comparative Neurology 2004, 468:311-321. This paper addresses specific aim 2; authorship is T.R. Clapp, R. Yang, C.L. Stoick, S.C. Kinnamon, J.C. Kinnamon. Chapter four contains preliminary experiments to determine whether $G\alpha_{gustducin}$ modulates components of the PLC signaling cascade and addresses specific aim 3.

Chapter 2

IMMUNOCYTOCHEMICAL EVIDENCE FOR CO-EXPRESSION OF TYPE III IP_3 RECEPTOR WITH SIGNALING COMPONENTS OF BITTER TASTE TRANSDUCTION

2.1 Abstract

Background: Taste receptor cells are responsible for transducing chemical stimuli into electrical signals that lead to the sense of taste. An important second messenger in taste transduction is IP_3, which is involved in both bitter and sweet transduction pathways. Several components of the bitter transduction pathway have been identified, including the T2R/TRB taste receptors, phospholipase C β_2, and the G protein subunits $G\alpha_{gustducin}$, $G\beta 3$, and $G\gamma_{13}$. However, the identity of the IP_3 receptor subtype in this pathway is not known. In the present study we

used immunocytochemistry on rodent taste tissue to identify the IP_3 receptors expressed in taste cells and to examine taste bud expression patterns for IP_3R3.

Results: Antibodies against Type I, II, and III IP_3 receptors were tested on sections of rat and mouse circumvallate papillae. Robust cytoplasmic labeling for the Type III IP_3 receptor (IP_3R3) was found in a large subset of taste cells in both species. In contrast, little or no immunoreactivity was seen with antibodies against the Type I or Type II IP_3 receptors. To investigate the potential role of IP_3R3 in bitter taste transduction, we used double-label immunocytochemistry to determine whether IP_3R3 is expressed in the same subset of cells expressing other bitter signaling components. IP_3R3 immunoreactive taste cells were also immunoreactive for $PLC\beta_2$ and γ_{13}. Alpha-gustducin immunoreactivity was present in a subset of IP_3R3, $PLC\beta_2$, and γ_{13} positive cells.

Conclusions: IP_3R3 is the dominant form of the IP_3 receptor expressed in taste cells and our data suggest it plays an important role in bitter taste transduction.

2.2 Introduction

Taste receptor cells are specialized epithelial cells, which are organized into discrete endorgans called taste buds. Typical taste buds contain 50-100 polarized taste cells, which extend from the basal lamina to the taste pore, where apical microvilli protrude into the oral cavity. The basolateral membrane forms chemical synapses with primary gustatory neurons (Fig. 2.1A). In mammals, lingual taste buds are housed in connective tissue structures called papillae.

Fungiform papillae are located on the anterior two-thirds of the tongue and typically contain 1-2 taste buds each. Vallate and foliate papillae are found on the posterior tongue and house several hundred taste buds each. Taste transduction begins when sapid stimuli interact with the apical membrane of taste cells, usually resulting in taste cell depolarization, calcium influx, and transmitter release onto gustatory afferent neurons. Simple stimuli, such as salts and acids depolarize taste cells by direct interaction with apical ion channels. In contrast, complex stimuli, such as sugars, amino acids, and most bitter compounds bind to G protein coupled receptors, initiating intracellular signaling cascades that culminate in Ca^{2+} influx or release of Ca^{2+} from intracellular stores (Lindemann, 1996; Gilbertson et al., 2000; Glendinning, 2000).

Inositol 1,4,5-trisphosphate (IP_3) is an important second messenger in both bitter and sweet taste transduction. In both pathways, activation of taste receptors stimulates a G protein-coupled cascade resulting in activation of phospholipase C (PLC), which cleaves phosphoinositol bisphosphate (PIP_2) to produce the second messengers IP_3 and diacylglycerol (DAG). The soluble messenger IP_3 binds to receptors located on calcium store membranes, causing release of calcium into the cytosol, while DAG remains in the membrane, where it can activate downstream effectors. While little is known about the role of IP_3 in sweet taste transduction, considerable data indicate that IP_3 plays an important role in bitter transduction. The first evidence for the involvement of IP_3 in bitter transduction was obtained by Akabas et al. (Akabas et al., 1988), who used Ca^{2+} imaging to show that the bitter stimulus denatonium causes release of Ca^{2+} from

intracellular stores. More recently, biochemical measurements have shown that several bitter compounds elevate IP_3 in taste tissue (Hwang et al., 1990; Spielman et al., 1994; Spielman et al., 1996; Huang et al., 1999).

Other studies, however, suggested that a decrease in cAMP, rather than an increase in IP_3, mediated bitter transduction. In 1992, a chemosensory specific G protein, $G\alpha_{gustducin}$, was identified in a subset of taste cells (McLaughlin et al., 1992). Alpha-gustducin, which is closely related to the rod and cone transducins, activates PDE to reduce intracellular levels of cAMP (Yan et al., 2001). Evidence for $G\alpha_{gustducin}$'s role in bitter taste came from knockout studies, in which a targeted deletion of $G\alpha_{gustducin}$ resulted in mice with a reduced sensitivity for bitter compounds (Wong et al., 1996). More recently, a variety of bitter compounds have been shown to activate $G\alpha_{gustducin}$ in biochemical assays (Ruiz-Avila et al., 1995; Wong et al., 1996; Ming et al., 1998), causing a decrease in intracellular cAMP levels via activation of PDE (Yan et al., 2001). It is now known that individual bitter receptors modulate both the IP_3 and cAMP pathways (Fig. 2.1B). Bitter compounds bind to a family of G protein-coupled receptors called the T2R (Adler et al., 2000; Chandrashekar et al., 2000) or TRB (Matsunami et al., 2000) receptors, which activate a heterotrimeric G protein consisting of $G\alpha_{gustducin}$ and a $\beta\gamma$ complex containing $\beta 3$ (Rossler et al., 2000) and γ_{13} (Huang et al., 1999). Alpha-gustducin activates PDE to decrease intracellular levels of cAMP (Yan et al., 2001), while its $\beta\gamma$ partners stimulate $PLC\beta_2$ to produce IP_3 and DAG (Rossler et al., 1998).

Although it is clear that IP_3 binds to receptors located on intracellular Ca^{2+} stores, the specific identity of IP_3 receptors in taste cells is not known. There are at least 3 known isotypes of IP_3 receptors encoded by different genes (Patel et al., 1999). Each protein product is about 300 kDa. Four subunits assemble to form a functional channel. Both homomultimeres and heteromultimeres have been reported (Patel et al., 1999). The N terminus of each subunit houses the IP_3 binding domain while the C terminus anchors the protein to the membrane, is involved in the formation of the tetrameric protein, and forms the Ca^{2+} pore region. The general structure of each isoform is similar, however they differ in primary sequence, distribution, regulation, and IP_3 affinity (Patel et al., 1999).

In this study we used immunocytochemical methods to determine which IP_3R isoform is expressed in taste cells and to examine the expression patterns of IP_3 receptors relative to other proteins known to be important for taste transduction. We report that the Type III IP_3 receptor is the dominant isoform expressed in rodent taste cells and that it is primarily found in the same subset of taste cells as other known signaling components of bitter transduction. A preliminary account of this work was published in abstract form (Clapp TR, 2000).

2.3 Materials and Methods

Animals: Adult male Sprague Dawley Rats and adult C57/B1 male or female mice were obtained from Charles River Laboratories (Wilmington, MA). Animals

were cared for in compliance with the Colorado State University Animal Care and Use Committee.

Tissue preparation: Rats or mice were deeply anesthetized by intraperitoneal injections of Sodium Pentobarbitol, 40 mg/Kg (Veterinary Laboratories, Inc., Lenexa, KS). Following anesthetization animals were injected intracardially with heparin (Elkins-Sinn, Inc., Cherry Hill, NJ) and 1% sodium nitrate. Rats were perfused with 80 ml of 4% paraformaldehyde and mice with 30 ml. Following perfusion tongues were removed and immediately placed into fresh 4% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) in 0.1 M phosphate buffer for approximately twenty minutes. Tongues were then put into a 20% sucrose solution in 0.1 M phosphate buffer overnight for cryoprotection. Forty micron sections were cut on a Leitz 1729 digital Kryostat and collected in 0.1 M phosphate buffered saline (PBS, pH7.2). Following sectioning, the slices were washed in PBS three times for ten minutes each at room temperature. Antigen retrieval was performed by placing sections into a 10 mM sodium citrate solution at 80°C for 30 minutes. This was done to help disrupt protein cross-bridges formed by formalin fixation and expose antigen binding sites. In some experiments the incubation time in sodium citrate was reduced to 5 minutes, and labeling was still apparent. All sections were incubated in blocking solution for 1-2 hours at room temperature. Blocking solution contained 0.3% Triton X-100, 1% normal goat serum, and 1% bovine serum albumin in 0.1 M PBS. All chemicals were purchased from Sigma Chemical Corporation (St. Louis, MO) unless otherwise noted.

Antibodies: Polyclonal antibodies raised in rabbit against the sequence N_{1829}KKKDDEVDRDAPSRKKAKE$_{1848}$ near the COOH-terminal domain of human IP_3R1 were purchased from Affinity Bioreagents, Inc. (Golden, CO, cat #PA1-901). Polyclonal antibodies raised in rabbit against a synthetic peptide with a sequence derived from the cytoplasmic, NH_2-terminal domain of the rat IP_3R2 protein (E_{317}LNPDYRDAQNEGKTVRD$_{334}$), were also purchased from Affinity Bioreagents, Inc. (Golden, CO, cat #PA1-904). Monoclonal mouse anti-IP_3 R3 was purchased from Transduction laboratories (Lexington, KY, cat #131220). Monoclonal mouse anti-IP_3R3 recognizes the peptide sequence 22-230. Rabbit anti-α-gustducin (cat #SC-395) directed against a peptide fragment containing amino acids 93-113 of α-gustducin; and rabbit anti-PLCβ$_2$ (cat #SC206) were obtained from Santa Cruz laboratories (Santa Cruz, CA). Rabbit anti-γ_{13} was prepared as described previously (Huang et al., 1999). All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). These included: Rhodamine Red X anti-rabbit (# 111-295-045), Rhodamine Red X anti-mouse (# 115-295-146), Cy-5 anti-mouse (# 115-175-146), Flourescein (FITC) anti-rabbit (# 711-095-152). Cy-5 anti mouse antibodies were tested on mouse tissue prior to these experiments to insure no background labeling.

Single label Immunocytochemistry: Sections from rat circumvallate papillae were incubated with primary antibodies overnight at 4°C. Controls in which the primary antibodies were excluded were included in each experiment. The primary antibodies rabbit anti-IP_3R1 and rabbit anti-IP_3R2 were diluted to 1:100 in

blocking solution. Mouse anti-IP_3R3 was used at 1:50 in blocking solution. Following overnight incubation in primary antibodies, sections were washed three times for ten minutes each in PBS at room temperature. Rabbit anti-IP_3R1, rabbit anti-IP_3R2, and no primary antibody control sections were then incubated with the secondary antibody Rhodamine Red X anti-rabbit (1:100 in blocking solution) at room temperature for two hours. Mouse anti-IP $_3$R3 labeled sections, were incubated in Rhodamine anti-mouse. Following incubation with secondary antibodies, sections were washed three times for ten minutes in PBS and mounted on RITE-ON micro slides (Becton, Dickinson and Company, Portsmouth, NH) using Flouromount-G (Southern Biotechnology Associates, Inc., Birmingham, AL, cat# 0100-01) and coverslipped (VWR Scientific, Media, PA). Slides were stored at 4°C.

Double Label Immunocytochemistry: Double-labeled experiments involved incubations with two primary antibodies and subsequent incubation with appropriate secondary antibodies. Rabbit anti-α-gustducin was used at 1:500 in blocking solution. Both rabbit anti-PLCβ_2 and rabbit anti-γ_{13} were used at 1:1000 in blocking solution. Following incubation with primary antibodies, sections were washed in PBS three times for ten minutes each. Both rabbit anti-IP_3R1 and rabbit anti-IP_3R2 labeled sections were then treated with Rhodamine Red X anti rabbit antibodies (1:100). For IP $_3$R3 double-labeled sections, Cy-5 anti-mouse secondary antibodies were applied. Rabbit anti-α-gustducin was visualized using FITC anti-rabbit (1:100) secondary antibodies. Both rabbit anti-PLCβ_2 and rabbit anti-γ_{13} were labeled with FITC anti rabbit (1:100). Incubations with secondary

antibodies were done at room temperature for two hours. Sections were then washed in PBS three times for ten minutes each and mounted using Flouromount-G and cover slips. Slides were stored at 4°C.

Imaging: Lingual sections were viewed with an Olympus Fluoview laser scanning confocal microscope. Sequential scanning techniques were used for some double-label experiments and showed no differences from simultaneous scans. There is no overlap between the excitation and emission spectra for the FITC and Cy5 secondary antibodies used in the double label experiments. Images were captured with an Olympus Fluoview Confocal Laser Scanning Microscope. Lasers included Argon 488 nm, HeNe 543 nm, and HeNe 622. Fluoview software was used for data acquisition. Images were processed and printed using Photoshop 6.0 software.

2.4 Results

IP_3 Receptor Isotypes: Forty-micron thick rat sections containing circumvallate papillae were exposed to either anti-IP_3R1, anti-IP_3R2, or anti-IP_3R3 antibodies and appropriate secondary antibodies. Laser scanning confocal microscopy of the resultant sections was used to examine the distribution of IP_3 receptor immunoreactivity in rat circumvallate taste buds. Labeling for IP_3R1 and IP_3R2 was negligible (Figs. 2.2A, B). In contrast, immunoreactivity to IP_3R3 was robust and present in a large subset of taste cells (Fig. 2.2C). Generally,

immunoreactivity extended throughout the cytoplasm of labeled cells. Immunoreactivity was found only in taste buds and was not present in the lingual epithelium surrounding taste buds. Primarily intragemmal taste cells were labeled, proliferative basal cells of the taste buds did not appear to be labeled. In addition, gustatory nerve fibers did not appear to be labeled. Controls in which the primary antibody was omitted showed no specific labeling (Fig. 2.2D).

Co-expression of IP_3R3 with known bitter signaling components:

To determine if IP_3R3 could be involved in bitter taste transduction, we performed double-label immunocytochemistry with antibodies to IP_3R3 and components of the bitter signaling pathway. For most experiments, mouse as well as rat tissues were examined. Exposure of tissue to antibodies against α-gustducin and IP_3R3 showed that all α-gustducin immunoreactive (IR) cells were also immunoreactive for IP_3R3, however, a subset of IP_3R3-IR taste cells lacked α-gustducin-IR (Fig. 2.3). Further analysis with antibodies to other signaling components of the bitter transduction pathway are shown in Figs. 2.4-2.5. Immunoreactivity for $PLC\beta_2$ and IP_3R3 showed nearly complete coincidence of labeling (Fig. 2.4). Immunoreactivity for $PLC\beta_2$ extended throughout the cytoplasm, as did immunoreactivity for IP_3R3. However, some taste cells appeared to differ in the degree of labeling for each antibody, with some cells showing more intense label for IP_3R3 and others for $PLC\beta_2$. We also compared IP_3R3 IR with γ_{13} IR in rat and mouse circumvallate taste buds. As shown in Fig. 2.5, nearly complete co-localization was again observed for both antigens. Results from $PLC\beta_2$ and γ_{13}

double label experiments were similar for mouse and rat taste buds. Taken together, these immunocytochemical data indicate that IP_3R3 is the predominant isoform of IP_3 receptor expressed in taste cells, and that it is found in the same subset of taste cells as other components known to be involved with bitter taste transduction.

2.5 Discussion

IP_3R3 is heavily expressed in a large subset of vallate taste cells of both mouse and rat, suggesting that IP_3R3 plays a similar role in both species. IP_3R3 appears to be located throughout the cytoplasm of taste cells, consistent with its expected location on the smooth endoplasmic membrane (Otsu et al., 1990). In other cells, IP_3 receptors have also been found on the plasma membrane (Patel et al., 1999), but because of heavy cytoplasmic labeling, we were unable to resolve whether it was also located on the plasma membrane. One caveat is that antigen retrieval was necessary to observe IP_3R3 labeling. However, using this method with α-gustducin, $PLC\beta_2$, and γ_{13} antibodies did not alter their immunoreactivities; similar results were obtained with and without antigen retrieval. Thus, we do not believe that antigen retrieval compromised our interpretation of the results.

Taste cells expressing IP_3R3 have an elongate, bipolar morphology, suggestive of Type II taste cells (Pumplin et al., 1997). Indeed, a subset of the IP_3R3 immunoreactive taste cells is also immunoreactive for $G\alpha_{gustducin}$, which has been identified exclusively in Type II cells (Yang et al., 2000b). However, whether

IP_3R3 is expressed exclusively in Type II cells awaits further investigation. It is noteworthy that a subset of taste cells does not express IP_3 receptors. This raises the question as to whether these cells have intracellular Ca^{2+} release mechanisms. Ryanodine receptors also mediate release of Ca^{2+} from intracellular stores, however a previous study showed no effect of ryanodine on bitter taste responses in *Necturus* taste cells (Ogura et al., 1997).

IP_3R3 immunoreactivity was expressed in the same subset of taste cells as PLCβ_2 and γ_{13}, and by inference from other data, $\beta 3$ (Huang et al., 1999). Antibodies against these proteins have been shown to inhibit IP_3 formation to bitter compounds in taste cells (Rossler et al., 1998; Huang et al., 1999; Rossler et al., 2000), suggesting that they are important components of the bitter-stimulated IP_3 pathway. It is of interest that only a subset of IP_3R3 IR cells express $G\alpha_{gustducin}$, a G protein known to be involved with bitter transduction. These data suggest that alpha subunit(s) in addition to $G\alpha_{gustducin}$ must be involved with the IP_3 signaling pathway in $G\alpha_{gustducin}$ negative cells. Several G protein alpha subunits have been identified in taste cells, and are potential candidates for this role. These include $G\alpha_{transducin}$ (McLaughlin et al., 1993), $G\alpha i$-2, $G\alpha i$-3, $G\alpha s$ (Kusakabe et al., 2000), and $G\alpha 15$ and $G\alpha q$ (Kusakabe et al., 1998). Further experiments will be required to identify the additional alpha subunits that couple to this pathway, and the receptors that activate these G proteins.

In addition to its role in bitter transduction, IP_3 is involved in the transduction of artificial sweeteners (Bernhardt et al., 1996). Interestingly, mice

lacking $G\alpha_{gustducin}$ are compromised in the detection of sweet compounds as well as bitter compounds, suggesting that sweet receptors may also couple to $G\alpha_{gustducin}$ (Wong et al., 1996). Approximately two-thirds of the $G\alpha_{gustducin}$ positive vallate taste cells express T2R/TRB receptors (Adler et al., 2000). It is possible that the remaining $G\alpha_{gustducin}$ positive vallate taste cells express receptors for synthetic sweeteners, and that they couple to the IP_3 signaling pathway. Thus, IP_3R3 may be involved with sweet as well as bitter taste transduction.

IP_3R3 is widely expressed in cells in a variety of tissues including adult pancreatic islets, kidney, gastrointestinal tract, salivary glands, and brain (Blondel et al., 1993; De Smedt et al., 1997). Many of these cell types, including taste cells, are polarized, where Ca^{2+} signals are initiated on the apical membrane and must propagate long intracellular distances. IP_3R3 is particularly well suited for this function, since it is the only IP_3 receptor isotype that is not inhibited at high Ca^{2+} concentrations (Hagar et al., 1998). In fact, under certain conditions, Ca^{2+} can stimulate IP_3R3, making it a likely candidate for participation in the propagation of Ca^{2+} oscillations. In taste cells Ca^{2+} oscillations have been observed in response to bitter stimuli (T. Ogura and S.C. Kinnamon, unpub. observations), and it's likely that IP_3R3 participates in these Ca^{2+} oscillations. Another interesting feature of IP_3R3 is that cAMP-dependent phosphorylation can inhibit its activity in pancreatic acinar cells (Giovannucci et al., 2000a; Giovannucci et al., 2000b). In these cells, cAMP-dependent phosphorylation decreases Ca^{2+} release from intracellular stores and slows the frequency of Ca^{2+} oscillations. These data suggest a possible role for $G\alpha_{gustducin}$ in bitter taste

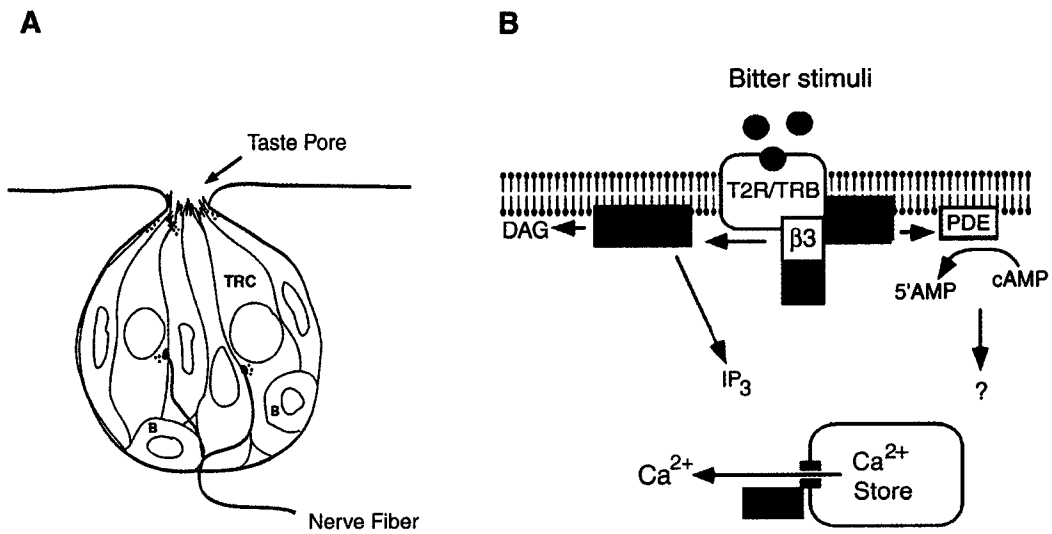
transduction. Specifically, activation of $G\alpha_{gustducin}$, which decreases intracellular cAMP by activation of PDE (Ruiz-Avila et al., 1995), may lead to a decrease in the cAMP-dependent phosphorylation of IP_3R3. This would disrupt the negative control of the receptor and potentiate the Ca^{2+} response. Interestingly, $G\alpha i$-2, another alpha subunit heavily expressed in taste cells (Kusakabe et al., 2000), also functions to decrease intracellular levels of cAMP and may lead to regulation of IP_3R3. Further experiments will be necessary to clarify the role of these alpha subunits in regulation of the IP_3 pathway in taste cells.

2.6 Conclusions

The principal finding in this study is the identification of IP_3R3 as the dominant isoform of the IP_3 receptor in taste cells. IP_3 has been shown to be an important second messenger in both bitter and sweet taste transduction, and IP_3R3 likely mediates the Ca^{2+} release from intracellular stores in response to IP_3. In bitter taste transduction, many signaling components have been identified, and IP_3R3 is co-expressed in the same taste cells (Fig. 2.6). Bitter stimuli bind to T2R/TRB taste receptors coupled to a heterotrimeric G protein complex consisting of $G\alpha_{gustducin}$ and its partners, $\beta 3$ and γ_{13}. Alpha gustducin activates PDE, causing decreases in intracellular cAMP, while its $\beta\gamma$ partners stimulate $PLC\beta_2$ to produce IP_3 and DAG. IP_3 subsequently binds to IP_3R3, causing increases in cytosolic Ca^{2+}, due to release from intracellular stores (Fig. 2.1B). The unique properties of IP_3R3, including its regulation by Ca^{2+} and cAMP dependent kinases, are

consistent with known characteristics of bitter signaling in taste cells.

Figure 2.1. Diagrammatic representation of a rodent taste bud and important components of the bitter transduction pathway. (A) A typical taste bud consists of 50-100 taste receptor cells (TRCs) that extend from the basal lamina to the taste pore. Taste stimuli interact with taste receptors on the apical membrane, while nerve fibers form chemical synapses with the basolateral membrane. Basal cells (labeled B) along the margin of the taste bud are proliferative cells that give rise to taste receptor cells. (B) Bitter stimuli interact with T2R/TRB receptors located on the apical membrane. These receptors couple to a heterotrimeric G protein consisting of α-gustducin, $\beta 3$, and γ_{13}. Alpha gustducin activates phosphodiesterase (PDE), causing decreases in intracellular cAMP, while $\beta 3\gamma_{13}$ activates phospholipase C β_2 ($PLC\beta_2$) to produce the second messengers' inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG). The IP_3 binds to receptors located on smooth endoplasmic reticulum, causing a release of Ca^{2+} into the cytosol. The purpose of this study was to identify the IP_3 receptor isoform that is expressed in taste cells.



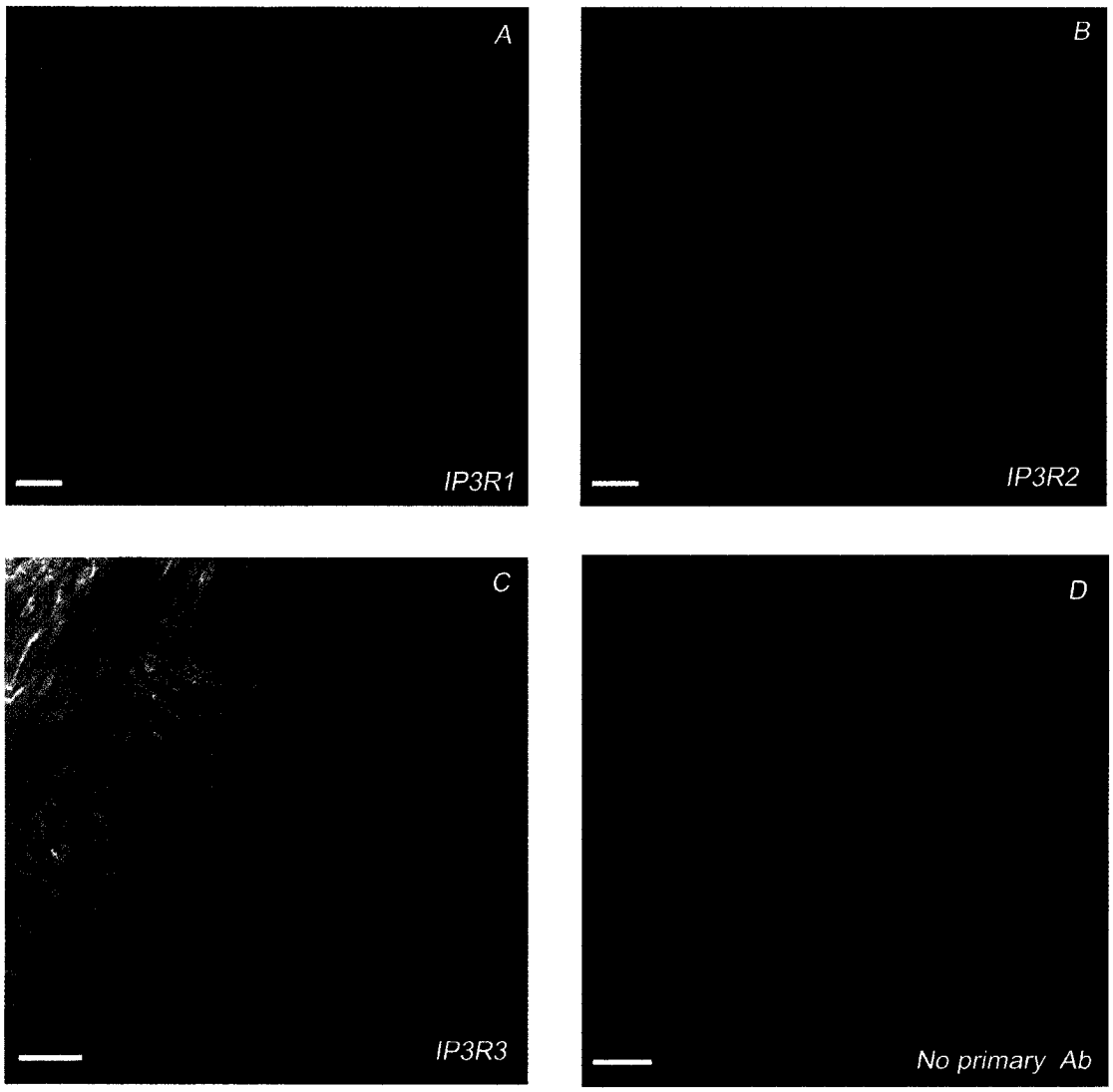


Figure 2.2. Laser scanning confocal micrographs (LCSMs) of rat circumvallate taste buds labeled with antibodies against the three isoforms of the IP_3 receptor. (A). IP_3R1 immunoreactivity (IR), (B) IP_3R2 IR, (C) IP_3R3 IR, (D) no primary antibody control for IP_3R3. The scale bar in each figure represents 10 μm.

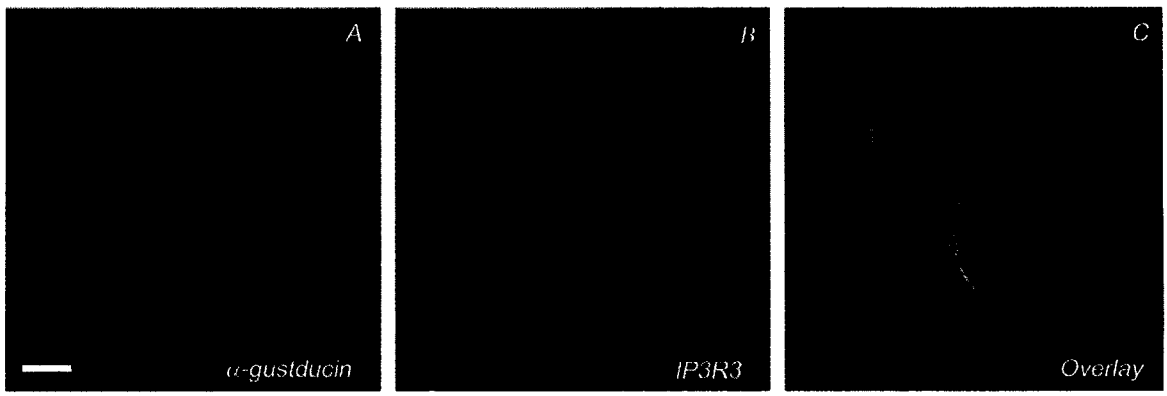


Figure 2.3. LCSMs of rat circumvallate taste buds double labeled with antibodies against $G\alpha_{gustducin}$ (green) and IP_3R3 (red). Panel C shows an overlay of A and B with the differential interference contrast image to enhance visualization of individual taste cells. Scale bar is 10 μm. Note that all $G\alpha_{gustducin}$ IR taste cells are also IP_3R3 IR, however not all IP_3R3 IR cells are $G\alpha_{gustducin}$ IR.

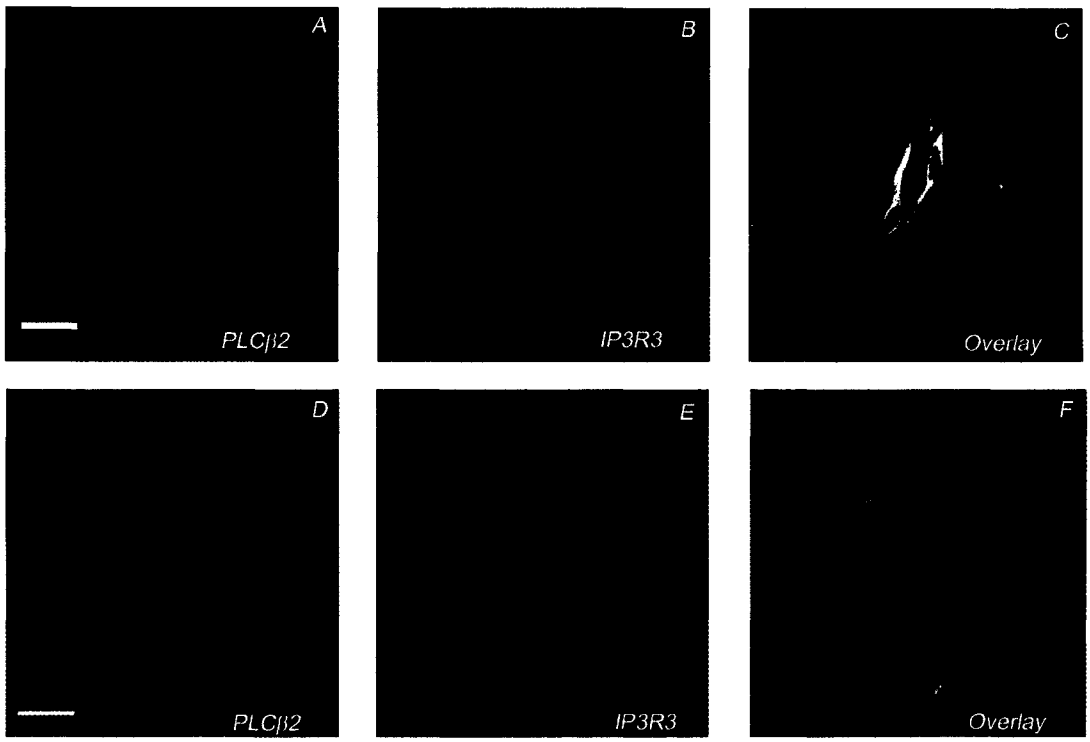


Figure 2.4. LCSMs of rodent circumvallate taste buds double labeled with antibodies against $PLC\beta_2$ (green) and IP_3R3 (red). Panels A-C are from mouse; D-F are from rat. Panels C and F represent the overlay, as described for Figure 2.3. Scale bar is 20 μm. Note almost complete co-localization of IP_3R3 IR and $PLC\beta_2$ IR.

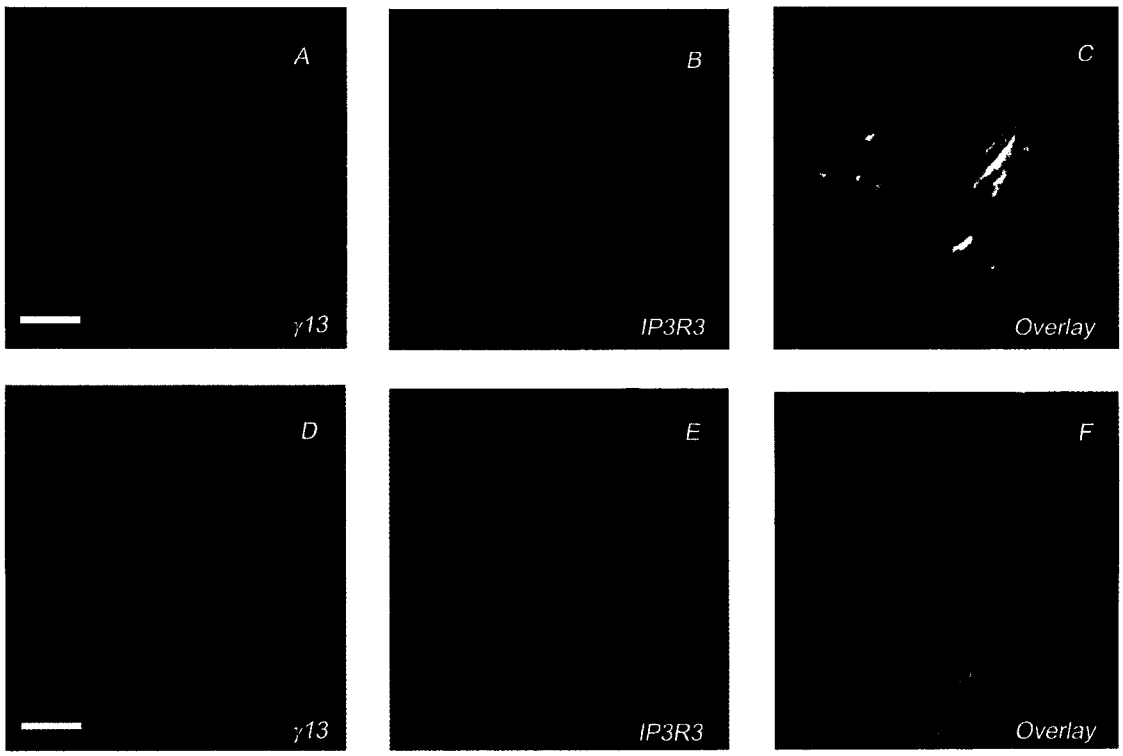


Figure 2.5. LCSMs of rodent circumvallate taste buds double labeled with antibodies against γ_{13} (green) and IP_3R3 (red). Panels A-C are from mouse; D-F are from rat. Panels C and F represent the overlay as described for Figs. 2.3-2.4. Scale bar is 20 μm. Note nearly complete co-localization of IP_3R3 IR and γ_{13} IR.

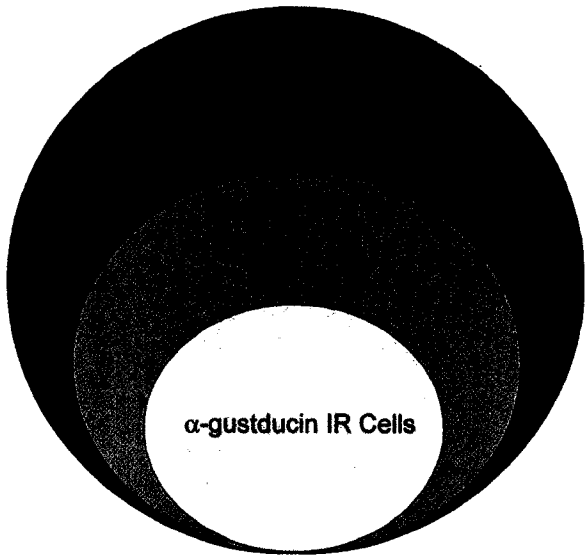


Figure 2.6. Qualitative representation of the co-expression patterns of IP_3R3, $G\alpha_{gustducin}$, $PLC\beta_2$, and γ_{13} in circumvallate taste buds.

Chapter 3

MORPHOLOGICAL CHARACTERIZATION OF RAT TASTE RECEPTOR CELLS THAT EXPRESS COMPONENTS OF THE PHOSPHOLIPASE C SIGNALING CASCADE

3.1 Abstract

Rat taste buds contain three morphologically distinct cell types that are candidates for taste transduction. The physiological roles of these cells is, however, not clear. Inositol 1,4,5 trisphosphate (IP_3) has been implicated as an important second messenger in bitter, sweet, and umami taste transduction. Previously, we identified the type III IP_3 receptor (IP_3R3) as the dominant isoform in taste receptor cells. In addition, a recent study shows PLCβ_2 is essential for

the transduction of bitter, sweet, and umami stimuli. Both IP_3R3 and $PLC\beta_2$ are expressed in the same subset of cells. In order to identify the taste cell types that express proteins involved in PLC signal transduction, we used DAB immunoelectron microscopy and fluorescent microscopy to identify cells with IP_3R3. Confocal microscopy was used to compare IP_3R3 or $PLC\beta_2$ immunoreactivity to that of some known cell type markers such as 5-HT, PGP 9.5, and NCAM. Here we show that a large subset of Type II cells and a small subset of Type III cells display IP_3R3 immunoreactivity within their cytoplasm. These data suggest that Type II cells are the principal transducers of bitter, sweet and umami taste transduction. However, we did not observe synapses between Type II taste cells and nerve fibers. Interestingly, we observed subsurface cisternae of smooth endoplasmic reticulum at the close appositions between the plasma membrane of Type II taste cells and nerve processes. We speculate that some Type II cells may communicate to the nervous system via subsurface cisternae of smooth endoplasmic reticulum in lieu of conventional synapses.

3.2 Introduction

Taste buds contain 50-150 polarized neuroepithelial taste receptor cells that are responsible for the transduction of sapid stimuli in the oral cavity and relay this information to nerve fibers (for reviews see (Witt and Reutter, 1996; Finger, 2000). Simple stimuli such as salts and acids can interact directly with

ion channels to cause depolarization and Ca^{2+} influx, whereas complex stimuli such as sugars and bitter compounds act via G protein-coupled receptors and a variety of second messengers (Kinnamon, 2000).

An important second messenger in taste transduction is inositol trisphosphate (IP_3), which has been implicated in the transduction of bitter, sweet, (for review see (Lindemann, 2001) and umami (Ninomiya et al., 2000) compounds. Recently we identified the downstream target of IP_3 in taste cells as the type III IP_3 receptor (IP_3R3) (Clapp et al., 2001; Miyoshi et al., 2001). This receptor is most often located on the smooth endoplasmic reticulum (SER) and allows Ca^{2+} to pass from the SER lumen into the cytoplasm upon activation. In taste cells IP_3R3 is co-expressed with other Ca^{2+} signaling molecules including $PLC\beta_2$, $G\gamma13$, and TRPM5, with $G\alpha_{gustducin}$ in a subset of these cells (Clapp et al., 2001; Miyoshi et al., 2001; Perez et al., 2002; Zhang et al., 2003).

At least four morphological types of cells have been identified in rodent taste buds. One cell type, the basal cell, is a round cell located near the base of the taste bud (Farbman, 1965). Basal cells are progenitor cells and are not believed to be involved in taste transduction (Murray, 1973; Delay et al., 1986; Roper, 1989). The other three cell types extend to the taste pore and are candidates for transducing taste stimuli. Type I ("dark") cells possess an electron-dense cytoplasm and are slender with several long microvilli of various lengths extending into the oral cavity. The nuclei of these cells may be invaginated and typically contains prominent heterochromatin adherent to the inner leaflet of the nuclear lamellae. Perhaps the most distinguishing feature of a

Type I cell is the presence of many 100-400 nm electron-dense granules in the apical cytoplasm of the cell. Type II ("light") cells are more electron-lucent, spindle- or pyriform-shaped cells with large, round nuclei that lack the heterochromatin and invaginations characteristic of Type I cells. The apical cytoplasm of Type II cells typically contains electron-lucent, swollen cisternae of smooth endoplasmic reticulum, but lacks the dense granules found in Type I cells. Several short microvilli of uniform length protrude from the apical region of Type II cells into the taste pore. Type III cells have a slender, spindle shape and also have an electron lucent cytoplasm. These cells can be distinguished from Type II cells by an elongate nucleus with prominent invaginations and the presence of large, dense-cored vesicles in the nuclear region of the cytoplasm. The most distinguishing ultrastructural feature of a Type III cell is the single, large, blunt microvillus that extends into the taste pore. Conventional synapses onto nerve processes have been observed from Type III cells in both mouse (Takeda, 1976) and rat (Takeda and Hoshino, 1975; Yee et al., 2001).

Type III cells also express immnocytochemical markers such as serotonin (5-HT), ubiquitin carboxyl terminase (PGP 9.5), and neural cell adhesion molecule (NCAM). Serotonin is present in a subset of rat Type III taste cells (Kim and Roper, 1995) and PGP 9.5 is found in a subset of both Type II and Type III cells (Yee et al., 2001). However, the PGP 9.5 immunoreactive Type III cells appear to be a separate population from the 5-HT immunoreactive cells (Yee et al., 2001). It is also believed that NCAM immunoreactivity is present in intermediate (likely Type III) cells (Nelson and Finger, 1993).

Despite numerous studies, the functional significance of morphologically distinct taste cell types is not clear. A recent study suggests that bitter, sweet, and umami taste transduction all involve activation of PLCβ_2 (Zhang et al., 2003). In the present study, we have used immunofluorescence and immunoelectron microscopy to identify the cell types that express proteins associated with the PLC signaling cascade. Our data show that most rat Type II taste cells and a small subset of Type III taste cells express PLCβ_2 and IP_3R3, suggesting that these cell types are involved in bitter, sweet, and umami taste transduction. Preliminary accounts of these data have been presented in abstract form (Clapp TR, 2002).

3.3 Materials and Methods

Confocal Laser Scanning Microscopy (CLSM)

Eleven adult male Sprague-Dawley Rats (250-350g) were obtained from Charles River Laboratories (Wilmington, MA). Animals were cared for in compliance with the Colorado State University and University of Denver Institutional Animal Care and Use Committees. Animals were deeply anesthetized using sodium pentobarbital (60mg/kg body weight) and perfused with room temperature 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.2). Following perfusion, tongues were removed and immediately placed into fresh 4% paraformaldehyde in 0.1M phosphate buffer for approximately one hour.

Tongues were then cryoprotected overnight with 20% sucrose in 0.1M phosphate buffer. Following sectioning, the 20-40 μm slices were washed three times for 10 minutes each in phosphate buffered saline (PBS) at room temperature. In experiments involving IP_3R3, antigen retrieval was performed using buffered sodium citrate (pH 9) at 80°C for 20 minutes. The antigen retrieval procedure helps to disrupt protein cross-bridges formed by paraformaldehyde fixation and exposes antigen-binding sites (Evers et al., 1998). In some experiments the incubation time in sodium citrate was reduced to 5 minutes and labeling was still apparent. All sections were incubated in a blocking solution consisting of 0.3% Triton X-100, 1% normal goat serum, and 1% bovine serum albumin in 0.1M PBS for 1-2 hours at room temperature. Monoclonal mouse anti-IP_3R3 was purchased from Transduction Laboratories (Lexington, KY) and used at 1:50 in blocking solution. Monoclonal mouse anti-IP_3R3 recognizes the N-terminal peptide sequence 22-230. Monoclonal mouse anti-serotonin was purchased from Biomeda (Foster City, CA) and used at 1:1 while polyclonal rabbit anti-$PLC\beta_2$ was purchased from Santa Cruz Laboratories (Santa Cruz, CA) and used at 1:1000. For double label experiments involving $PLC\beta_2$ and serotonin animals were injected with 5-hydroxytryptophan (5-HTP, 80mg/kg, I.P.) one hour before sacrifice. Rabbit anti-PGP 9.5 was used at 1:100 and purchased from Biogenesis (Poole, England). Rabbit anti-Neural cell adhesion protein (NCAM) was used at 1:200 and purchased from Chemicon (Temecula, CA). All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). These included Cy-5 anti-mouse, Cy-5 anti-

rabbit, Fluorescein (FITC) anti-mouse, and FITC anti-rabbit. Sections from rat circumvallate papillae were incubated with primary antisera overnight at 4°C. Following incubation with primary antisera, sections were washed in PBS and incubated with the appropriate secondary antisera at room temperature for two hours. Sections were then washed in PBS and mounted on slides using Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, AL). Double-label experiments involved incubations with two primary antibodies raised in different species and subsequent incubation with appropriate secondary antibodies. Controls for immunofluorescence microscopy consisted of omitting the primary antiserum. No immunoreactivity was seen under these conditions. Sections were viewed with an Olympus Fluoview confocal laser-scanning microscope. Sequential acquisition at the different wavelengths were used for some double-label experiments and showed no differences compared with simultaneous scans. Images were processed with Adobe Photoshop 6.0 software.

DAB Staining for Electron Microscopy

Eight adult Sprague-Dawley rats (250 – 350g) were anesthetized with sodium pentobarbital. (60-mg/kg body weight, i.p.) For DAB staining of electron microscopy, rats were perfused for 10 sec through the left ventricle with 0.1% sodium nitrite, 0.9% sodium chloride, and 100 units of Heparin in 100ml of 0.1M phosphate buffer (pH 7.3). This was followed by 4% paraformaldehyde in 0.1M

phosphate buffer (pH 7.3) for ten minutes (Weedman et al., 1996). All perfusates were warmed to 42°C before use. After perfusion the tongues were removed and the excised circumvallate papillae were fixed in the above fresh fixatives for 2-3 hours.

Sections 80 μm thick were sliced with a vibratome (Vibratome Series 1000, Ted Pella, Inc.), placed in 10mM sodium citrate (pH 9) at 80° C for 5 minutes, and then blocked with 5% NGS, 1% BSA, and 0.1% fish gelatin in 0.1M PBS, (pH 7.3), for one hour at room temperature. This was followed by incubation with IP_3R3 primary antiserum at a dilution of 1:50 in 0.1M PBS for 24 hours at 4°C. After rinsing in PBS, the sections were incubated with affinity purified biotinylated goat anti-mouse IgG (Jackson Laboratories cat # 115-065-100) diluted to 1:200 in PBS for 2 hours at room temperature. The sections were washed and then incubated with avidin-biotin complex (Elite Vectastain, Vector Laboratories) in PBS for 1 hour at room temperature. After rinsing, sections were treated with 0.05% DAB for 10 minutes. The label was visualized by floating the sections for 2-4 minutes in a fresh aliquot of DAB mixture activated with 0.002% hydrogen peroxide. Sections were then washed in Tris buffer, followed by washes in 0.1M PB (pH 7.3) and postfixed in 1% OsO_4 in 0.1M PB for 15 min. After rinsing in 0.05M sodium maleate buffer (pH 5.2), the sections were en bloc stained in 1% uranyl acetate in 0.025M sodium maleate buffer (pH 6.0) overnight at 4°C, followed by a dehydration in an alcohol series, processed through propylene oxide, and embedded with Eponate (Ted Pella, Inc.). The sections were re-embedded using the technique of Crowley and Kinnamon (HH Crowley,

1995). Thin sections were cut with a diamond knife on a Reichert Ultracut E ultramicrotome and examined with a Hitachi H-7000 transmission electron microscope at 75kV. Controls for electron microscopy consisted of omitting the primary antiserum, the secondary antibody or the avidin-biotin complex. No immunoreactivity was seen under these conditions.

3.4 Results

Double labels studies

Double label immunocytochemistry was used to determine any overlap between IP_3R3 and that of known Type III taste cell markers, including serotonin (5-HT), ubiquitin carboxyl terminal hydrolase (PGP 9.5), and neural cell adhesion molecule (NCAM). However, the antibody that we proposed to use for 5-HT immunocytochemistry was raised in mouse, as was the IP_3R3 antibody. To avoid cross-reactivity of secondary antibodies, we used a polyclonal $PLC\beta_2$ antibody raised in rabbit for double label experiments with 5-HT. This was possible due to earlier studies showing nearly complete co-localization of IP_3R3 and $PLC\beta_2$ (Clapp et al., 2001; Miyoshi et al., 2001).

Most $PLC\beta_2$-like immunoreactivity (LIR) cells have large, round, non-immunoreactive nuclei resembling Type II cells (Fig. 3.1). $PLC\beta_2$-LIR is present in the cytoplasm of the immunoreactive cells extending from the basal lamina to the taste pore. $PLC\beta_2$-LIR was seen only in taste cells and was not observed in

the non-gustatory lingual epithelium surrounding taste buds. Control sections in which the primary antibody was omitted showed no labeling (data not shown). Similar results were obtained with an antibody directed against IP_3R3 (Fig. 3.2). In contrast, antibodies directed against 5-HT labeled only a few cells per taste bud (Fig. 3.1). Most 5-HT-LIR cells are more slender than the $PLC\beta_2$-LIR cells, and are heavily labeled throughout the extent of the cell, including the nuclei. PGP 9.5-LIR appears throughout both the cytoplasm and nuclei of labeled taste cells, and is also present in nerve fibers within the taste bud (Fig. 3.2A, D). NCAM-LIR is present in the membranes of taste cells that tend to have an elongated nucleus characteristic of Type III taste cells (Fig. 3.2G).

$PLC\beta_2$ and 5-HT. $PLC\beta_2$-LIR is present in a larger set of cells than the set of 5-HT-LIR cells. There is, however, a small subset of cells that display both $PLC\beta_2$-LIR and 5-HT-LIR (Figs. 3.1C, F). To quantify the co-localization of $PLC\beta_2$ and 5-HT we counted approximately 300 taste buds and 2000 immunoreactive cells. Cells were scored as immunoreactive only if an enlarged area consistent with a nuclear region along with an apical extension was present. We analyzed over 1500 immunoreactive $PLC\beta_2$ cells and found that approximately 3.5% of them were also 5-HT immunoreactive, while 12% of 5-HT immunoreactive cells are also $PLC\beta_2$ immunoreactive. Because nearly all IP_3R3 immunoreactive cells are also $PLC\beta_2$ immunoreactive (Clapp et al., 2001), we believe these data are representative of the population of taste cells that express both IP_3R3 and 5-HT.

IP_3R3 and PGP 9.5. To determine if the non-serotonin-LIR population of Type III cells is immunoreactive for PLC signaling components, we performed double-label immunocytochemistry with IP_3R3 and PGP 9.5. A complete overlap of IP_3R3 and PGP 9.5 would indicate that the non-serotonin-LIR Type III cells all exhibit PLC signaling components. Some cells are double-labeled, but IP_3R3-LIR is present in a larger subset of cells than PGP 9.5-LIR (Fig. 3.2C, F). Taste cells that exhibit both IP_3R3-LIR and PGP 9.5-LIR tend to be apically located in the taste bud, pyriform in shape, and have large round nuclei typical of Type II cells. However, some double-labeled cells are spindle-shaped (e.g., Fig. 3.2F), suggesting some Type III cells may also express IP_3R3. Because cell type of these double-labeled cells could not be determined with certainty, no further quantification was attempted.

IP_3R3 and NCAM. NCAM-LIR is reported to be present only in elongate Type III taste cells (Nelson and Finger, 1993; Yee et al., 2001). As shown in Fig. 3.2I, IP_3R3-LIR and NCAM-LIR appear to label completely separate populations of cells. However, NCAM labels surface membranes, making it difficult to resolve double-labeled cells when the other marker is a cytosolic protein. Thus, we cannot conclude that there is absolutely no overlap in the immunoreactivity of these two proteins.

Ultrastructual features of IP_3R3-LIR taste cells

The data above showed only a small overlap of PLC signaling components and Type III cell markers (e.g. NCAM and 5-HT) (Figs3.1, 3.2). To provide further insight into the morphological cell types expressing $PLC\beta_2/IP_3R3$, we examined the ultrastructural characterisitcs of IP_3R3-LIR cells. A large set of rat circumvallate taste cells display IP_3R3-LIR (Fig. 3.3). Most immunoreactive taste cells are either spindle-shaped or pyriform, with large, ovoid to circular nuclei (Fig. 3.3), characteristic of Type II taste cells. Based on the ultrastructural features of the apical microvillar processes (Fig. 3.4), two distinct morphological types of IP_3R3-LIR taste cells exist: taste cells with numerous, short microvilli of uniform length characteristic of Type II taste cells, and taste cells with a solitary thick microvillus characteristic of Type III cells. We saw no evidence of labeling in Type I cells (Fig. 3.4).

Relationship between IP_3R3-LIR taste cells and nerve processes

Although Type II taste cells appear to possess many of the necessary proteins for signal transduction, conventional synapses have not yet been observed between Type II cells and nerve processes of the rat. In the present study, we observed conventional synapses only between Type III cells and nerve processes (Fig. 3.5A). These synapses had numerous synaptic vesicles along with increased pre and post-synaptic membrane densities (Fig. 3.5B). We were

unable to identify any IP_3R3-LIR taste cells containing conventional synaptic specializations.

Interestingly, we found twenty-eight subsurface cisternae of smooth endoplasmic reticulum (SSC) at close appositions between IP_3R3-LIR taste cells and nerve processes (Figs. 3.5A, C and 3.6A-C). The SSC are narrow lamellar structures (Fig. 3.5C), but some are more dilated, presumably artifacts of fixation (Figs. 3.6B and C). The outer membrane of the SSC is separated from the inner leaflet of the taste cell membrane by a narrow gap, and numerous mitochondria are often present in the adjacent nerve processes, which lack IP_3R3-LIR (Figs. 3.5C and 3.6A-C). SSC were identified prior to cell type identification. We found that all SSC were present in immunoreactive cells, which were identified as Type II cells based primarily on their nuclear profile (Fig. 3.5A, C). Future studies using serial reconstruction will be needed to determine whether or not all Type II cells possess SSC.

3.5 Discussion

The principal finding in this study is that IP_3R3, and by inference, $PLC\beta_2$, TRPM5, and $G\gamma13$ are expressed in a large subset of Type II and a small subset of Type III vallate taste cells in rat. These proteins are all integral components of the PLC signaling pathway recently demonstrated to be essential for the transduction of bitter, sweet, and umami taste stimuli (Zhang et al., 2003). Thus, it is likely that

Type II (and a small subset of Type III) cells are involved in bitter, sweet, and umami transduction.

IP_3 is an important second messenger in bitter, sweet, and umami taste transduction (Bernhardt et al., 1996). Only recently, however, has the target of IP_3 in taste receptor cells been identified as IP_3R3 (Clapp et al., 2001; Miyoshi et al., 2001). Previous studies have shown IP_3R3 is co-expressed with other PLC signaling proteins, including $PLC\beta_2$, TRPM5, and $G\gamma13$ (Clapp et al., 2001; Miyoshi et al., 2001; Perez et al., 2002; Zhang et al., 2003). Thus, we assume that IP_3R3 immunoreactive cells are representative of $PLC\beta_2$ immunoreactive cells and vice versa. Using both fluorescence immunocytochemistry and DAB immunoelectron microscopy, we have provided compelling evidence that IP_3R3 is present in the majority of Type II cells and a small subset of Type III cells, giving insight into the physiological roles these cell types may play.

Unfortunately no immunocytochemical marker encompasses the whole population of Type II or Type III cells to date. NCAM appears to be present in many intermediate cells (Nelson and Finger, 1993), which we now believe to be equivalent to Type III cells. Serotonin-LIR and PGP 9.5-LIR are found in mutually exclusive subsets of Type III cells, but PGP 9.5-LIR can also be seen in some Type II cells (Yee et al., 2001). Brain-derived neurotrophic factor (BDNF) is present in all Type III cells, but is also present in a small subset of Type II cells (Yee et al., 2003). Finally, our results suggest that PLC signaling components are present in the vast majority of Type II cells, but are also present in a small subset of Type III cells. The fact that no known immunocytochemical marker

completely describes a particular cell type raises the question as to whether distinct cell types really exist within taste buds. It is possible that the different cell types represent a continuum, with the different cell types sequentially derived from a common precursor. Tritiated thymidine labeling shows a sequential pattern of labeling, which is consistent with this hypothesis (Delay et al., 1986), However, other studies suggest that the cell types represent distinct lineages (Pumplin et al., 1997; Stone et al., 2002). Further studies will be needed to resolve this issue.

Type I taste cells do not exhibit immunoreactivity to IP_3R3 or other components of the PLC signaling cascade. Also, mouse Type I cells lack voltage-gated Ca^{2+} channels (Medler et al., 2003), and we have not observed conventional synapses between Type I cells and nerve processes. Electron micrographs of Type I taste cells in some species often show these cells wrapping around other cell types much like glia (Murray, 1973; Pumplin et al., 1997). In addition, immunocytochemical data suggest that Type I cells have a glial-like function (Lawton et al., 2000). Our data showing a lack of Ca^{2+} signaling transduction components in Type I cells are consistent with this role as a glial-like cell.

The great majority of Type II taste receptor cells express the proteins involved in the PLC signaling cascade. Whether all Type II cells display IP_3R3-LIR could not be determined, since apical processes were not available in all sections and serial sectioning was not performed. Specifically $PLC\beta_2$ is essential for the transduction of bitter, sweet, and umami compounds (Zhang et al., 2003).

Based on evidence shown here and previous evidence that IP_3R3 and $PLC\beta_2$ are co-expressed, we believe that Type II cells are responsible for the transduction of bitter, sweet, and umami tastes.

Although Type II cells express proteins needed for taste transduction, classical synapses have not been found associated with Type II cells in rats. In fact there are very few conventional synapses located in rat taste buds (Kinnamon et al., 1988). Interestingly, we found numerous examples of subsurface cisternae (SSC) of smooth endoplasmic reticulum associated with IP_3R3 immunoreactive Type II cells. SSC are found exclusively at close appositions between a taste cell and a nerve process, and we have observed numerous 40-70 nm vesicles in these cells. This intimate association suggests that a functional connection may exist at those close appositions. IP_3 receptors are predominantly located in the membrane of endoplasmic reticulum so we predict that these receptors are present in the SSC of taste cells. We speculate that the SSC may be associated with sites of transmitter release onto nerve fibers, since these areas would experience localized high Ca^{2+} concentrations. Subsuface cisternae have been observed in other sensory cells as well as in the CNS. In the auditory system SSC are found associated with synapses in outer cochlear hair cells (Canlon and Dulon, 1993; Sridhar et al., 1997; Spicer et al., 1998; Koyama et al., 1999; Mammano et al., 1999; Frolenkov et al., 2000; Llano et al., 2000). The putative roles of SSC in hair cells are varied, but Ca^{2+} sequestration near synapses is a proposed function for this organelle. Studies indicate that SSC display immunoreactivity to antibodies directed against both

IP_3 receptors and calmodulin-dependent protein kinase IV (CaMK-IV) (Frolenkov et al., 2000). By modulating local cytosolic Ca^{2+} levels, SSC may alter the temporal properties of the receptor potential of the hair cell (Sridhar et al., 1997; Spicer et al., 1998). Another possible function is the control of local current flow at the synapse, possibly enhancing transmitter release (Saito, 1983). Llano et al., (2000) found that intracellular stores of Ca^{2+} could elicit neurotransmitter release in the absence of external Ca^{2+} in hair cells and Krizaj et al., (1999) observed that release of Ca^{2+} from intracellular stores may modulate release of transmitters in rod photoreceptors (Krizaj et al., 1999). Local participation in protein synthesis has also been suggested as a possible function for SSC (Racca et al., 1997), as well as to control metabolic exchange (Watanabe and Burnstock, 1976) or cell-cell coupling (Braekevelt, 1992).

Alternatively, SSC may function in the efferent modulation of taste cells (Ide and Munger, 1980). In outer hair cells, SSC are a functional component of the efferent synapse involving cholinergic nerve fibers (Sridhar et al., 1997). Taste cells also respond to acetylcholine and the response involves activation of the PLC signaling pathway (Ogura, 2002). However, the source of endogenous acetylcholine in taste cells is unknown. Further studies will be required to determine if SSC in taste cells have an afferent or an efferent function.

Type III taste receptor cells were first characterized in rabbit (Murray, 1973) and later shown in rat (Takeda and Hoshino, 1975; Yee et al., 2001). Two subsets of Type III cells have been identified, those that accumulate 5-HT and those that express PGP 9.5, which is also expressed in a subset of Type II cells

(Yee et al., 2001). NCAM is also believed to be present in the membranes of Type III cells (Nelson and Finger, 1993). Our data show that a small subset of taste cells are immunoreactive for both PLCβ_2 and 5-HT, and a larger subset is immunoreactive for IP_3R3 and PGP 9.5, which is expected because PGP 9.5 labels both Type II and Type III cells. Interestingly, many of the double-labeled serotonin-LIR cells are pyriform in shape and are apically located in the taste bud (Fig. 3.1C,F), characteristics of some Type II taste cells. It is possible that 5-HT is accumulated in some Type II cells, but this has not been demonstrated previously. Further, we did not observe co-expression of IP_3R3 and NCAM, suggesting again that the majority of Type III cells do not express PLC signaling components. However, we do not believe that PLC signaling components are confined solely to Type II cells. Our immunoelectron microscopy revealed one putative Type III cell with IP_3R3-LIR, identified by its single blunt microvillus (Fig. 3.4). The paucity of IP_3R3-LIR Type III cells is not surprising, as only about 3.5% of the PLCβ_2-LIR cells also display 5-HT-LIR. Type III cells are the only cell type in rat known to be associated with conventional synapses to date. Most Type III taste receptor cells do not express IP_3R3 and the associated proteins of the PLC signaling pathway, however, they do express voltage gated Ca^{2+} channels (Medler et al., 2003). It is not clear whether Type III cells directly transduce any taste stimuli, since most do not express PLC signaling components. It is possible, however, that Type III cells are involved in transduction of other stimuli such as salts and acids. Alternatively, Type III cells may relay signals from the PLC-

expressing Type II cells to afferent nerve fibers. Further work will be required to distinguish between these alternatives.

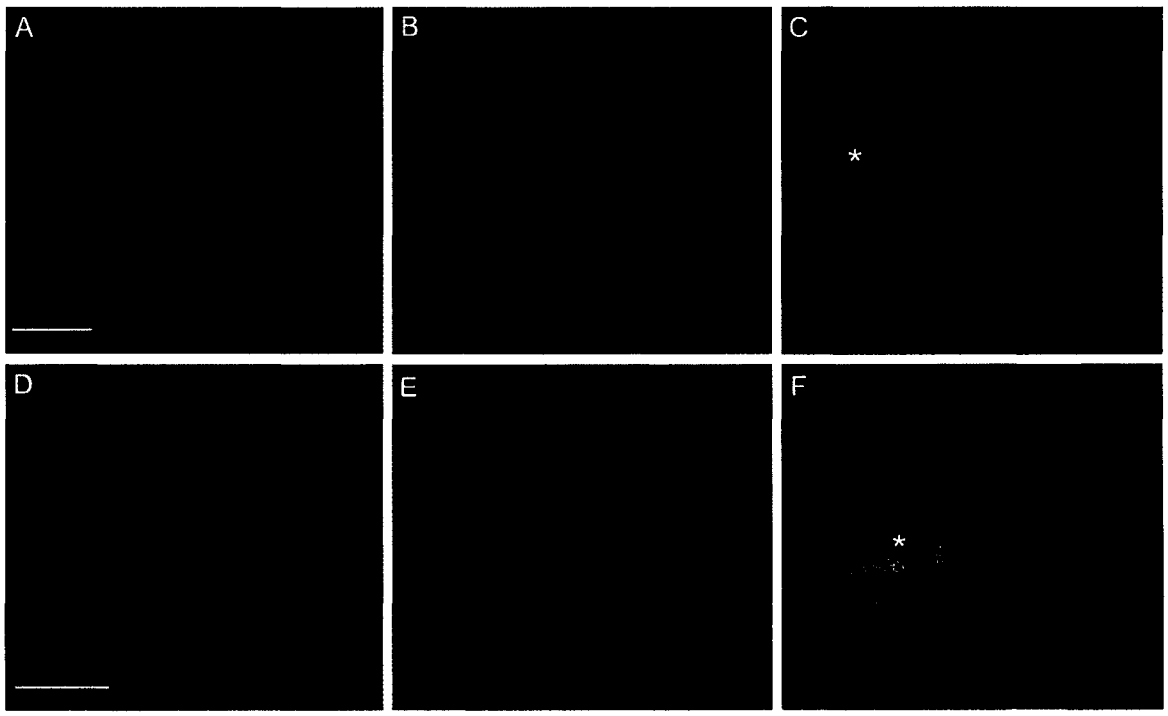


Figure 3.1. Laser scanning confocal micrograph of rat circumvallate taste buds labeled with antibodies directed against phospholipase $C\beta_2$ ($PLC\beta_2$) and serotonin (5-HT). Green indicates 5-HT-like immunoreactivity (LIR; **A, D**) and red indicates $PLC\beta_2$-LIR (**B, E**). Most of the 5-HT-LIR cells appear spindle shaped, with heavy labeling throughout the cytoplasm and nucleus (**A, C, D, F**). $PLC\beta_2$-LIR is present in many more cells than is 5-HT-LIR and does not exhibit nuclear staining. The asterisks indicate cells that appear immunoreactive for 5-HT and $PLC\beta_2$. Some of the cells that display $PLC\beta_2$-LIR and 5HT-LIR are more apically located and pyriform in shape. Scale bars = 20 μm.

Figure 3.2. Laser scanning confocal micrograph of rat circumvallate taste buds labeled with antibodies directed against inositol 1,4,5-triphosphate receptor 3 (IP_3R3), protein gene-regulated peptide 9.5 (PGP 9.5), and neural cell adhesion molecule (NCAM). Green denotes PGP 9.5-like immunoreactivity (LIR) (**A, C, D, F**) and NCAM-LIR (**G, I**). Red indicates IP_3R3-LIR (**B, C, E, F, H, I**). PGP 9.5-LIR is present in nerve fibers and the cytoplasm of taste cells. IP_3R3-LIR resembles phospholipase $C\beta_2$-LIR (Fig. 3.1). IP_3R3-LIR is found in many cells in the taste bud, with prominent cytoplasmic labeling and lack of any nuclear staining. IP_3R3-LIR was not observed in non-gustatory lingual epithelium. Asterisks indicate a few cells showing PGP 9.5-LIR and IP_3R3-LIR (C, F). NCAM-LIR is seen in the membranes of some taste cells and nerve fibers (G). We did not see any obvious overlap between NCAM-LIR and IP_3R3-LIR in taste cells, but it is difficult to be certain because NCAM-LIR is membrane associated and IP_3R3-LIR is cytosolic (I). Scale bars = 20 μm.

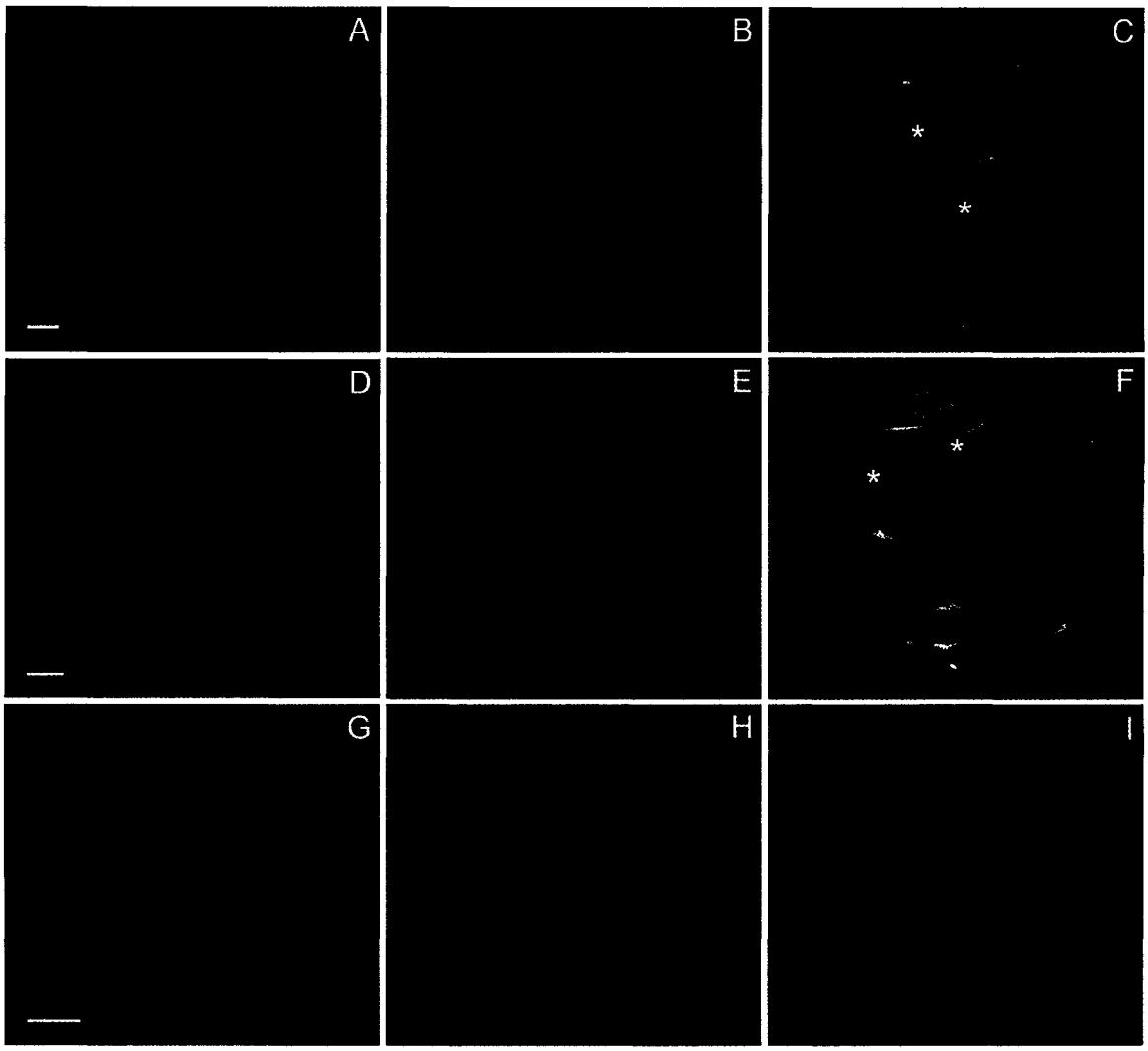


Figure 3.3. Immunoelectron micrograph showing inositol 1,4,5-triphosphate receptor 3-like immunoreactivity (IP_3R3-LIR) in a large subset of type II cells (II). IP_3R3-LIR appears throughout the cytoplasm and is not found in the nuclear region. IP_3R3-LIR cells have a large, round nucleus typical of type II cells. Type I (I) cells do not display IP_3R3-LIR. TP, taste pore. Scale bar = 2 μm.

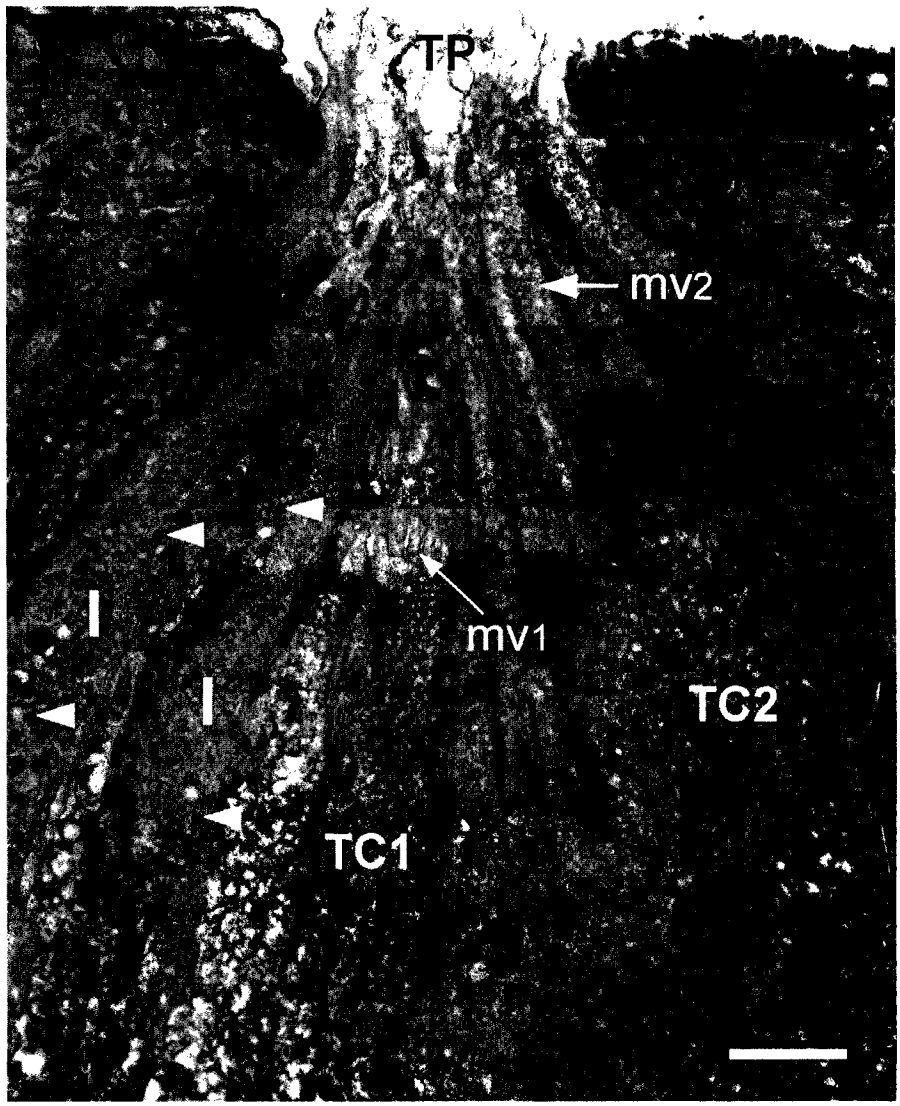


Figure 3.4. Immunoelectron micrograph showing the apical region of a rat circumvallate taste pore (TP). TC1 and TC2 are taste cells exhibiting inositol 1,4,5-triphosphate receptor 3-like immunoreactivity (IP_3R3-LIR). Type I cells (I) with many dense-core granules (arrowheads) do not display IP_3R3-LIR. The apical region of TC1 shows a tuft of microvilli (mv1) characteristic of type II cells. The apical region of region of TC2 exhibits a single blunt microvillus (mv2) typical of type III cells. Scale bar = 1 μm.

Figure 3.5. Transverse section through a taste bud. **A:** Type I (I) cells do not exhibit inositol 1,4,5-triphosphate receptor 3-like immunoreactive (IP_3R3-LIR). Type II (II) cells with very large, round nuclei display IP_3R3-LIR in the cytoplasm and often contain subsurface cisternae (SSCs; box C). The type III (III) cells in this section do not show IP_3R3-LIR. Type III cells have pronounced nuclear invaginations and can be associated with nerve processes via conventional synapses (box B). **B:** Conventional synapses (s) display pre- and postsynaptic densities and numerous synaptic vesicles (sv). **C:** SSCs are in close apposition between a taste cell (TC) and a nerve process (N). These associations include the membrane of smooth endoplasmic reticulum in close proximity to the plasma membrane of taste cells and the membrane of a nerve process. Arrows denote the lumen of the smooth endoplasmic reticulum. m, mitochondria. Scale bars = 2 μm in A; 0.5 μm in B and C.

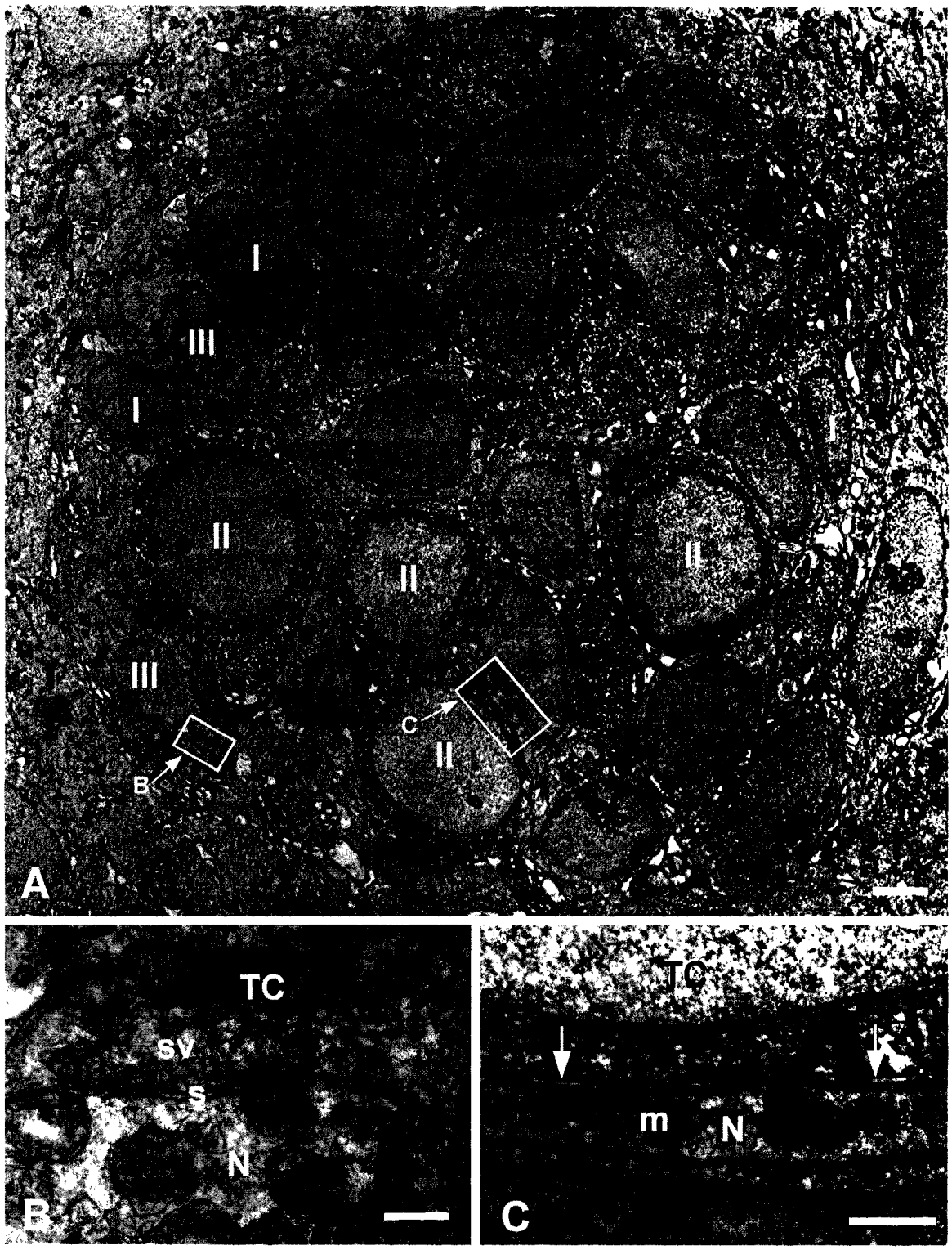
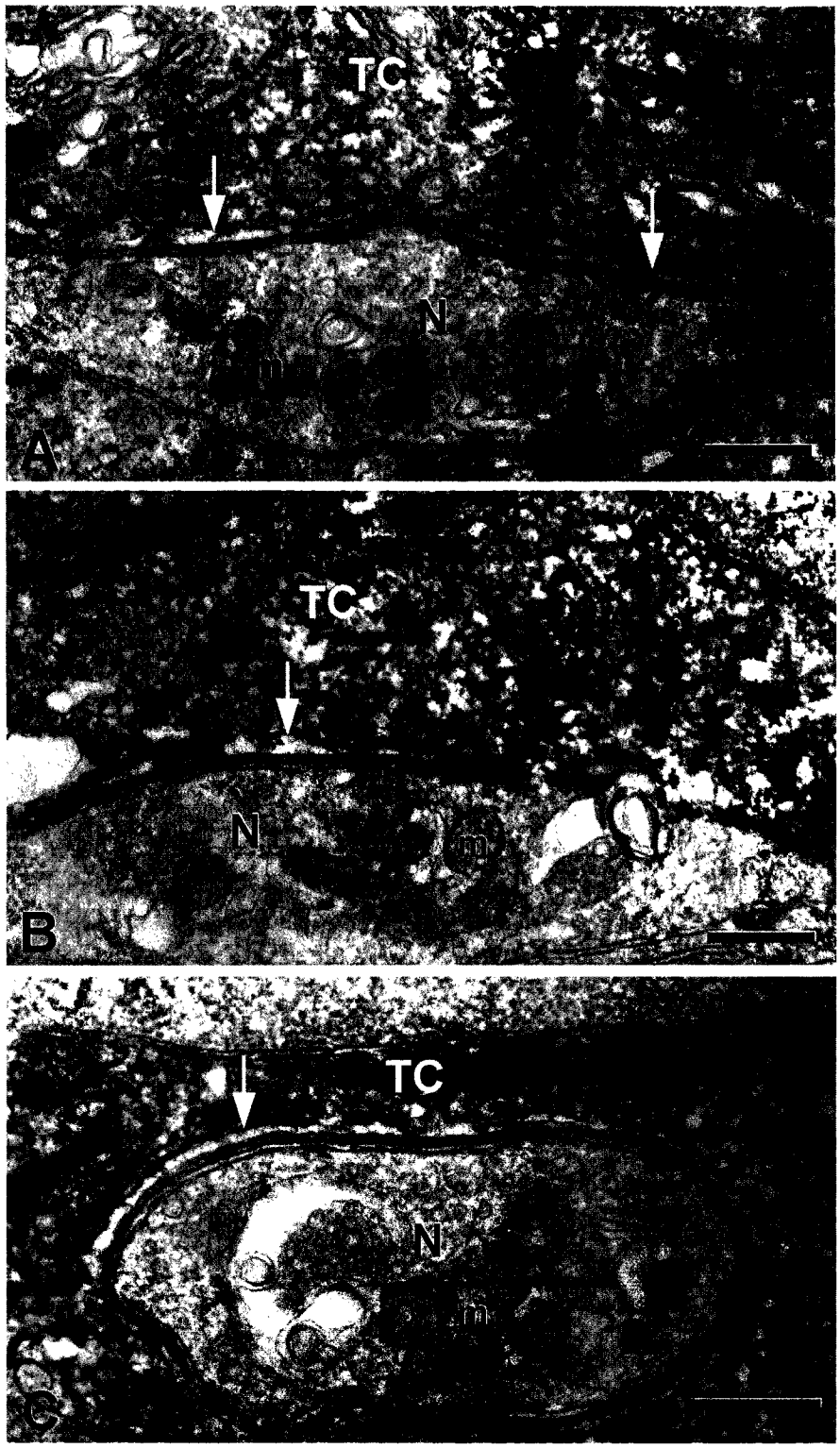


Figure 3.6. A-C: We found several examples of inositol 1,4,5-triphosphate receptor 3-like immunoreactive (IP_3R3-LIR) type II cells containing subsurface cisternae (SSCs). The SSCs (arrows) are in close apposition to the membrane of an IP_3R3-LIR taste cell (TC) and the membrane of a nerve process (N). m, mitochondrion. Scale bars = 0.5 μm



Chapter 4

REGULATION OF THE PLC SIGNALING PATHWAY IN TASTE RECEPTOR CELLS BY $G\alpha_{gustducin}$ AND PHOSPHORYLATION

4.1 INTRODUCTION

The sensation of taste has evolved to allow organisms the capability to detect chemical stimuli in their environment. Taste plays an important role both in identifying nutritional foodstuffs that may be sweet and salty and harmful substances that may be bitter or sour.

In mammals the perception of taste begins in the oral cavity where the cells ultimately responsible for the detection of chemical stimuli are located. Taste receptor cells are organized into structures called taste buds, which can contain between 50 and 150 polarized cells (for reviews see (Witt and Reutter, 1996; Finger, 2000). Taste buds are further organized into connective tissue structures called papillae that are located in discrete locations of the oral pharyngeal cavity. Fungiform papillae are located in the anterior tongue, contain one to two taste buds, and are innervated by the chorda tympani branch of cranial nerve VII. Foliate and circumvallate papillae are in the posterior lateral and medial regions of the tongue, respectively. Branches of both cranial nerve VII and IX innervate foliate papillae while the glossopharyngeal (CN IX) innervates the circumavallate papilla (Lindemann, 1996).

Neuroepithelial taste receptor cells mediate the transduction of tastants and the information is propagated to the nervous system via primary afferents. Simple ionic stimuli such as salts and acids interact directly with ion channels in the membrane whereas more complex stimuli such as bitter, sweet, and umami compounds activate second messenger systems by way of G-protein coupled receptors (Kinnamon, 2000). Bitter taste transduction is the best understood of the G-protein activated pathways (Lindemann, 1996; Gilbertson et al., 2000; Glendinning, 2000). Bitter stimuli bind T2 receptors (T2R); also called taste receptor B's (TRB) located in the apical portion of taste receptor cells (Adler et al., 2000; Chandrashekar et al., 2000; Matsunami et al., 2000). T2R's/TRB's activate a heterotrimeric G-protein most often consisting of $G\alpha_{gustducin}$ (Ruiz-Avila

et al., 1995; Ming et al., 1998) and its partners, $\beta 3\gamma_{13}$ (Rossler et al., 1998; Huang et al., 1999), although other alpha subunits have been implicated (Caicedo et al., 2003). $\beta 3\gamma_{13}$ activate phospholipase $C\beta_2$ that in turn cleaves phosphotydil inositol bisphosphate into inositol trisphosphate (IP_3) and diacyl glycerol (DAG)(Rossler et al., 1998). While the role of DAG is unknown in taste receptor cells, IP_3 activates inositol trisphoshpate receptors (IP_3R's) most often located in the membrane of smooth endoplasmic reticulum (SER)(Otsu et al., 1990). The $G\alpha_{gustducin}$ subunit activates phosphodiesterase (PDE) that converts cyclic adenosine monophosphate (cAMP) into 5' adenosine monophosphate (5'AMP), resulting in a decrease of cAMP (Yan et al., 2001). The downstream target of the decreased intracellular cAMP in taste receptor cells is currently unknown. Interestingly, in pancreatic acinar cells IP_3R3, which is also the predominant isoform found in taste receptor cells (Clapp et al., 2001; Miyoshi et al., 2001), is inhibited by cAMP dependent phosphorylation (Giovannucci et al., 2000a; Giovannucci et al., 2000b), as is $PLC\beta_2$ in COS-7 cells (Liu and Simon, 1996). In taste cells we hypothesize that $G\alpha_{gustducin}$ reduces phosphorylation of PLC signaling components, including IP_3R3 and $PLC\beta_2$, enhancing release of Ca^{2+} from intracellular stores (Fig. 4.1). This chapter describes our preliminary experiments designed to test this hypothesis using Ca^{2+} imaging with pharmacological agents to experimentally manipulate the phosphorylation state in taste receptor cells.

4.2 Materials and methods

Isolation of mouse taste receptor cells and taste buds

Animals were cared for in compliance with the Colorado State University Institutional Animal Care and Use Committees. Taste buds from both C57/BL and 129 strains of mice were isolated from circumvallate papillae using the protocol of Béhé et al., (1990). Animals were anesthetized with CO_2 and sacrificed by cervical dislocation. The tongues were removed and rinsed with freshly made cold Tyrodes solution. The tongue was trimmed at the median eminance and approximately 1cc of enzyme containing Dispase II (3 mg/ml; Boehringer Mannheim), Trypsin inibitor (1 mg/ml; Sigma), and Collagenase B (0.8 mg/ml; Boehringer Mannheim) in Tyrodes was injected below the epithelium. This was then placed in fresh Tyrodes and perfused with oxygen for 30 minutes. The epithelium was carefully removed and pinned in a sylguard dish containing Ca^{2+} free media for 30 minutes. Individual taste buds and cells were removed by gentle suction a glass pipette and placed on cover slips coated with Cell-Tak (BD Biosciences, Bedford, MA).

Intracellular calcium measurement

Intracellular Ca^{2+} measurements were obtained using the membrane permeable Ca^{2+} sensitive dye fura-2 AM (Molecular Probes). Cover slips were

attached to a perfusion chamber (Warner Instrument Corp) and cells were bathed in 2μM fura-2AM + 0.8% DMSO (Sigma) + 0.02% pluronic (Molecular Probes) in Tyrodes for 20 minutes. Cells/buds were then washed for 20 minutes with Tyrodes to allow for adequate de-esterification of the dye. Images were acquired with a CCD camera (IC100-ICCD, Paultec Imaging) through a 40X oil immersion lens (Fluor X40, 1.3 NA, Nikon) of an inverted Nikon Diaphot TMD microscope. Images from the camera were captured using Axon Imaging Workbench software with an Axon Image Lightning 2000 video capture board in a PC. Fura-2 loaded cells were excited at both EX350nm and EX380nm and flourescent images were taken at EM510nm. A set of images was captured every 5 seconds to allow experiments to run up to 30 minutes without significant photobleaching. Data were plotted as a ratio of F350/F380 over time and analyzed using Origin 4.1 software or GraphPad Prizm 3.0.

Cells were constantly perfused with Tyrodes solution until challenged. Stimuli included: $bt_3Ins(1,4,5)P_3/AM$ (50μM, Alexis Biochemicals) a membrane permeant form of IP_3, ATP (10μM, Sigma), ACh (10μM, Sigma), Thapsigargin (1μM, Sigma), cyclopiazonic acid (20μM, Calbiochem), PKAi (20μM, Calbiochem), KT5720 (1μM, Calbiochem), H-89 (200μM, Calbiochem), forskolin (10μM, Tocris), IBMX (100μM, Sigma), and SQ 22536 (50μM, Sigma).

Solutions

Tyrodes: 140mM NaCl, 5mM KCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 10mM HEPES, 10mM glucose, and 1mM pyruvic acid, pH 7.4. Ca^{2+} free Tyrodes:

140mM NaCl, 5mM KCl, 10mM HEPES, 2mM EGTA, pH=7.4. High K^+ Tyrodes: 90mM NaCl, 55mM KCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 10mM HEPES, 10mM glucose, 1mM pyruvic acid, pH=7.4.

4.3 Results

To examine possible modulation of IP_3R3 by phosphorylation the Ca^{2+} responses to membrane permeant IP_3 in the presence and absence of cAMP and PKA modulators were examined. Under these conditions, any phosphorylation effects would be confined to IP_3R3 or a downstream target, such as TRPM5. Bath perfusion of IP_3-AM in normal Tyrodes induced sharp and reversible increases of intracellular Ca^{2+} in two cells, however only at concentrations of 50μM (Fig. 4.2). We had a very limited supply of IP_3-AM, which was extremely expensive making more experiments impractical using 50μM conccentrations. However, it is possible that chronic phosphorylation was inhibiting responses to lower concentrations of IP_3 and PKA inhibitors would unmask that release. Out of thirty-nine cells there were no reproducible Ca^{2+} response to 25μM IP_3-AM alone or with the PKA inhibitors PKAi, KT5720, or H89. The SERCA pump inhibitors thapsigargin and cyclopiazonic acid (CPA) were used in attempt to mimic intracellular Ca^{2+} release by IP_3R's. Thapsigargin did increase intracellular Ca^{2+} in some cells but the effect was very small, slow

and irreversible. No measurable increase in intracellular Ca^{2+} was recorded from the application of CPA to twelve cells.

The inability to efficiently evoke Ca^{2+} responses with membrane permeant IP_3 lead to investigations with other stimuli that activate components of the PLC signaling pathway and attempts to manipulate phosphorylation in these cells. My working hypothesis is that most bitter sensitive cells express the $G\alpha_{gustducin}$ subunit, which decreases intracellular cAMP, eventually modulating the PLC signaling pathway via phosphorylation. Thus, bitter stimuli are an obvious choice, however, bitter sensing cells do not appear to be broadly tuned to different bitter stimuli but instead respond to single discrete stimuli (Caicedo et al., 2003). This makes it very tedious to randomly select a cell that responds to one particular bitter compound. A bitter "cocktail" that included several bitter compounds was also tried but did not evoke predictable Ca^{2+} responses.

Previous work has demonstrated that acetylcholine (ACh) causes sharp and reversible increases in cytosolic Ca^{2+} and does so via the PLC signaling pathway (Ogura, 2002). The previous results with ACh as the stimulus have been accurately reproduced in thirty-four cells showing that ACh induces large Ca^{2+} responses that can be washed out and repeated (Fig. 4.3). The presumption is ACh activates the same transduction components used by bitter stimuli in taste receptor cells. Thus, when ACh binds its receptor, I am assuming the $G\beta\gamma$ subunits activate the PLC pathway, while $G\alpha_{gustducin}$ activates PDE, decreasing cAMP. To investigate the effects of phosphorylation the adenylate cyclase activator forskolin was used in an attempt to increase intracellular cAMP

levels and presumably PKA activity in ACh sensitive cells. Forskolin irreversibly inhibited the Ca^{2+} response in all eighteen cells stimulated with ACh (Fig. 4.3).

Adenosine trisphosphate (ATP) also has been shown previously to activate the PLC signaling pathway and elicit Ca^{2+} release from intracellular stores in taste receptor cells (Baryshnikov et al., 2003). Similar to ACh, bath perfusion of ATP evoked Ca^{2+} responses in a large number of cells (85), and the presence of forskolin decreased the response to ATP in thirteen out of fifteen cells tested (Fig. 4.4). Since forskolin is known to have non-specific effects, intracellular cAMP levels were also manipulated using membrane permeant 8Br-cAMP, along with the PDE inhibitor IBMX to prevent breakdown of cAMP. In 13 cells bath perfusion of both 8-Br-cAMP and IBMX failed to decrease the Ca^{2+} response to ATP (Fig. 4.5). It is possible that isolated taste cells already have high levels of cAMP and that PLC signaling components are maximally phosphorylated under our recording conditions. To test this hypothesis the AC inhibitor SQ22356 was used, which should decrease intracellular cAMP and the activity of PKA dependent phosphorylation, but SQ22536 was unable to alter the Ca^{2+} response to ATP in thirteen cells (Fig. 4.5).

4.4 Discussion

Thus far in this in this study it has been demonstrated that IP_3-AM can cause Ca^{2+} release in taste receptor cells presumably by activation of IP_3R3 and

this does not appear to be affected by inhibition of PKA. Both ACh and ATP evoke a fast and reversible Ca^{2+} response in many taste receptor cells, which is attenuated in the presence of the adenylate cyclase activator forskolin. These data suggest that phosphorylation of PLC signaling components may affect Ca^{2+} signaling in taste receptor cells. However, because forskolin has non-specific effects such as inhibition of K^+ channels (Herness et al., 1997), whether or not phosphorylation affects IP_3R3 or $PLC\beta_2$ remains to be determined.

Upon activation, $G\alpha_{gustducin}$ activates a PDE decreasing intracellular cAMP levels (Hoon et al., 1995; Ruiz-Avila et al., 1995). $G\alpha_{gustducin}$ knockout mice are insensitive to bitter stimuli, suggesting this α subunit is important for bitter taste transduction (Wong et al., 1996; Ruiz et al., 2003). A role for the decreased cAMP in bitter taste transduction has not been reported but possible targets include PKA and cyclic nucleotide gated (CNG)(Misaka et al., 1997)or blocked (Kolesnikov and Margolskee, 1995) channels, however GFP-tagged $G\alpha_{gustducin}$ expressing mouse taste receptor cells do not appear to respond to membrane permeant cAMP (Medler, 2001). Thus, it is not likely that the target of the reduced cAMP is a plasma membrane ion channel. In other systems such as pancreatic acinar cells PKA dependent phosphorylation modulates the sensitivity of IP_3R3 such that decreases in PKA activity enhance Ca^{2+} release through IP_3R3 (Giovannucci et al., 2000a; Giovannucci et al., 2000b). IP_3R3 is also the isoform expressed in taste receptor cells (Clapp et al., 2001; Miyoshi et al., 2001). IP_3-AM has been used to investigate the effects PKA dependent phosphorylation on IP_3R3 in taste receptor cells but thus far I have not completed

a comprehensive study due to the high concentration needed to evoke a Ca^{2+} response and its limited supply. The preliminary results suggest that inhibition of PKA activity does not increase the sensitivity of IP_3R3. No responses were seen to IP_3-AM at concentrations of 5μM, 10μM, and 25μM or in the presence of PKA inhibitors such as PKAi, KT5720, and H89. IP_3-AM includes an ester group that needs to be cleaved in order to be active. It is possible that the endogenous intracellular esterases are relatively slow and elicit only a small, slow, sustained amount of IP_3, which may cause a change in Ca^{2+} too small to measure.

$PLC\beta_2$ is also a target PKA dependent phosphorylation (Liu and Simon, 1996) and recent evidence suggests that $PLC\beta_2$, the only PLC isoform currently reported in taste receptor cells, mediates bitter taste transduction (Rossler et al., 1998; Huang et al., 1999; Asano-Miyoshi et al., 2000; Zhang et al., 2003). Perhaps $PLC\beta_2$ is a target of the $G\alpha_{gustducin}$-mediated decrease in cAMP. In order to investigate the effects of phosphorylation on PLC signaling components an effective stimulator of this pathway was needed. Initially bitter compounds were used to stimulate taste receptor cells but this turned out to be impractical. Bitter sensing cells do not appear to be broadly tuned to different stimuli but instead most often respond to only a single discrete bitter substance (Caicedo and Roper, 2001) and I could not accurately evoke responses in enough cells.

The use of ATP and ACh seemed feasible because they both activate the PLC signaling pathway in a significant number of cells (Ogura, 2002; Baryshnikov et al., 2003). My hope is that by triggering the PLC signaling cascade with ATP and ACh transduction machinery is activated that includes

PLCβ_2 and IP_3R3. Application of ACh or ATP causes large increases in cytosolic Ca^{2+} that are similar in size and shape, however Ca^{2+} responses to ATP are slightly more repeatable than those evoked by ACh, thus ATP was examined in more detail. Forskolin is a cell permeable direct activator of AC, which increases cAMP, although it can have nonspecific effects on other cellular machinery (for review see (Insel and Ostrom, 2003). Both ACh and ATP responses are reduced in the presence of forskolin even though occasionally a cell responding to ATP that did not appear to be affected by forskolin. Although I presume these data are a result of increased cytosolic cAMP, the inhibition could be due to some nonspecific effect of forskolin such as a blockage of K^+ channels (Herness et al., 1997). The possibility that forskolin is affecting something other than cAMP lead to other methods that can manipulate cAMP levels including cell permeable 8Br-cAMP and the PDE inhibitor IBMX, which prevents cAMP breakdown. Application of 8Br-cAMP and IBMX did not appear to alter the Ca^{2+} response to ATP. Although ATP activates the PLC pathway it may do so in a manner independent of phosphorylation such as activation of a PLC isoform other than PLCβ_2, which does not include phosphorylation domains.

In support of my hypothesis some recent data have emerged suggesting that phosphorylation inhibits Ca^{2+} release in a subset of ATP sensitive taste receptor cells (Baryshnikov et al., 2003). These data state that application of 8Br-cAMP and IBMX decreases the ATP response in taste receptor cells by 10-30% (Baryshnikov et al., 2003). I have attempted to reproduce these data but have not been able to see any change in the ATP response in the presence of

8Br-cAMP and IBMX. However, Baryshnikov et al., (2003) does state that these data may not be conclusive because repeated ATP responses can show desensitization, which may account for the 10-30% decreased ATP response they report. This study also reported the application of the AC inhibitor SQ22536 increased the Ca^{2+} response to ATP (Baryshnikov et al., 2003) and again I have been unable to duplicate this. The data using the AC activator forskolin concurs with data from Barshnikov et al., (2003) suggesting phosphorylation decreases the Ca^{2+} response to ATP. No positive controls for the 8Br-cAMP, IBMX, and SQ22536 were carried out to make sure they are acting as we believe. It is possible that the effects of these compounds are not seen because they are not getting to their targets properly. Further experiments are needed to prove the efficacy of 8Br-cAMP, IBMX, and SQ22536 with my experimental setup. It is also important to note that the experiments involving forskolin were not performed in the traditional manner. Unfortunately, forskolin was never applied to a cell in the presence of a stimulus (ACh or ATP), rather it was applied immediately prior. These experiments need to be repeated so that forskolin is applied both before and during the application of a stimulus. However, based on current data I do believe forskolin reduces the Ca^{2+} response to both ACh and ATP.

Finally, it is possible that phosphorylation of PLC signaling is not important in for bitter transduction. Mice lacking $G\alpha_{gustducin}$ have a reduced sensitivity to bitter compounds but are not completely insensitive. Perhaps the only role of $G\alpha_{gustducin}$ is to hold the $\beta\gamma$ subunits in place. In this case the $G\alpha_{gustducin}$ knockout

may be compromised because the βγ subunits are not properly located for activation. It may also be that without $G\alpha_{gustducin}$ the βγ subunits are causing persistent activation of $PLC\beta_2$, decreasing the sensitivity to bitter stimuli.

4.5 Future directions

The effects of forskolin appear promising, however we have not done the control experiments to make sure it is not blocking K^+ channels. To test the specificity of forskolin we will use dideoxyforskolin, which is a forskolin analog that does not activate adenylate cyclase but can block K^+ channels as forskolin does in some cells. There is a good chance that many of the cells being imaged do not possess bitter taste transduction machinery. We now know that $PLC\beta_2$ is essential in bitter taste transduction (Zhang et al., 2003) and that IP_3R3 and TRPM5 are expressed in these same cells (Clapp et al., 2001; Miyoshi et al., 2001). We have recently received a transgenic GFP-IP_3R3 mouse and a transgenic GFP-TRPM5 mouse that I am currently testing to ensure GFP appears in the correct cells. These lines will allow us to select and image only Type II cells expressing IP_3R3, TRPM5 and by inference $PLC\beta_2$. These mice should provide more dependable results by allowing consistent selection of the correct cell type expressing bitter taste transduction proteins. We also have $G\alpha_{gustducin}$ knockout mice that express GFP. I will use Ca^{2+} imaging to determine

if these cells have altered resting levels in Ca^{2+}, which might indicate constitutive $\beta\gamma$ activity.

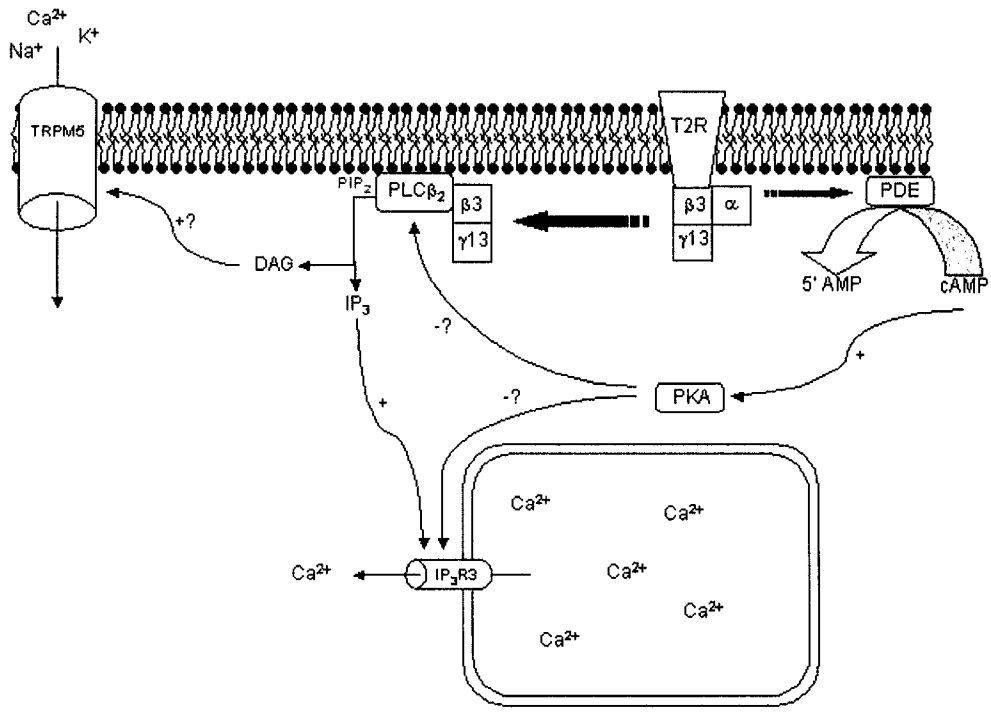


Figure 4.1. Our hypothesis is that the $G\alpha_{gustducin}$ subunit attached to T2R's plays a role in modulating the PLC signaling pathway. It has been shown to activate a PDE and decrease intracellular cAMP however the role of this diminished cAMP is currently unknown. We propose that as the G-protein is activated two pathways are activated, which converge to regulate Ca^{2+} release. The $G\beta\gamma$ subunits activate IP_3R3 by way of $PLC\beta_2$ and IP_3 and the $G\alpha$ subunits reduce PKA activity to potentiate Ca^{2+} release.

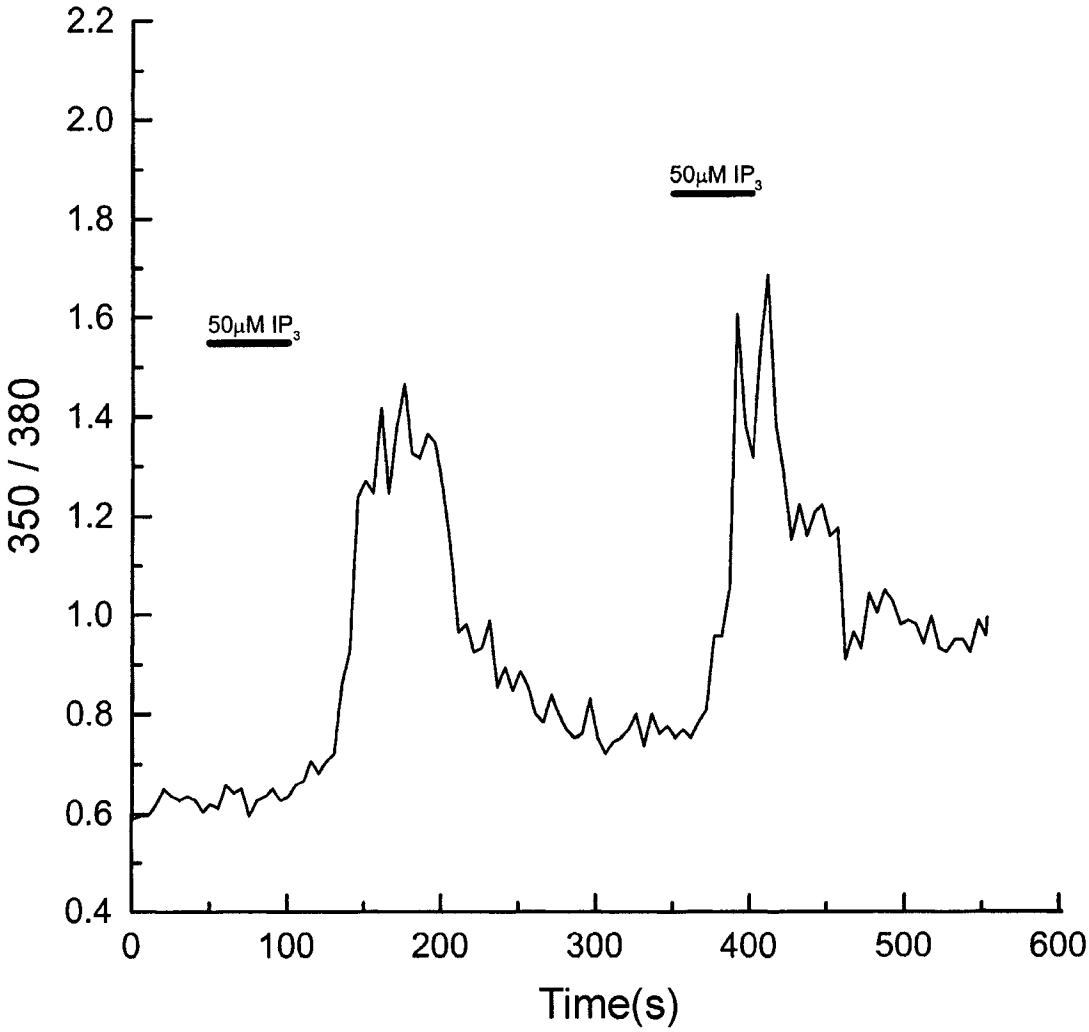


Figure 4.2. IP_3-AM induced Ca^{2+} transients as measured by fura2-AM fluorescence. 50μM IP_3-AM causes a Ca^{2+} transient that can be washed out.

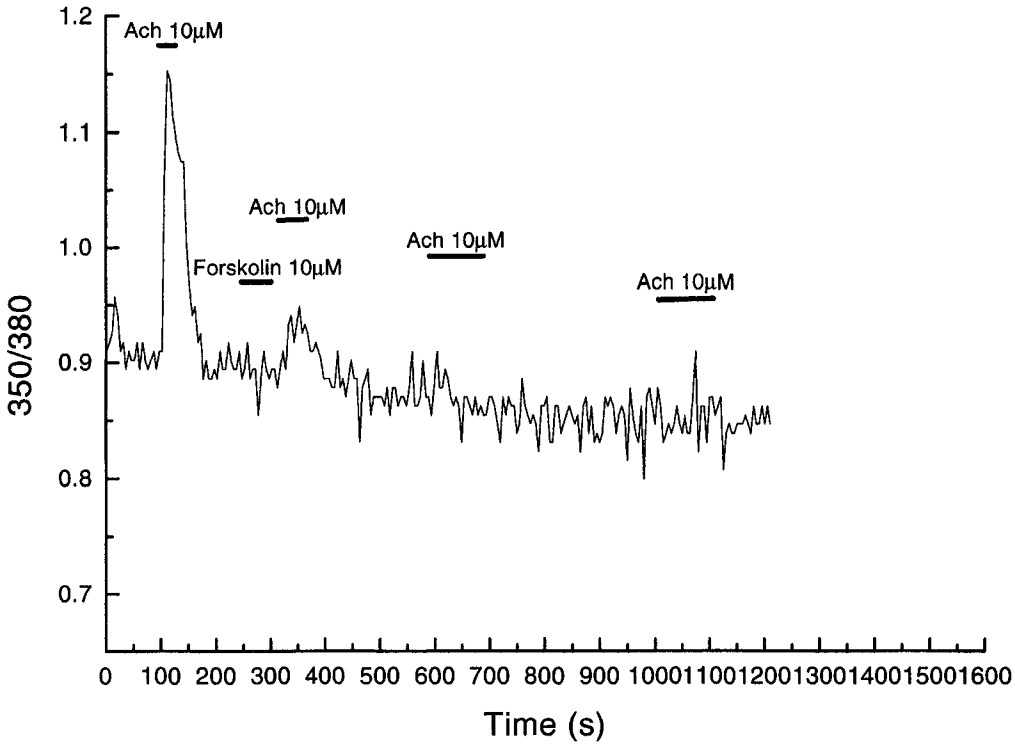


Figure 4.3. Acetylcholine (ACh) induced Ca^{2+} transients in mouse taste receptor cells. 10μM ACh causes a sharp increase in intracellular Ca^{2+}. This ACh response can be inhibited by prior application of 10μM forskolin.

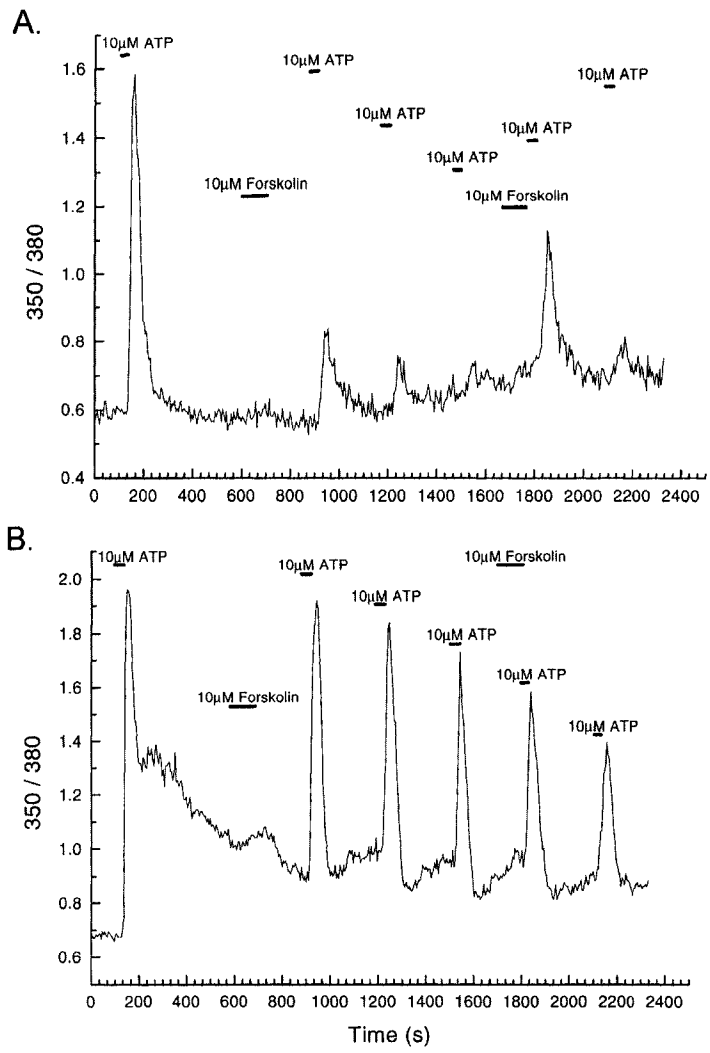


Figure 4.4. ATP induced Ca^{2+} transients in mouse taste receptor cells as measured by fluorescence of fura2-AM. **A.** 10μM ATP causes rapid and reversible Ca^{2+} influx that is inhibited by prior application of forskolin in most cells. **B.** Occasionally an ATP responsive cell did not appear to be inhibited with 10μM forskolin.

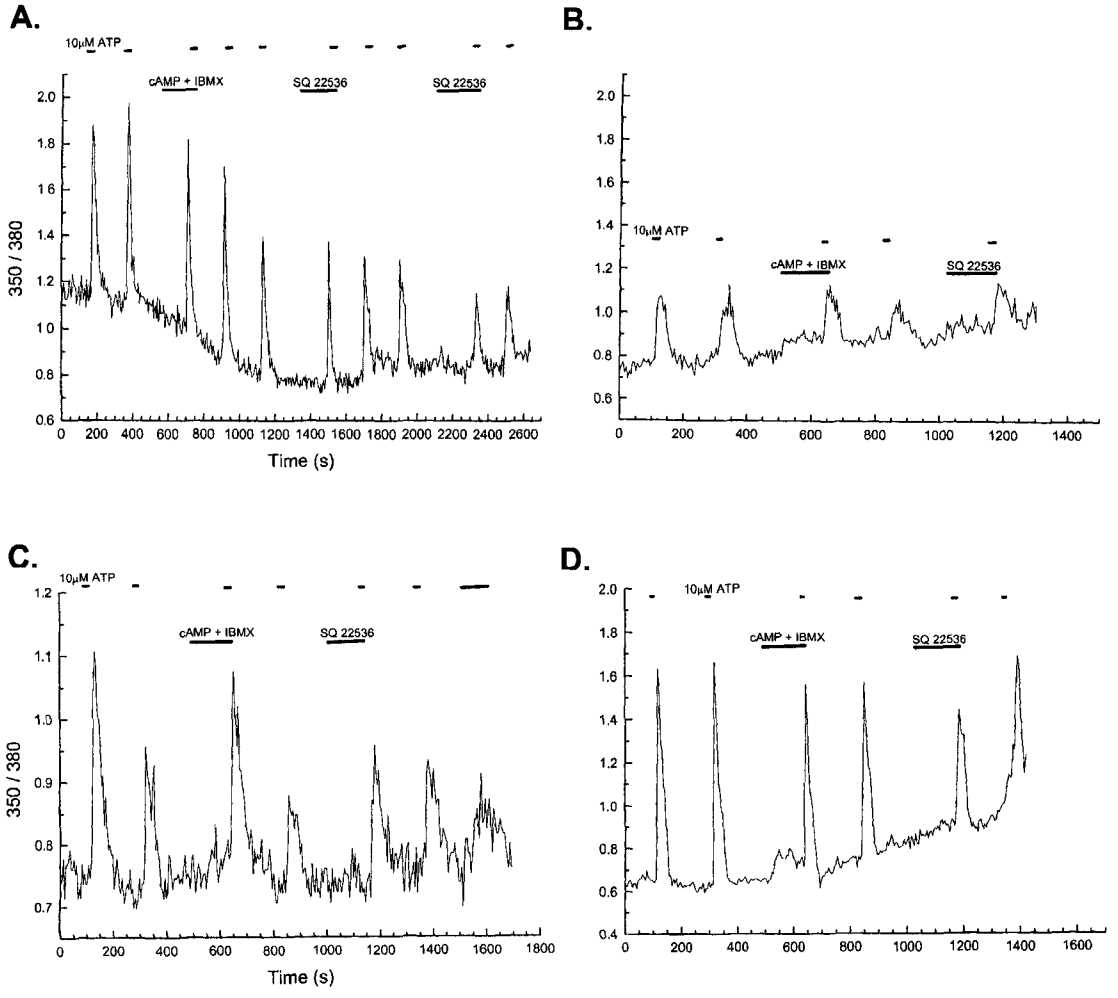


Figure 4.5. Effects of cAMP agonists/antagonists on Ca^{2+} release stimulated by ATP. Agonists/antagonists of cAMP do not appear to have an effect on Ca^{2+} influx in taste receptor cells as measured by fluorescence of fura2-AM. Even small responses to ATP as shown in **B.** do not seem to change in the presence of cAMP agonists/antagonists. 400μM 8Br-cAMP; 100μM IBMX; 50μM SQ 22536.

Chapter 5

SUMMARY

It has been shown for some time that inositol trisphosphate (IP_3) is produced in taste receptor cells responding to certain stimuli. The majority of the data comes from work with bitter compounds and this is also what we have focused on. In attempt to further the understanding of bitter taste transduction we first identified the target of IP_3 as the type III IP_3 receptor (IP_3R3) and show that this receptor is co-expressed in cells with other proteins involved in bitter taste transduction such as $G\alpha_{gustducin}$ and $PLC\beta_2$.

It was shown over thirty years ago that there are morphologically distinct cell types in mammalian taste buds. Rodents have four distinct cell types three of which extend from the basal lamina to the taste pore and are designated Type I, II, and III. Some evidence suggests a supportive role for the Type I cells

leaving Type II and III as probable candidates for actual transduction of taste stimuli. Recent work showed that PLCβ_2 is essential for the transduction of bitter, sweet, and umami stimuli (Zhang et al., 2003) and we have presented evidence suggesting IP_3R3 is co-expressed with PLCβ_2. Using immunocytochemistry and immuno-electron microscopy we have been able to show that Type II cells express both IP_3R3 and PLCβ_2 suggesting this cell type is responsible for the transduction of bitter, sweet, and umami stimuli. However, Type III cells are the only cells that make conventional synapses with the nervous system. If indeed Type II cells are the principle transducers of bitter, sweet, and umami, stimuli they must have a way to communicate this information to the nervous system. One thought is that Type II cells relay information to Type III cells, which can then relay information via conventional synapses. Interestingly, we found that many, if not all; Type II cells expressing IP_3R3 and PLCβ_2 possess subsurface cisternae (SSC) of smooth endoplasmic reticulum. These are close appositions between the membrane of the endoplasmic reticulum of the taste cell, the plasma membrane of the taste cell, and the membrane of a nerve cell. In other systems such as the auditory system these structures are a functional component of a synapse. We believe it is possible Type II cells use SSC as their way of communicating with the nervous system.

Taste receptor cells predominantly express the type III IP_3 receptor. Interestingly this isoform of the IP_3R's is very sensitive to phosphorylation. In pancreatic beta cells it has been shown that activity of cyclic adenosine monophosphate (cAMP) dependent protein kinase A (PKA) significantly reduces

the ability of IP_3R3 to release Ca^{2+} into the cytosol. Taste cells responding to bitter stimuli not only generate IP_3 but also decrease cAMP levels via $G\alpha_{gustducin}$. The purpose or effect of $G\alpha_{gustducin}$ and the subsequent decrease in cAMP is not yet known but we believe it may play a role in decreasing the activity of PKA leading to a decrease in the phosphorylation state of IP_3R3. Using the adenylate cyclase (AC) activator forskolin to increase cytosolic cAMP levels we have been able to decrease the Ca^{2+} response to stimulators of the PLC signaling pathway. Although these preliminary data are encouraging it does not show a direct link between $G\alpha_{gustducin}$ and IP_3R3. There are several points along the transduction pathway that we haven't investigated, one of which is phosphorylation of $PLC\beta_2$. Our hope is that with the use of Ca^{2+} imaging techniques and pharmacological agents we can manipulate the phosphorylation state of the PLC signaling pathway giving insight into the role of $G\alpha_{gustducin}$.

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