Intracellular pH, the Proximate Signal for Cell Volume Changes that are Mediated by the Actin Cytoskeleton

William Pasley

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INTRACELLULAR pH, THE PROXIMAL SIGNAL FOR CELL VOLUME
CHANGES THAT ARE MEDIATED BY THE ACTIN CYTOSKELETON.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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August 2005
Acknowledgements

The author would like to thank several people who have assisted, contributed to, and supported this work. I would like to thank my wife, Rachel, who has been loving, supportive, and understanding of the time I’ve needed to commit to this project. I would like to thank my parents for their encouragement to persevere and complete my degree. I thank Dr. Lyall for all his guidance, motivation, and teaching that has enabled me to bring this project together. I would like to thank Dr. DeSimone for his confidence in me. I would also like to thank Shobha for all of her assistance in teaching me the techniques necessary to do this research. Lastly, I would like to recognize Vicky, Tam, Dr. Heck, and Dr. Vinnikova who have each contributed to this project.
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<td>NH₄Cl</td>
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Chapter 1: Introduction

1.1 An Overview of Taste

"Melts in your mouth," "Too good to be true," or maybe just "Wow!" Those are just some of the ways that people describe the way something tastes. Our sense of taste, along with seeing, hearing, smelling, and touching, make up the five special senses that humans and other vertebrates discriminate. Gustation, the sense of taste, is perceived and transduced by the chemoreceptive taste cells on the tongue. Humans recognize four primary taste qualities: sour, sweet, salt, and bitter. However, in recent years, other taste qualities have been recognized: water, electric taste and umami (the taste of monosodium glutamate; MSG) (44, 45).

Gustation has a much more basic function than allowing humans to distinguish between the different styles of food preparation. That function is to determine whether an ingested compound is beneficial or harmful (33). At the most primitive level, compounds that stimulate sweet taste receptors, such as simple sugars and carbohydrates are perceived as beneficial or good based upon their taste perception. Other compounds that have an intense bitter taste quality would be rejected as potentially harmful (17). Of course, most food perception is not this simple. However, what is perceived as tasting good is eaten, while those foods that taste bad are rejected (44).
1.2 Taste Receptor Cells

1.2.1 Taste Receptor Cell Function

Dispersed throughout the surface of the tongue, taste receptor cells (TRCs) detect the presence of thousands of different taste compounds ingested daily (45, 78). Each individual compound excites one or more of the four or five basic taste qualities inducing a chemical signal that must be ultimately converted into an electrical signal that forms a response pattern that the brain can interpret. Interaction of these response patterns in the brain allows humans to differentiate substances with very similar taste qualities (19, 35, 11).

1.2.2 Taste Receptor Cell Morphology

Highly specialized, elongated chemotaxic TRCs are responsible for gustation. Through apical plasma membrane receptor proteins, ion channels, and intracellular signaling cascades, TRCs detect and respond to taste compound or tastants. Although in many ways these cells are similar to epithelial cells, they have the ability to elicit action potentials and, as a result, are similar to neurons, but unlike neurons, TRCs can regenerate if damaged (11). This dual similarity to two types of clearly defined cells has resulted in TRCs being termed the neuroepithelial cells (33).

Grouped together with TRCs are two other groups of cells: basal cells and supporting cells. Basal cells are the precursors to TRCs because as need arises they will differentiate into new TRCs. Supporting cells have no clearly defined role in relation to TRCs, but it has been observed that they demonstrate paracrine-like activity (11). Basal cells, supporting cells, and TRCs jointly form the taste bud (Figure 1.1).
Figure 1.1 Cartoon of a taste bud. Diagram illustrates the cellular makeup of a single taste bud.
1.3 Taste Buds

1.3.1 Taste Bud Morphology

Taste buds are collections of 50-100 individual cells that have the appearance of an onion bulb (35). Approximately 55 $\mu$m in height and 30-70 $\mu$m in width, taste buds have microvillar processes that extend from the apical neck into the opening of the taste pore (39, 75). The apical taste pore is very narrow, 5-7 $\mu$m, and is the opening through which gustatory stimuli are detected. The microvilli allow for an increased surface area to detect stimuli from the oral environment.

1.3.2 Taste Bud Distribution

Taste buds are distributed throughout the oral cavity, but the largest collection is found along the dorsal surface of the tongue. These lingual taste buds are located within specialized papillae. Papillae are “elevations of the oral epithelium and lamina propria that assume various forms and functions” (35). Four types of papillae are present on the tongue: filiform, fungiform, foliate, and circumvallate. However, only three of the four are involved in taste perception. Fungiform, foliate, and circumvallate have gustatory properties (Figure 1.2).

Fungiform papillae have a mushroom shape with their taste buds located along the surface of the papillae. These papillae are distributed irregularly along the dorsal surface of the tongue, but most are centered around the anterior tip (11, 35). Foliate papillae are poorly developed in humans. They are located along the lateral edges back towards the posterior edge of the tongue. These papillae have parallel ridges and furrows. The taste buds are located within these surfaces (35). Along the base of the tongue arranged in
Figure 1.2 Cartoon of the surface of the human tongue. Illustrates the three types of functional papillae and their distribution on the lingual surface.
rows are the circumvallate papillae. They are smallest in number, but contain about half of the total number of taste buds. A trench containing the taste buds surrounds each of these papillae (11).

1.4 Innervation of the Lingual Taste Buds

Three cranial nerves (CN) serve as pathways for the perception of taste. They are the facial nerve (CN VII), the glossopharyngeal nerve (CN IX), and the vagus nerve (CN X) (11). Taste buds within the tongue are innervated by only two of these nerves, CN VII and CN IX.

The chorda tympani (CT) is the branch of the facial nerve responsible for innervating the anterior two-thirds of the tongue. This innervation includes the fungiform papillae. A branch of the glossopharyngeal nerve, the lingual tonsilar branch, innervates the posterior third of the tongue where the foliate and circumvallate papillae are located. These two nerves together with the vagus nerve, which innervates the back of the throat and epiglottis, ascend from the tongue in the solitary tract, and terminate in the solitary nucleus of the medulla. Second-order nerve fibers within the medulla project ipsilaterally to the ventral posteriomedial nucleus of the thalamus. Taste information is then relayed via third-order neurons which terminate in regions of the cortex responsible for taste interpretation (11).

1.5 Sour Taste

Of interest to this study is the response of a subset of rat TRCs to acid stimuli. Sourness stimuli evoke a response through dissociable $H^+$ ions. Many people have assumed that $pH_0$ dictates sour taste response; however, this assumption is not true. If
pH\textsubscript{e} controls sour taste, then two different acids at the same pH would taste the same; however, even from practical experience, sourness perception does not correspond to stimulus pH (71, 43, 2). For example, when using a CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-} buffer system, studies in TRCs have shown that it is unnecessary to have pH\textsubscript{e} changes to decrease TRC pH\textsubscript{i} (54). CO\textsubscript{2} entry into TRCs is driven by its concentration gradient and is independent of stimulus pH (51). Intracellular carbonic anhydrase catalyzes the conversion of CO\textsubscript{2} to H\textsubscript{2}CO\textsubscript{3} inside the cell (40, 13, 29).

Early neural recordings support the evidence that sourness is not driven by stimulus pH (28, 3, 4). More recent experimentation has found that increasing concentrations of CO\textsubscript{2} at physiological pH are able to reversibly modulate CT nerve activity (51). The evidence overwhelmingly supports pH\textsubscript{i} as the proximate stimulus for sour taste response (51).

While studies in isolated TRCs, intact lingual preparations, and CT nerve responses have proven that pH\textsubscript{i} is the promixate stimulus for sour taste transduction, several different mechanisms whereby H\textsuperscript{+} ions enter the cell have been proposed and tested. Lyall et al. (52) have found that there is an apical H\textsuperscript{+} channel that is responsible for the H\textsuperscript{+} entry for strong acids. The divalent cations, Zn\textsuperscript{2+} and Cd\textsuperscript{2+} block this channel, but Ca\textsuperscript{2+} does not. Furthermore, this apical channel shows increased activity when TRCs are treated with cAMP before the acid stimulus is applied (52). Weak acids are demonstrated to have high lipid solubility and are able to diffuse across the apical membrane into TRCs as undissociated neutral molecules. Once inside the cells, the neutral molecule dissociates into H\textsuperscript{+} and the corresponding anion. As mentioned above,
CO$_2$ diffuses across the cell membrane, but instead of dissociating into a neutral molecule, carbonic anhydrase catalyzes its conversion to carbonic acid which then dissociates into H$^+$ and the corresponding anion (51). Passive diffusion down a concentration gradient and the dissociation constant are the driving forces behind the generation of H$^+$ inside the cell.

Ugawa et al. (85) have discovered a heteromeric acid sensing channel (ASIC2) that is gated by external H$^+$. Reverse-transcriptase polymerase chain reaction (RT-PCR) and immunohistochemical testing revealed that this channel is expressed in circumvallate, foliate, and fungiform papillae. Stimulation of this channel revealed that it had several important qualities, namely, it generated maximal inward currents at a pH $\leq$ 2.0, which is in agreement with the *in vivo* pH sensitivity of rat taste cells, and that the amiloride sensitivity of the heteromer decreased with decreasing pH and was almost completely abolished at a pH of 2.0. These findings provide persuasive explanations for the amiloride insensitivity of acid-induced responses of rat taste cells (85). This channel is activated by external H$^+$ but is not a pathway for H$^+$ entry. Upon H$^+$ activation, the ASIC channel allows Na$^+$ ions to enter the cells. However, recent studies suggest that acid responses are independent of external salt concentration and pH$_o$ (51). Thus, the ASIC is most likely not the sour sensing ion channel in TRCs.

Not all researchers agree that the ASIC channel is involved in the entry of strong acid H$^+$ ions. Richter et al. (73) were unable to detect the expression of this channel in mice by RT-PCR. Also, in ASIC2 knock-out mice, there was no change in intracellular Ca$^{2+}$ upon physiological stimulation (73). Richter et al. (73) report a sour response
dependence upon extracellular $\text{Ca}^{2+}$ influx in mice which was seen to occur when acid stimuli depolarize gated calcium-channels allowing an influx of $\text{Ca}^{2+}$. Experimentation using $\text{Cs}^+$ and amiloride did not affect acid-evoked $\text{Ca}^{2+}$ responses leading to the conclusion that neither hyperpolarization-activated cyclic nucleotide-gated cation (pacemaker) channels nor epithelial $\text{Na}^+$ channels, respectively, transduce sour taste (73).

Richter et al. (73) stated, “Consequently, it is difficult to differentiate between events related to sour taste transduction \textit{per se} and unrelated effects of protons.” In referring to the multiple activities that TRCs must regulate involving protons, they have suggested that \textit{in vivo} evidence must support whatever conclusion is drawn from \textit{in vitro} experimentation. (In actuality it is the in vitro evidence that must support the in vivo observations, not vice versa.) Consequently, I believe that the $\text{H}^+$ channel proposed by Lyall \textit{et al.} (52) more accurately depicts what actually occurs in intact lingual epithelial cells because when this apical $\text{H}^+$ channel is modulated, the neural response recorded in the CT is altered also.

1.6 Cellular Volume

Many researchers have studied how a primary change in cellular volume can regulate numerous membrane transporters, which in turn can result in cell volume compensation (32, 1, 83, 74, 16, 55). In almost all cases these studies have used an osmotic disequilibrium approach to change cellular volume and then monitor changes in various membrane transporters. To quote one researcher, “After exposure to anisotonic conditions, cells undergo compensatory volume changes that are mediated by active transport and passive movement of ions and solutes” (48). With this basic definition of
how compensatory volume changes (CVC) are mediated, researchers have begun to take a
closer look at specific cellular processes to obtain a more accurate picture of how
compensatory volume changes are mediated.

Several excellent candidates for CVC have been investigated. One candidate, the
cellular cytoskeleton, which imparts structural stability to the cell, has provided excellent
data to support its role in CVC. Specifically, HL-60 cells, Ehrlich Ascites Tumor Cells,
and trout hepatocytes have been used to study the role of the F-actin cytoskeleton in
regulatory volume decrease (RVD) and regulatory volume increase (RVI) (31, 65, 66,
22). Overwhelmingly, the evidence has pointed to F-actin content as inversely varying
with cellular volume increases and decreases. The support for these findings has come
from using cytochalasins to disrupt F-actin (66). Before becoming too enthralled with F-
actin's role in CVC, it must be pointed out that, as of yet, there is no conclusive
mechanism whereby changes in the cytoskeleton, specifically in the F-actin content,
occurs.

While studying cellular volume changes, researchers have noticed that changes in
volume are accompanied by changes in pHᵢ. Cells must strictly regulate their pHᵢ
because large changes will result in decreased cellular activity and possible cell death.
Quite naturally, researchers began to investigate how compensatory changes in cellular
volume affected pHᵢ and how changes in pHᵢ could, in turn, affect the mechanisms
important in CVC. It was found that pHᵢ was reduced by hypoosmolar-induced swelling,
and that the reduced pHᵢ was maintained as long as the cells were in hypoosmotic
medium. This reduction in pHᵢ could be inhibited by Na⁺-free extracellular medium, but
could not be inhibited by amiloride, a sodium channel blocker (48). These results indicated that perhaps sodium, but not sodium channels directly, played a role in the regulation of pHᵢ during CVC.

1.7 Sodium-Hydrogen Exchangers

With this evidence that sodium, but not sodium channels played a role in pHᵢ during CVC, investigation turned to sodium ion exchangers, specifically sodium-hydrogen exchangers (NHEs). NHEs were a logical choice for further investigation because these not only exchange sodium, but are a major pH-regulating system (63).

Investigation into the relationship between NHEs, CVC, and the cytoskeleton has yielded much valuable data in understanding how this cellular process functions. NHEs are among the major ion transporters involved in cell volume regulation. In all known biological systems, the influx or efflux of water is tied to a cell’s ability to transport sodium. By coupling sodium transport to the transport of H⁺ ions, cell volume regulatory NHEs may lead to changes in pHᵢ, which supports the previously observed change in pHᵢ when cells undergo CVC (74).

Recent studies have shed further light on NHEs’ role in CVC by demonstrating a link between NHE1, one of the six NHE isoforms, and actin filaments. There is evidence of pH-dependent actin filament dynamics; however, NHE1 acts as an anchor for actin filaments to control the integrity of the cytoskeleton independently of its role as an ion translocator (16). Building off this discovery of a role for NHE1 in cytoskeletal function, investigation has focused on NHE1 as a platform from which intracellular signaling
occurs. Early results indicate that NHE1 serves as a scaffold from which extracellular signals coordinate divergent signaling pathways that regulate the cellular response (1).

1.7.1 NHE1 & NHE3

To date, there are six known NHE isoforms that help mediate transepithelial Na\(^+\) transport processes and housekeeping functions including regulation of organellar pH and volume (83). Of interest to researchers in the chemosensory field of gustation are the ubiquitously expressed NHE1 in the basolateral membrane of epithelial cells and NHE3 which is found in the apical membrane of many transporting epithelia. Both isoforms are present in TRCs (88).

Research outside the field of gustation has yielded important discoveries into the functions and cellular location of these two NHEs. As mentioned above, NHE1 is a major pH-regulator, anchor for actin filaments, and platform for intracellular signaling. Polarized cells have a great need to maintain and control pH\(_i\) fluctuations, and this need has enticed researchers to look at its role and location of NHE1 within these types of cells. Research into four different polarized epithelial cell lines using immunofluorescence and confocal microscopy has revealed that NHE1 is located at both apical and basolateral membranes (63). However, functional Na\(^+\) studies revealed that 70-80% of NHE1 expression was located at the basolateral membrane.

NHE3 differs greatly from NHE1. First, its function is less involved in pH-regulation; its role much more specialized in transepithelial Na\(^+\) transport. Second, localization of the NHE3 is exclusively restricted to the apical side of polarized epithelial cells (63). Third, these two exchangers have been found to be activated under different
conditions. NHE1 is activated under cell-induced shrinkage such as is seen when a cell is placed in a hyperosmotic environment, while NHE3 is inhibited by cell shrinkage. Indeed, depolymerization of the actin cytoskeleton inhibits NHE3 (74). In the renal thick ascending limb, a closer look has revealed that NHE1 regulates NHE3 and $\text{HCO}_3^-$ absorption by controlling the organization of the cytoskeleton (89). Inhibition of NHE1 on the basolateral membrane causes a decrease in apical NHE3 regulated $\text{Na}^+/\text{H}^+$ exchange that results in a decrease in $\text{HCO}_3^-$ absorption.

1.7.2 TRCs, NHE1, & NHE3

Research within the gustatory field has revealed similar properties, but also some distinctive properties for NHE1 and NHE3. Using immunofluorescence and confocal microscopy, localization of NHE3 occurs strictly at the apical membrane. This finding matched all previously studied in polarized epithelial cells. However, unlike other polarized cells, NHE1 was not found to be localized at both membranes, but rather only at the basolateral membrane in rat fungiform and circumvallate TRCs (88).

Functionally, NHE1 is still the major pH-regulator, and NHE3 is a transepithelial $\text{Na}^+$ transport. In some epithelia, such as renal thick ascending limb epithelia, the apical NHE3 is coupled with a $\text{Cl}^-/\text{HCO}_3^-$ exchange (89). This results in net apical entry of $\text{Na}^+$ and $\text{Cl}^-$. Since one $\text{H}^+$ is exchanged for one $\text{HCO}_3^-$, this process does not result in a change in pH$_i$. Both systems work together to regulate $\text{Na}^+$ flux, but only NHE1’s $\text{Na}^+$ flux across the basolateral membrane is coupled to changes in pH$_i$. This role of NHE1 involving $\text{Na}^+$ and pH$_i$ was determined using sodium-free solutions and pH and sodium-sensitive dyes, and specific blockers of NHE1. The data indicated that NHE1 is the
major pH-regulator in TRCs, but that it only account for approximately 80% of pH regulation. As of yet, additional mechanisms are responsible for the remaining 20% of pH regulation (88). The relation of NHE1 to CVC and the cytoskeleton has yet to be determined.

1.8 Objective of this Study

Acid stimulation of TRCs produces a neural response in the CT that has both phasic and tonic phases. Currently the mechanism for the phasic response is not known (55, 56). However, experimentation using carbonic anhydrase blockers to prevent the conversion of CO₂ to H₂CO₃ and subsequently free H⁺ and HCO₃⁻ decreases both the phasic and tonic components of the CT neural response. This means that the decrease in pHᵢ brought about by the dissociation of a weak acid into free H⁺ and the corresponding anion is the proximate signal for both the phasic and tonic CT response.

I propose that the initial cellular response due to a primary change in pHᵢ will be a linear change with time in volume and that volume and pHᵢ recovery will occur, in part, due to the processes mediated by NHE1 activity in the basolateral membrane. Inhibition of NHE1 activity will result in an inability of TRCs to recover from changes in pHᵢ and the pHᵢ-induced volume change. In addition, NHE1 activity has been shown to modify the actin cytoskeleton; therefore, inhibiting changes in the actin cytoskeleton will result in a physiologically significant decrease in volume change associated with a change in pHᵢ. Confirmation of the physiological significance of my findings will be determined by correlating cellular in vitro activity of TRCs with in vivo rat CT responses monitored during the same conditions.
Chapter 2: Materials & Methods

2.1 Animal Model

2.1.1 Animal Protocol

Female Sprague-Dawley rats weighing approximately 185 g were housed one per cage and were fed a standard laboratory diet (Harlan Teklad). For experimentation, each rat was individually anesthetized with isoflurane (1-chloro-2, 2, 2-trifluoroethyl difluoromethyl ether), a non-flammable, non-explosive inhalation anesthetic. After approximately 15 min of exposure to isoflurane, the rats were sacrificed by making a midline incision in the chest wall that allowed the aorta to be severed in accordance with the protocol set forth by the Institutional Animal Care and Use Committee (IACUC) of Virginia Commonwealth University. The animal tongues were quickly removed from the oral cavity and placed in nominally HC03⁻-free Normal Ringer's (NR) solution (pH 7.4, 4°C) consisting of (in mM): 140 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 Na-pyruvate, 10 glucose, and 10 HEPES (N-[2-hydroxyethyl]piperazine-N-[2ethanesulfonic acid]).

2.1.2 Retrieval of the Lingual Epithelium

Using a dissecting stereomicroscope, collagenase treatment was used to prepare the lingual epithelium for isolation. Stock collagenase treatment contains 40 mg Collagenase A (Boehringer-Mannheim, Indianapolis, ID), 20 mg Trypsin Inhibitor, and 20 ml of KCM (high-K⁺ culture medium) which consists of (in mM): 103 KCl, 0.42 CaSO₄, 0.41 MgSO₄, 8.43 Na₂HPO₄, 10 Glucose, and 25.2 HEPES. Using a 25-guage
needle, approximately 2 ml of stock collagenase was injected subepithelially by carefully
guiding the needle through the superficial tongue muscle, just below the epithelium. Care was taken to ensure that the needle did not puncture the epithelium and that there was a uniform distribution of the solution along both the ventral and dorsal surfaces of the tongue. Without uniform distribution of the solution, the epithelium could not be easily separated from the remaining tissue. After injection of the collagenase treatment, the tongues were returned to NR solution and bubbled with 100% O₂ for 60-70 min with the solution being changed after the first 15 min. Solution change was necessary to prevent collagenase leaked from the injection site from interacting with the receptors on the dorsal surface. This time period allowed for the digestion of the collagen connecting the mucosa tissue to the overlying lingual epithelium by the collagenase treatment. After digestion of the connecting tissue, the intact lingual epithelium was gently peeled away from the underlying tissue.

2.2 Polarized Preparation

2.2.1 Microscopy Chamber

Mounting a single, bipolar fungiform papilla required a special microscopy chamber (Figure 2.1). A central hole was bored in a 2 mm thick aluminum block. This central receiving hole allowed for the mounting of the tissue in a bipolar preparation. A perfusion channel was bored across each side of the hole. Its entry and exit ports were located on opposing sides of the chamber.

To support the tissue preparation, a small plastic ring, with a 0.2 mm thickness, having a central hole, with a diameter of approximately 500 μm was fitted to the central
Figure 2.1 Microscopy chamber. Depicts the specialized microscopy chamber used to mount a piece of isolated lingual epithelium.
receiving hole on the bottom side of the chamber with cyanoacrylate ester (Loctite, Rocky Hill, CT). Since the perfusion channel lay flush with the receiving hole on this side, a narrow perfusion channel, about 1 mm deep, was etched across this plastic ring using a Dremel hand tool. The etching of this perfusion channel allowed for the uninterrupted laminar flow of solution from one side of the perfusion channel across the central hole to the other side. This ring served a support ledge upon which the epithelium could be mounted from above. Finally, the backside of the chamber was sealed with a 0.16 mm thick glass cover slip (Thomas Scientific, Thomas Red Label Micro Cover Glasses) using a non-fluorescence paraffin wax.

2.2.2 Mounting the Lingual Epithelium

An approximately 4 mm height difference between the receiving hole and the perfusion channel existed on the upper side of the chamber. This side of the chamber was used to mount the epithelium. A small amount (2 ml) of NR solution was placed upon the chamber’s receiving hole and perfusion channel. The isolated lingual epithelium was placed upon the chamber in this solution to keep the cells from drying out and dying. Using a Styrofoam platform beneath a stereomicroscope, the tissue was stretched taunt using supporting pins to hold the outermost edges in place against the surface of the chamber. With the tissue held securely in place, the chamber could be gently moved beneath the tissue until a single fungiform papilla with its taste bud was located above the central hole of the supporting ring within the chamber’s receiving hole.

Final mounting of the isolated lingual epithelium required that another plastic ring, with a 0.2 mm thickness, and a central hole, with a diameter of about 500 μm be
fitted into the central receiving chamber to secure the epithelium in place. This securing plastic ring held the epithelium in place by fitting snugly against the walls of the receiving hole. It was unnecessary to etch a narrow perfusion channel in this plastic ring since it lay on a lower plane than the bottom of the chamber’s perfusion channel. With both rings in place, solution reached the epithelium by flowing through the perfusion channel and into the central hole of each ring. Once both rings were in place, the excess epithelium was trimmed away from the receiving hole. This ensured that no other tissue or debris would compromise the isolated lingual epithelium. Lastly, a glass cover slip was glued onto the upper side of the chamber with cyanoacrylate ester. This glue sets rapidly (5-10 s) and produces minimum damage to the tissue. With both sides of the chamber sealed, the volumes of the two sides were approximately 25 μl. The chamber with the mounted tissue was then transferred to a covered bucket and placed on ice.

### 2.2.3 Loading with pH-sensitive Dye

pH$_i$ of the TRCs was monitored using the pH-sensitive fluoroprobe BCECF (2, 7-bis-[2-carboethyl]-5-[and-6-]carboxyfluorescein, acetoxymethyl ester). The normal cytoplasm pH range of live cells is between 6.8 and 7.4. BCECF has a pK$_a$ of 6.98, which because it falls within the normal range, makes BCECF ideally suited for measuring pH$_i$. 50 μg of BCECF-AM dye was dissolved in 50 μl of DMSO (dimethyl sulfoxide). This solution, in turn, was mixed with 3 ml of NR solution to a concentration of 27 μM. After crossing the cell membrane, the AM form of the dye is hydrolyzed to its fluorescent form (BCECF-acid) by intracellular esterases. BCECF-acid possesses multiple negative charges making it less likely to leak out of the cells. Only live cells
with intact cell membranes are able to hydrolyze BCECF-AM and maintain the fluorescent dye.

The dye solution was injected every 15-20 min into both sides of the chamber's perfusion channels for 80-100 min. This much time was necessary to sufficiently load the TRCs so that images can be taken at exposure times of around 5-10 ms to prevent bleaching. Air bubbles were removed from overtop of the papilla to ensure that the tissue was exposed to the dye. Loading of the dye occurred on ice, and the chamber was covered due to the light sensitivity of the fluoroprobe.

2.3 Experimental Procedure

2.3.1 Overview of the Imaging System

An imaging system built around a Zeiss Axioscope 2 microscope was used to monitor the pH\textsubscript{i} and volume of polarized TRCs (Figure 2.2). The pH\textsubscript{i} was monitored through fluorescence changes of the dual-excitation ratiometric BCECF fluoroprobe while the volume was monitored through the single, isosbestic excitation wavelength. After securing the chamber to the stage of the microscope, the TRCs inside the fungiform papilla were viewed from their basolateral side through the 40x water objective lens (Zeiss; 0.8 NA). A cooled, charge-coupled device (CCD) camera (Imago, TILL Photonics, Applied Scientific Instrumentation, Eugene, OR) along with an attached image intensifier (VS4-1845 Videoscope, Washington, DC) transmitted the images of the taste bud to the computer where they were viewed and analyzed using TILLvisION v3.1 imaging software.
Figure 2.2  Zeiss Axioscope 2 microscope.
Excitation of the TRCs at the proper wavelengths (490 nm and 440 nm) for measurement with BCECF required an epifluorescent light source (Polychrome IV, TILL Photonics). In addition a 535 nm emission filter and a 515 nm dichroic beam splitter (Omega Optical, Brattleboro, Vermont) were used to ensure accurate measurements were obtained. BCECF has two main excitation wavelengths. Excitation at a wavelength of 490 nm is pH-dependent and causes an emission of light at 535 nm. Excitation at a wavelength of 440 nm is pH-independent. Excitation at this latter wavelength also happens to be the isosbestic wavelength. The imaging acquisition software records the emitted light that the dye emits as it drops down to its ground state after being excited by the excitation wavelength. A comparison of the ratio of the emitted light from these two wavelengths allows for the measurement of \( \text{pHi} \).

In order to prevent the recording of the excitation light, a dichroic beam splitter was used to separate the emitted light from the source light. The beam splitter reflects the light at a wavelength lower that 515 nm and allows higher wavelengths to pass. Along with the splitter, neutral density filters reduced the intensity of the light homogenously by combining reflection and absorption. Using these filters in conjunction with the image intensifier prevents bleaching of the dye by allowing experiments to be done in low light levels.

2.3.2 Preparatory Procedure

While preparations were made to begin the experiment, the tissue was perfused with Control Ringer’s (CR) solution (150 mM NaCl, 5 mM KCl, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 glucose, and 10 HEPES) at pH 7.4. This perfusion allowed for the complete
hydrolysis of the dye to BCECF-acid form and to remove any unhydrolyzed BCECF-AM form. Perfusion of the chamber occurred by gravity flow from syringes and was electronically controlled by a pinch valve system (Warner Instrument Corp., Hamden, CT). Pipette tips inserted into the sides of the chamber controlled the flow rate at about 1 ml/min. Elevation of the exit port syringe on the basolateral side exerted a positive pressure that kept the tissue in place during the continuous perfusion and prevented its movement throughout the experiment.

Through the TILLvisION v3.1 image acquisition software, an initial region of interest (ROI) (Figure 2.3) was taken that encompassed the entire taste bud prior to the start of the experiment and was used to monitor temporal changes in fluorescence and fluorescence intensity ratio (FIR) during the experiment.

2.3.3 Intracellular pH Measurement of Polarized TRCs

Alternate excitation of the TRCs of the taste occurred at wavelengths of 490 nm and 440 nm, the BCECF wavelengths for monitoring pH$_i$ (61). The FIR, 490 nm/440 nm, was used to monitor the relative changes in TRC pH$_i$ (Figure 2.4). Both squamous epithelial cells and TRCs within the papilla absorb the dye, but for as yet unknown reasons, the receptor cells absorb the dye better. It is likely that the mechanisms for dye extrusion, such as multidrug resistance protein (MDR) or other anion transporters are not active in TRCs (34). The camera, exposure time, polychrome and the acquisition of the images was automated by writing a protocol in the TILLvisION image analysis program. The protocol acquisition ensured all measurements were taken from the BCECF loaded receptor cells within the taste bud region. During continuous perfusion, images were
Figure 2.3 Transmission image of a taste bud. Single isolated fungiform papillae with its taste bud. The circle represents the RIO from which fluorescent recordings are taken.
Figure 2.4 Fluorescent image (490) of a taste bud. The taste bud, the inner yellow circle, is located within the papillae, the outer yellow and red circle.
acquired at 15 s intervals. Throughout the experimental procedure, the dual images of the ROI taken at the excitation wavelengths of 490 nm (Figure 2.4) and 440 nm (Figure 2.5) and were used to calculate the FIR.

The changes in TRC pH\textsubscript{i} recorded with FIR were calibrated by using an extracellular buffer that equilibrated pH\textsubscript{i} to itself. Bilateral perfusion of two high-K\textsuperscript{+} nigericin solutions having a pH of roughly 7.8 and 6.5, respectively, allowed for equilibration. These potassium rich solutions contained (in mM): 140 KCl, 4.6 NaCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 glucose, 10 HEPES, and 0.01 nigericin (39, 27). This solution is designed to imitate the low sodium/high potassium intracellular environment. Nigericin is used in the calibration of BCECF’s fluorescence because it is a K\textsuperscript{+}/H\textsuperscript{+} ionophore and equilibrates the intracellular and pH\textsubscript{o} by exchanges an internal K\textsuperscript{+} for an external H\textsuperscript{+} (84).

Only experiments that successfully calibrated and in which the dye bleaching and loss was minimal were used. Thus in my experiments there was no need to correct the F\textsubscript{440} images for time-dependent bleaching or dye loss. In my experiments following a decrease in cell volume F\textsubscript{440} increased and then recovered to almost 90-95% of the control value upon volume recovery.

After completion of the experiment, a detailed analysis of the changes in volume and pH\textsubscript{i} were made in multiples ROIs within the taste bud (Figure 2.6). Each ROI contained 2-3 TRCs and thus represents mean FIR or F\textsubscript{440} from 2 or 3 cells. In taste bud at least 6-8 ROIs were chosen. The calibration of pH\textsubscript{i} was made in each ROI and the temporal relationship between cell volume and pH\textsubscript{i} were made in each individual region.
Figure 2.5 Fluorescent image (440) of a taste bud. Images taken from the 440 fluorescence appear less intense as is seen from the lack of red in the surrounding papillae.
Figure 2.6 Transmitted image of a taste bud. Multiple ROIs were taken from the initial image and used to calculate the data gathered from the experiment.
TILLvision v3.1 software was used to analyze these regions' changes in FIR. The raw FIR data was then processed using SigmaPlot v8.0 to convert them to mean pH$_i$.

### 2.3.4 Cellular Volume Measurement of Polarized TRCs

BCECF has the desirable characteristic of having an excitation wavelength at 440 nm that is pH-independent. This pH-independence enables cell volume measurements to be observed (61). Unlike pH$_i$ measurements, volume measurements were made using only a single wavelength emission. Cell volume measurements were recorded as increasing or decreasing light intensity emission for the selected initial ROI. The basis for this measurement is if a fixed amount of dye exists, then changing the volume of solution in which that dye is dissolved will result in a new dye concentration, for a fixed amount of dye, assuming that no dye is lost as the volume is changed. Therefore, cell volume is inversely proportional to intracellular dye concentration. Since you can calibrate the pH$_i$ by using nigericin and high K$^+$ solutions of known pH values it is an indication that you are dealing with BCECF in the cytosolic compartment. Secondly, the dye loading is uniform within the cell. Most of the time when the dye is compartmentalized the loading looks spotty.

Light emission from excitation at this wavelength is already recorded in the process of measuring pH$_i$. The changes in F$_{440}$ reflect relative changes in TRC volume. However, in these experiments I did not calibrate the relationship between cell size and fluorescence intensity. Such a calibration can only be done using confocal microscopy where temporal changes in cell size can be directly related to changes in F$_{440}$. Thus the
data presented in my studies reflects only relative changes in cell volume from control as a function of F_{440}.

The experiment was begun once a steady-state cell volume was obtained from which all changes would be compared. Since volume measurements were obtained using a single wavelength and were made assuming that the dye concentration remained constant, only experiments that successfully calibrated (necessary for accurate pH_{i} measurement) and retained the dye without bleaching were used.

The same multiple ROIs within the initial ROI as were used for pH analysis were used for data analysis. TILLvislON v3.1 software was used to analyze the regions' changes in 440 nm excitation. This raw data was then processed using SigmaPlot v8.0 to convert it to a percentage change in fluorescent 440 nm (F440) relative to control.

2.4 Stimulus Solutions

A variety of stimulus solutions were made to test the cells response to changes in acid stimuli. All solutions were made using CR as the standard solution. CR contained (in mM): 150 NaCl, 5 KCl, 1 CaCl_{2}, 1 MgCl_{2}, 10 glucose, and 10 HEPES. To make a solution containing X mM of acid, an equivalent amount of NaCl was removed (Table 2.1). This ensured that the osmolarity of that solution and of all the solutions remained constant throughout the experiment. Solutions containing micromoles of other chemicals or drugs did not affect osmolarity.

To test the volume response to changing pH_{0}, solutions were mixed according to standard CR solution. Once the solution was made, the pH was adjusted to 7.0, 7.4, or 7.6. All solutions were buffered using HEPES.
To test the volume response to acid at a constant pH, solutions were mixed according to standard CR. The desired concentration of acid was added with an equivalent amount of NaCl being removed. After addition of acid, the pH was adjusted to 7.4. Again, all solutions were buffered using HEPES.

For solutions contained cytoskeleton inactivating drugs, the drug was added after the solutions were made, but before adjustment of pH.

2.5 Statistical Analysis

All raw data was processed using SigmaPlot v8.0 to convert it to its standard scientific form. For pH this involved changing a ratio emission to its usable, standard pH scale. Dye concentration emission was changed to percent change in F440 allowing for a percentage change in volume to be calculated. Individual results for each ROI were calculated as well as the mean of all ROIs in that experiment. All results are presented, unless otherwise indicated, as means ± standard error of the mean (SEM) of the number of ROIs selected in a taste bud. Comparison between the mean values for different experiments was analyzed using paired and unpaired t-tests.
<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Ringer’s (NR)</td>
<td>140 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, 10 Glucose, and 10 Na-Pyruvate</td>
</tr>
<tr>
<td>Control Ringer’s (CR)</td>
<td>150 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 Glucose</td>
</tr>
<tr>
<td>Zero-Sodium</td>
<td>CR with 150 N-Methyl D-Gluconate (NMDG) substituted for 150 NaCl</td>
</tr>
<tr>
<td>Ammonium Chloride (NH₄Cl)</td>
<td>CR with 25 NH₄Cl and 125 NaCl</td>
</tr>
<tr>
<td>Sodium Acetate (Na-Ac)</td>
<td>CR with 20 Na-Ac and 130 NaCl</td>
</tr>
<tr>
<td>Phalloidin</td>
<td>CR with 10 µM Phalloidin</td>
</tr>
<tr>
<td>Cytochalasin-B</td>
<td>CR with 10 µM Cytochalasin-B</td>
</tr>
<tr>
<td>High Potassium Calibrating</td>
<td>140 KCl, 4.6 NaCl, 2 CaCl₂, 1 MgCl₁, 10 HEPES, 10 glucose, and 0.01 Nigericin</td>
</tr>
</tbody>
</table>

Table 2.1 Stimulus Solutions & Compositions
Chapter 3: Results

3.1 Perfusion Chamber

The perfusion chamber used allows for in vitro experimentation to more accurately imitate actual in vivo polarized TRCs. Mounting of an intact fungiform papilla in the perfusion chamber allows experimental access to both the apical and basolateral membrane of TRCs. Thus, the polarity of the TRCs is preserved. Stimuli as well as agonists and antagonists of taste transduction receptor channels can be applied to the apical side exclusively, just as occurs in vivo. This method of mounting and perfusion allows for modulation of the solution perfusing the basolateral membrane also. With the accessibility of the exposed basolateral side to drugs and different ionic composition of solutions, the regulation of both pH$_i$ and volume can be studied directly.

3.2 BCECF Loading

BCECF allows for simultaneous recording of pH$_i$ and cell volume because of its dual wavelength emissions at F$_{490}$ (pH-sensitive) and F$_{440}$ (pH-insensitive) (61). Changes in pH$_i$ were measured by recording the FIR (F$_{490}$/F$_{440}$). Relative changes in volume were measured by recording at the isosbestic wavelength, F$_{440}$.

BCECF is an ideal marker for both pH$_i$ and cell volume. pH$_i$ is measured using a dual wavelength (FIR) emission that is not plagued by many of the problems associated with single wavelength dyes such as bleaching, changes in focus, dye loss, and tissue movement. The measurement of cell volume was made using the isosbestic wavelength
(440 nm) of BCECF. There are many disadvantages to using a single wavelength (F440) as compared to a dual wavelength ratio (e.g. FIR) to measure change in cell volume. These are bleaching, change in focus, dye loss, and tissue movement. Problems associated with these artifacts can be minimized by using optimum dye loading conditions that permit fluorescence images to be taken using a very short exposure time (3-5 ms) and allowing a longer time interval between images (15 s). Proper mounting of the epithelium, stretching the epithelium taunt before fixing in place with mounting ring, and applying hydrostatic pressure on the tissue by raising the height of the exit tube (3 inches above the chamber) will restrict movement of the tissue that will result in less focus change. Finally, clearing all air bubbles from the perfusion lines will ensure that neither excess bleaching, focus change, dye loss, and/or tissue movement occurs.

Taking care to ensure that these precautions are taken allows us to monitor changes on a routine basis in intracellular Na\(^+\) and Ca\(^{2+}\) activities in TRCs using single wavelength dyes, such as, sodium green and calcium green, respectively (52, 50, 56). Additionally, relative TRC volume changes using single wavelength dyes, such as calcein and sodium green (55, 56) and, now in this study, pH\(_i\) fluorescence of BCECF (Results Section). BCECF was chosen to monitor relative changes in TRC volume because it allows for the simultaneous measurement, in polarized TRCs, of the temporal changes in pH\(_i\) and relative changes in volume.

3.3 BCECF as a Live Cell Marker

For the loading of TRCs with BCECF we used the non-fluorescent membrane permeable –AM form of the dye. The –AM form enters the cell by moving passively
down its concentration gradient. Once inside the cell, intracellular esterases hydrolyze BCECF-AM and convert it to its fluorescent non-membrane permeable acid form. In this form, BCECF’s fluorescence shows a linear change between pH 6.5 and 7.8. BCECF-acid is relatively less membrane permeable because it has a large number of negative charges that help to retain it inside the cell. For successful loading to occur, BCECF-AM must be cleaved by intracellular esterases and the cells must have intact membranes.

In my studies, I was able to successfully load BCECF into polarized TRCs and then to monitor changes in pH\textsubscript{i} and cell volume for 2-4 hours. The results I obtained from polarized TRCs demonstrate both pH\textsubscript{i} and volume regulation. These results strongly indicate that despite harvesting the lingual epithelium by collagenase injections, mounting and stretching it in the perfusion chamber, and loading the polarized TRCs with BCECF on ice for 1-2 hours, cell viability and function were not compromised.

3.4 Application of Acid Stimuli

Initial studies investing the relationship between TRC pH\textsubscript{i} and cell volume were done by applying the acidic stimuli on the basolateral side. At the start, both the apical and basolateral sides of the mounted lingual epithelium were perfused with CR solution before the acid stimulus was applied to the basolateral side. Finally, the acid stimulus was also applied on the apical side to mimic acid stimulation \textit{in vivo}. Using TILLvisION image acquisition software, the pH\textsubscript{i} and relative cell volume of the TRCs were monitored from the basolateral side of the tissue.
3.5 Effect of Osmolarity on TRC pH\textsubscript{i} and Cell Volume

First, it was necessary to establish the validity of the methodology. This was achieved by exposing the basolateral membrane to a hypertonic stimulus and monitoring changes in both pH\textsubscript{i} (FIR; \(F_{440}/F_{440}\)) and relative cell volume (\(F_{440}\)) in polarized TRCs. The method used will only work if it can be demonstrated that independent monitoring of pH\textsubscript{i} and relative cell volume can occur during an osmotic stimulus.

CR solution was perfused across both the apical and basolateral sides of the lingual epithelium preparation (150 mM NaCl at pH 7.4). Table 2.1 gives a list of the chemical makeup of all solutions to which TRCs were exposed. After establishment of a steady baseline from which to measure changes in pH\textsubscript{i} and cell volume, the baseline perfusion solution was changed to a hypertonic solution containing 500 mM NaCl. Figure 3.1 shows that this solution produced a very rapid and transient increase in \(F_{440}\) (A-B; blue line) reflecting an osmotically-induced decrease in relative cell volume. Decreasing the cell volume causes an increase in dye concentration leading to an increase in fluorescent intensity. While 500 mM NaCl solution was still being perfused across the basolateral membrane, \(F_{440}\) spontaneously decreased to a new baseline (B-C). This decrease represents a spontaneous volume recovery. Returning to basolateral perfusion with CR solution led to another rapid transient decrease in \(F_{440}\) (C-D) which is the result of a rapid increase in cell volume upon removal of the osmotic stimulus. An increase in cell volume will decrease the dye concentration inside the cells leading to a decrease in the fluorescence intensity. After this rapid decrease, \(F_{440}\) spontaneously increased back to its original baseline established at the beginning of the experiment (D-E). This temporal
Figure 3.1 500 mM NaCl
increase in F440 suggests that TRCs are capable of regulatory volume recovery. The spontaneous recovery of F440 following exposure to 500 mM NaCl, indicates RVI and demonstrates RVD after return to control solution. This volume regulation is a characteristic of the behavior of many cells exposed to an osmotic stimuli (55).

The data further indicates that a spontaneous increase in pH (I-II; black line) was observed during RVI when the TRCs were exposed to 500 mM NaCl. Similarly, after changing back to 150 mM NaCl from 500 mM NaCl solution, pH spontaneously decreased (II-III) during RVD. Just as the volume changes in TRCs were consistent with observed cell behavior to osmotic stimuli in other cell types, the changes in pH were also consistent with previously observed results during volume compensation (55).

However, in some experiments I did find small changes in pH associated with cell shrinkage. Figure 3.2 represents another experiment with osmotically-induced shrinkage of TRCs. In this experiment a sudden temporal drop in pH (I-II; black line) occurred with the temporal F440 increase. The mechanism responsible for this drop in pH is as yet unknown. During the initial period (45 s), i.e. during the time when changes in pH and volume occur without any compensation, (A-B; blue line) both pH and F440 changed linearly with time ($r^2 = 0.9681 \pm 8.3910e-3; n = 5$). Despite this unexplained change in pH, RVD (B-C) and spontaneous pH recovery (II-III) were not affected and occurred as seen before in Figure 3.1. However, similar to the data shown in Figure 3.1, the largest changes in pH occurred during RVI. During spontaneous recovery, analysis of the taste bud also showed a linear change with time (210 s) of pH and F440 ($r^2 = 0.9167 \pm 0.0235; n = 5$). Upon return of the basolateral solution to 150 mM NaCl, a
Figure 3.2 500 mM NaCl
brief, sudden increase in pH\textsubscript{i} (IV-V) accompanied the decrease in F\textsubscript{440} (D-E). Analysis of the initial time period (60 s) of pH\textsubscript{i} and F\textsubscript{440} change also indicated a linear change with time ($r^2 = 0.9904 \pm 4.9534 \times 10^{-3}$; $n = 5$). This highly significant correlation of pH\textsubscript{i} and F\textsubscript{440} seen during RVI and RVD suggests that TRC pH-regulatory mechanisms are involved in both processes. In Figure 3.2, the small interruption in the record is due to the passage of a small air bubble within the perfusion chamber during image acquisition. Appearance of the air bubble in the field does not give reliable fluorescence measurements. Therefore, the data during this time period was not plotted. However, after the air bubble moved out of the field both pH\textsubscript{i} values and F\textsubscript{440} values demonstrated temporal relationship shown in Figure 3.1. Repeat of this experiment substituting 500 mM mannitol for 500 mM NaCl resulted in fundamentally similar results although the degree of RVD seen during 500 mM NaCl exposure was not as great (Figure 3.3). These data suggest that different osmotic stimuli induce changes in cell volume but may activate different membrane mechanisms during RVD and RVI.

The increase in F\textsubscript{440} and subsequent spontaneous decrease upon exposure to hypertonic solutions and the decrease in F\textsubscript{440} with spontaneous increase upon return to normal osmolarity are both consistent with the proposed RVD and RVI that would occur in any cell upon exposure and then removal of a hypertonic solution. Indeed, these results are consistent with previous studies in which Lyall \textit{et al.} used calcein and Na-green to study changes in TRC volume (55, 50). The studies with calcein are more relevant to my study. Calcein is both pH- and -Ca\textsuperscript{2+} independent. In addition, calcein fluorescence is quenched upon cell shrinkage (increase in intracellular dye concentration)
Figure 3.3 500 mM Mannitol
and unquenched upon cell volume increase. Thus upon cell shrinkage the calcein fluorescence is decreased and upon cell swelling the calcein fluorescence is increased. Since relative changes in volume with calcein, a volume marker, are similar to those reported here with F440 of BCECF, consistency of these results with previous studies indicates that measurement of F440 and FIR in BCECF loaded TRCs is a reliable way to monitor successive changes in pH$_i$ and cell volume.

3.6 Zero-Na$^+$, TRC pH$_i$ & Cell Volume

_Hypothesis:_ At constant pH$_o$, pH$_i$ regulates the change and recovery of cell volume through the sodium-hydrogen exchanger isoform 1 (NHE1) in the basolateral membrane (88). A decrease in pH$_i$ activates NHE1. Accordingly, in the absence of extracellular sodium, any change in pH$_i$ and associated change in cell volume at constant external osmolarity will not show spontaneous recovery until reintroduction of sodium to the extracellular solution.

A zero-sodium environment was obtained and maintained by substituting an equivalent concentration of N-Methyl D-Gluconate (NMDG) for sodium to maintain a constant osmolarity. NMDG is a useful sodium-substitute because much like mannitol, it is too large to diffuse through the cell membrane or be taken by channels within the membrane and it does not activate extracellular receptors. These qualities allow it to be used as an osmolarity substitute for sodium without causing any unwanted side-effects.

NHE1 has been shown to be the sodium-hydrogen exchanger (NHE) located in the basolateral membrane of TRCs (88). A zero-sodium (150 mM NMDG) isosmotic environment should effectively eliminate the role that NHE1 plays in initial pH-recovery
and cell volume recovery. NHE1 operates by an exchange of one sodium ion for one hydrogen ion (70). With the removal of sodium, NHE1 should initially exchange intracellular Na\(^+\) for extracellular H\(^+\) which will lower pHi. Recovery should not be possible since there will be no Na\(^+\) to exchange for intracellular H\(^+\).

As shown in Figure 3.4, removal of sodium brought about the expected decrease in pHi (I-II; black line). Spontaneous recovery from the decrease in pHi did not occur for the 16+ min that the sodium-free solution was being perfused in the basolateral membrane compartment (I-V). Recovery of pHi to baseline levels (V-VI) occurred following the isosmotic addition of 150 mM sodium. An increase in F\(_{440}\) (A-B) accompanied the removal of sodium and decrease in pHi. Analysis of the initial 80 s of change showed a linear change with time for both pHi and F\(_{440}\) \((r^2 = 0.9131)\). F\(_{440}\) also showed no signs of spontaneous recovery (A-E) while sodium-free solution was present. With the isosmotic addition of 150 mM NaCl, pHi decreased back to baseline levels (E-F). These results demonstrate that TRC RV1 is linked to pHi recovery during isosmotic changes. Furthermore, NHE1 is demonstrated to be the mechanism whereby TRC pHi regulation occurs and is unable of regulating pHi upon removal of extracellular sodium.

3.7 Weak Acid Stimuli

3.7.1 Ammonium-Chloride, TRC pHi & Cell Volume

*Hypothesis:* At constant pH\(_0\), an ammonium pulse will induce intracellular alkalinization and cell swelling. Likewise, a decrease in pHi will activate NHE1 to restore both pHi and volume to baseline levels. In a zero-sodium environment, an ammonium pulse will further decrease pHi and increase F\(_{440}\). However, no spontaneous
Figure 3.4 Zero-Na$^+$ Environment (NH$_4$Cl Pulse)
pH\textsubscript{i} and volume recovery to baseline levels would take place due to NHE1's inability to regulate pH\textsubscript{i} in a zero-sodium environment. An ammonium pulse is used to produce a rapid and graded change in pH\textsubscript{i} at constant external pH. In solution, NH\textsubscript{4}\textsuperscript{+} dissociates into NH\textsubscript{3} and H\textsuperscript{+} according to its equilibrium constant (pK\textsubscript{a} = 9.2). NH\textsubscript{3} rapidly diffuses across the cell membrane and binds to free intracellular H\textsuperscript{+} to form NH\textsubscript{4}\textsuperscript{+} causing a rapid alkalinization of pH\textsubscript{i}. This is followed by a slower entry of NH\textsubscript{4}\textsuperscript{+} that will dissociate intracellularly to NH\textsubscript{3} and H\textsuperscript{+} causing a slight drop in pH\textsubscript{i} with time. During the ammonium chloride (NH\textsubscript{4}Cl) pulse cells accumulate NH\textsubscript{4}\textsuperscript{+}. Upon NH\textsubscript{4}Cl washout, NH\textsubscript{3} rapidly diffuses out of the cells and the cells are left with excess H\textsuperscript{+} that causes the pH\textsubscript{i} to drop below its resting value. This decrease in pH\textsubscript{i} is transient and recovers spontaneously due to the presence of pH-regulatory mechanisms present in cell membranes (76, 53, 52).

As before, the epithelial preparation was perfused on both sides with CR solution (150 mM NaCl at pH 7.4) to establish baseline levels from which measurement of pH\textsubscript{i} and F\textsubscript{440} could be made. Addition of NH\textsubscript{4}Cl (replacing an equivalent amount of NaCl in CR; pH 7.4) resulted in a rapid alkalinization of TRC pH\textsubscript{i} (Figure 3.5, I-II). Washout of the ammonium pulse with CR did result in an acidification of pH\textsubscript{i} that fell below baseline (II-III) before fully recovering to resting baseline level (III-IV). This full recovery of acidic pH\textsubscript{i} to baseline pH\textsubscript{i} is due to the activation of basolateral NHE1 in TRCs (88, 50).

Application of the ammonium pulse (pH 7.4) caused alkalinization and a rapid decrease in F\textsubscript{440} (Figure 3.6, A-B; blue line). Analysis of the initial minute of application showed that both pH\textsubscript{i} and F\textsubscript{440} had a linear change with time ($r^2 = 0.9606 \pm 0.0189; n = 9$) indicating cell swelling induced by an increase in pH\textsubscript{i}. Removal and washout of the
Figure 3.5 NH₄Cl Pulse & pHᵢ Behavior
Figure 3.6 NH₄Cl Pulse & Volume Behavior
ammonium pulse produced a rapid transient increase in F_{440} (B-C) that overshot its baseline value just as pH\textsubscript{i} did (I-II; black line). Yet again, both pH\textsubscript{i} and F_{440} changed linearly with time (r\textsuperscript{2} = 0.9826 \pm 5.5589e-3; n = 9). As above, F_{440} spontaneously recovered toward its baseline value (C-D). These results imply that during the rapid pH\textsubscript{i} segment of an ammonium pulse, the increase in pH is associated with a decrease in TRC volume. Immediately upon washout of the ammonium, the decrease and overshoot in pH\textsubscript{i} is associated with an increase and overshoot in TRC volume before recovery of both pH\textsubscript{i} and volume.

Additionally, further experiments were done in which an ammonium pulse was combined with a zero-sodium environment. CR solution perfusion of both apical and basolateral membranes established steady baselines for both pH\textsubscript{i} and F_{440} before an ammonium pulse (pH 7.4) was given (Figure 3.7). An ammonium pulse (pH 7.4) resulted in an increase in pH\textsubscript{i} (I-II; black line) and a decrease in F_{440} (A-B; blue line). Replacement of the ammonium pulse with zero-sodium resulted in a spontaneous permanent decrease in pH\textsubscript{i} (II-III; black line) that showed no signs of spontaneous recovery as seen in the presence of sodium-normal solutions. Occurring along with this sudden change in FIR was a spontaneous permanent increase in F_{440} (B-C). Analysis of the initial minute of change showed a linear change with time of pH\textsubscript{i} and F_{440} (r\textsuperscript{2} = 0.9205 \pm 0.0216; n = 5). Neither pH nor volume showed any indication of regulatory recovery that characterized their actions in sodium-normal solutions. The spontaneous increase in F_{440} in the absence of sodium, suggests that some cells may have sodium-independent mechanisms that are involved in partial volume recovery that do not involve changes in
Figure 3.7 Effects of Na\(^+\) Removal Upon pH\(_i\) & Volume
pH$_i$. This may involve Na$^+$-K$^+$-2Cl$^-$ or KCl co-transporters. Addition of CR solution (150 mM NaCl at pH 7.4) resulted in immediate recovery of pH and volume toward baseline levels (not pictured) that, once again, showed a linear change with time ($r^2 = 0.9644 \pm 4.2719e-3; n = 7$). These results demonstrate that in the presence of external sodium, NHE1 controls pH$_i$ recovery and RVI.

Figure 3.4 also demonstrates that an ammonium pulse (pH 7.4) given during sodium-free basolateral perfusion also results in an increase in pH$_i$ (II-III; black line). Removal of the ammonium pulse results in a return to the decreased pH$_i$ (III-IV) less than established by the zero-sodium environment. F$_{440}$ (B-C & C-D) changed as expected in the presence of an ammonium pulse and return to zero-sodium ($r^2 = 0.9505$).

### 3.7.2 Sodium-Acetate, TRC pH$_b$, & Cell Volume

**Hypothesis:** At constant pH$_{o}$, a sodium acetate (Na-Ac) pulse will decrease pH$_i$, bring about an increase in F$_{440}$, and activate NHE1 to restore both pH$_i$ and volume to baseline levels.

After establishment of pH$_i$ and F$_{440}$ baseline levels, the basolateral side was perfused with a Na-Ac solution (Na-Ac replaced an equivalent amount of NaCl in CR; pH 7.4). Figure 3.8 shows that addition of Na-Ac resulted in a rapid acidification of TRC pH$_i$ (I-II). Entry of lipid-soluble un-dissociated acetic acid followed by its subsequent dissociation into CH$_3$COO$^-$ and free intracellular H$^+$ which is responsible for the rapid decrease in pH$_i$ (76, 53, 52). The transient nature of this pH$_i$ change is seen in the spontaneous recovery due to the activation of basolateral NHE1 (52, 88, 50). With Na-Ac washout, pH$_i$ immediately became more alkaline and overshot its resting value (III-
Figure 3.8 Na-Ac Pulse & pH$_i$ Behavior
IV) resulting from the rapid diffusion of un-dissociated acetic across the cellular membrane and a decrease in pH\textsubscript{i}. Recovery of the pH\textsubscript{i} (IV-V) to its baseline level in the absence of any further stimulus is the result of as yet an uncharacterized pH-recovery mechanism.

There was a rapid increase in F\textsubscript{440} following the perfusion of Na-Ac (Figure 3.9, A-B; blue line). F\textsubscript{440} began a slow spontaneous recovery towards baseline before Na-Ac washout occurred (B-C). Immediately upon washout, a rapid, but transient decrease in F\textsubscript{440} occurred (C-D) followed by a slow spontaneous recovery towards baseline (D-E). Clearly, these changes in F\textsubscript{440} can be seen to parallel the acid-induced changes in pH\textsubscript{i} (black line). These results demonstrate that acidification of TRC pH\textsubscript{i} is associated with a decrease in cell volume, and that alkalinization, due to washout of Na-Ac, is associated with an increase in cell volume.

3.8 \textit{pH\textsubscript{o}, TRC pH\textsubscript{i}, & Cell Volume}

\textit{Hypothesis:} pH\textsubscript{o} changes on the basolateral and/or the apical membranes result in a change in pH\textsubscript{i} which is the proximate signal for a change in cell volume. Spontaneous recovery of neither pH\textsubscript{i} nor cell volume will be seen at acidic basolateral pH\textsubscript{o} because external acidification inhibits NHE1 activity responsible for pH\textsubscript{i} recovery (88), and cell volume has already been shown to not recover when pH\textsubscript{i} recovery does not occur. Spontaneous recovery of pH\textsubscript{i} and cell volume may occur when acid stimulation comes from the apical membrane because NHE1 is located on the basolateral side (88) and not directly affected by the acid stimulus.
Figure 3.9 Na-Ac Pulse & Volume Behavior
3.8.1 Effect of Basolateral pH<sub>o</sub> on TRC pH<sub>i</sub> & Cell Volume

A summary of the data presented in Figure 3.10 shows that lowering the pH<sub>o</sub> from 7.4 to 7.0 produced a sustained acidification in TRC pH<sub>i</sub> (I-II; black line). Return of basolateral pH<sub>o</sub> to 7.4 completely reversed TRC acidification with no resulting overshoot (III-IV). An increase in F<sub>440</sub> accompanied acidification of TRC pH<sub>i</sub> as a result of a decrease in pH<sub>i</sub> (A-B; blue line). Reversal of the increase in F<sub>440</sub> accompanied the return of pH<sub>i</sub> toward baseline (C-D). Numerical analysis of the initial 60 s period showed pH<sub>i</sub> and F<sub>440</sub> changed linearly with time \( r^2 = 0.9729 \pm 0.0112; n = 8 \) and \( r^2 = 0.9496 \pm 0.0120; n = 8 \), respectively. Figure 3.11 shows alkalinization of TRCs (I-II; black line), due to alkaline pH<sub>o</sub>, above resting baseline is also accompanied by a decrease in F<sub>440</sub> (A-B; blue line) \( r^2 = 0.9841 \).

Furthermore, this linear relationship seen between pH<sub>i</sub> and cell volume does not just occur over a small pH<sub>i</sub> change. A linear relationship that changes with time is seen over a decrease in pH<sub>i</sub> from approximately 7.6 to 7.0 (III-IV & C-D; \( r^2 = 0.9940 \)). This relationship is highly significant both statistically as well as physiologically (Figure 3.12). TRCs are exposed to a wide variety of acidic and osmotic stimuli through normal eating and drinking. They must be able to adapt to, respond to, and recover from these stimuli without dying. These data show that TRCs are equipped to regulate their pH<sub>i</sub> and volume over a wide pH<sub>i</sub> range.

3.8.2 Apical pH<sub>o</sub> Change

Under physiological conditions acid stimuli come in contact with only the apical membrane of TRCs. The pH<sub>i</sub> associated changes in F<sub>440</sub> must be reproducible from the
Figure 3.10 pH\textsubscript{i} & Volume Behavior Upon Lowering pH\textsubscript{o} from 7.4 to 7.0
Figure 3.11 $pH_i$ & Volume Behavior Between $pH_o$ 7.6 & 6.7
Figure 3.12 Linear Relationship of pH$_i$ & Volume as pH$_o$ Varies Between 7.6 & 6.7
apical side for these results to have any physiological importance. Figure 3.13 demonstrates the same TRC pH_i and F_{440} responses seen above. Lowering the pH_o to 3.0 produces a decrease in pH_i (I-II) which has an associated increase in F_{440} (A-B). Reversing the basolateral pH_o to 7.4 resulted in an increase in pH_i (III-IV) along with a decrease in F_{440} (C-D). These results indicate that a decrease in pH_i is seen to be the proximate signal for a change in cell volume even when the acidic stimuli is applied on the apical side.

3.9 Cytoskeleton Inhibitors, TRC pH_i & Cell Volume

_Hypothesis_: pH_i is the proximate signal for cell shrinkage. Changes in pH_i regulate cell volume through polymerization and depolymerization of the actin cytoskeleton. Introduction of the cytoskeleton modifying drugs, phalloidin and cytochalasin-B, into TRCs will cause partial and/or total decoupling of changes in cell volume associated with a change in pH_i.

Several studies have suggested that the actin cytoskeleton plays an important role in cell volume regulation (58, 59, 7). In almost all cell types studies, swelling and shrinkage are associated with an increase and decrease, respectively, in F-actin content (32, 31, 5, 12, 42, 60). These findings have been based upon osmotically induced cell swelling and shrinkage. Recently, confocal microscopy studies using rhodamin-phalloidin to mark both the filamentous (F)-actin and monomeric (G)-actin inside the cell indicate that changes in TRC pH_i produce changes in the relative distribution of F- and G-actin in TRCs (Unpublished observations; Lyall et al., 2005).
Figure 3.13 Apical pH₀ Change & pHᵢ/Volume Behavior
Cytochalasins are a class of drugs that have been demonstrated to depolymerize F-actin and inhibit the RVD process in many cell types, such as, gallbladder epithelial cells, Ehrlich ascites tumor cells, and rabbit proximal tubules (25, 9, 10, 47, 21, 30, 77). Cytochalasin-B serves as an actin inhibitor by capping and cleaving F-actin. This capping action does not permit actin polymerization (86) and destabilizes the cytoskeleton.

Phalloidin is a well-characterized stabilizer of F-actin that shifts the equilibrium from G-actin to F-actin by inhibiting both the release of phosphate as a product of ATPase and inhibiting depolymerization of F-actin (14, 15, 23). Both these drugs are easily introduced into the cell by passive diffusion down their concentration gradient.

3.9.1 Phalloidin

Baseline pH_{i} and F_{440} levels were established in CR at pH 7.4. Before testing the effects of phalloidin on TRC response to acid stimuli, a control test was performed from which all changes were gauged. Control pH_{i} and F_{440} measurements (Figure 3.14) were virtually identical to those results presented in section 3.5.3. Perfusion of the basolateral membrane with CR at pH 7.0 resulted in a sustained acidification in TRC pH_{i} (I-II; black line) that showed full recovery upon return to pH 7.4 (II-III). An F_{440} increase (A-B; blue line) and decrease (B-C) accompanied the TRC pH_{i} changes. Again, both pH_{i} and F_{440} changed linearly with time (r^2 = 0.9587 ± 9.1749e-3; n = 6 and r^2 = 0.9548 ± 0.0105; n = 6, respectively).

In this experiment phalloidin was perfused on both the apical and basolateral side for 15 min. Lowering the pH_{o} to 7.0 (i-ii; dark yellow line) and raising pH_{o} back to 7.4
Figure 3.14 pHₐ & Volume Behavior in the Presence of 10 μM Phalloidin
(ii-iii) produced virtually identical acidification of TRC $pH_i$ when compared to the control stimulus. As in the control, an $F_{440}$ increase (a-b; dark blue line) accompanied TRC $pH_i$ acidification, and an $F_{440}$ decrease (b-c) accompanied TRC $pH_i$ alkalinization. Phalloidin did not interfere with the linear change with time of $pH_i$ and $F_{440}$ ($r^2 = 0.9728 \pm 0.0124; n = 6$ and $r^2 = 0.9773 \pm 5.6527e-3; n = 6$, respectively). However, a change did occur between the percentage response in cell volume between control and drug. The accompanying $F_{440}$ response to acidifying TRC $pH_i$ was 4.3 % smaller than the control response. This $F_{440}$ response equals a 49.26% difference in relative volume change between control and drug. The significance of this difference between control and drug was established using an unpaired t-test ($t = 19.02; n = 9$). Return of $pH_o$ to 7.4 and the resulting TRC $pH_i$ alkalinization brought about a normal return of $F_{440}$ to its baseline level. These results clearly indicate that the accompanying increase in $F_{440}$ to an acid stimulus is decreased in the presence of phalloidin. $F_{440}$ recovery upon return to baseline pH is not hindered due to drug interaction with the cytoskeleton.

### 3.9.2 Cytochalasin-B

Perfusion of both the apical and basolateral membranes with CR at 7.4 allowed establishment of baseline levels of $pH_i$ and $F_{440}$. As with the phalloidin experiments, control data was collected from which all changes were gauged. Control $pH_i$ and $F_{440}$ exhibited normal responses to the acid stimulus (Figure 3.15; black & blue lines). Drug control $pH_i$ and $F_{440}$ exhibited normal responses to the acid stimulus also (dark yellow & dark blue lines).
Figure 3.15 pH$_i$ & Volume Behavior in the Presence of 100 μM Cytochalasin-B
In this experiment Cytochalasin-B was perfused on both the apical and basolateral side for 30 min. Cytochalasin-B produced almost identical results to phalloidin. Acidification of TRC pH to a change in pH resulted in an accompanying increase in F440 that was 3.4 % smaller than the control increase. This percent change in F440 response equaled a 56.0 % relative volume difference between control and drug. TRC pH alkalization resulted in an accompanying decrease in F440 to the baseline level. The results from an unpaired t-test showed a large significant difference between the control and drug responses (t = 42.03; n = 9). Results from testing with cytochalasin-B indicate that inhibition of the cytoskeleton will cause a much smaller response in F440 to a TRC pH change. This drug does not hinder return of cell volume to baseline levels once the acid stimulus is removed.

3.10 Summary of Results

1. Osmotic shrinkage of TRCs demonstrates characteristic RVD and RVI that is linked to a spontaneous decrease and increase in pH, respectively.

2. In an isosmotic zero-sodium environment, spontaneous sustained decrease in pH occurs. This sustained decrease has an associated sustained increase in F440 that changes linearly with time. Reintroducing sodium causes spontaneous return of pH and F440 to baseline levels, strongly suggesting that NHE1 is regulating pH and the associated volume change.

3. Isosmotic weak acid stimuli lead to pH changes with corresponding linear F440 changes. Recovery of both pH and F440 requires the presence of sodium, which again suggests that NHE1 regulates recovery.
4. pHo stimuli from the basolateral membrane cause a sustained decrease in pHi that is reversible upon removal of extracellular acid stimulus. Spontaneous pHi and volume recovery are not seen because the acid stimulus inhibits NHE1.

5. Apical pHo stimulus demonstrates that the mechanism whereby pHi and cell volume are regulated is a physiologically viable mechanism and is not just able to be induced from basolateral membrane.

6. The cytoskeleton is responsible, at least in part, for pHi-induced cell volume shrinkage. Fixing F-actin and G-actin cytoskeleton content does not allow complete shrinkage of the cytoskeleton in response to an extracellular acid stimulus.

7. Overall, initial response of TRCs to acid stimuli produces a linear change with time in both pHi and F440 whose recovery is mediated by NHE1. pHi induces changes in cell volume by altering the actin cytoskeleton. Inhibition of the actin cytoskeleton causes a smaller volume change, but does not affect the linear change with time in both pHi and F440.
Chapter 4: Discussion

An acid-induced decrease in TRC pH$_i$ is the proximate signal for acid taste transduction, however, there is much contention as to what downstream events caused by a decrease in pH$_i$ are responsible for the phasic and tonic parts of the CT neural response to acidic stimuli. An increase in intracellular Ca$^{2+}$ causing activation of NHE1, which plays an essential role in maintaining and regulating the tonic phase of the CT response to acidic stimuli (50). My results demonstrated that a linear relationship exists between the acid-induced pH$_i$ change and the associated cell volume change. pH$_i$ regulation, RVI, and RVD show spontaneous recovery that is mediated by NHE1. Furthermore, since the magnitude of the pH$_i$-induced decrease volume is attenuated in the presence of actin-blockers, it suggests that the pH$_i$ associated volume change is regulated by the actin cytoskeleton. Consistent with my in vitro studies, CT recordings done in Dr. DeSimone’s lab demonstrate that an acid-induced change in TRC pH$_i$ serves to regulate the phasic part of the CT response through an associate TRC volume change. Consequently, my studies on the pH$_i$-induced changes in cell volume of TRCs provide a physiological mechanism that is responsible for the phasic component of the CT to acidic stimuli.

4.1 Relationship between TRC pH$_i$ & Cell Volume

One interesting phenomenon was seen to occur consistently during washout of weak acids. Rapid exit of NH$_3$ resulted in a rapid, transient acidification of TRCs that always overshot the pH$_i$ baseline. Recovery from this overshoot was dependent upon
NHE1 activity. The surprising result was the strict linear fashion in which cell volume tracked pH$_i$ movement (Figure 4.1). Statistical analysis of this double overshoot revealed important significance between pH$_i$ and volume ($r^2 = 0.9957 \pm 8.6935\text{e}-4$; $n = 11$). This phenomenon was not limited to ammonium washout. It also occurred during washout of Na-Ac (Data can be seen in Figure 3.6b). This consistent overshoot illustrates the transient nature of these pH$_i$ and volume changes induced by weak acid stimuli. Recovery of both pH$_i$ and volume was largely dependent upon the activation of NHE1 (88) unless sodium-free solution was used to inhibit NHE1 (Figure 3.5).

Another important observation is the adaptability which TRCs displayed to the broad range of acidic stimuli. TRCs showed a consistent ability to regulate and return to their baseline levels of pH$_i$ and volume despite being repeatedly stimulated (Figure 4.2). This ability to quickly adapt to different stimuli and return to maintainable homeostatic conditions is a necessary function for cells that have constant exposure to multiple rapid stimuli (e.g. eating and drinking).

4.2 Correlation of TRC pH$_i$ Measurements with CT Neural Response

The CT branch of the facial nerve, CN VII, innervates the anterior two-thirds of the tongue in which the fungiform papillae are located. Upon application of an acid stimulus to the lingual surface of the tongue, an increase in neural activity of the CT is seen. There is a direct response between the activity within TRCs due to an acid stimulus and the nerve response that activity elicits (18, 79, 64).
Figure 4.1 Overlay of pH\textsubscript{i} & Volume Overshoot & Recovery
Figure 4.2 pH<sub>i</sub> Regulation at Varying pH<sub>e</sub> & Weak Acid Stimuli
4.2.1 Osmolarity & CT Nerve Responses

*In vivo* experiments gave CT recordings that showed an almost identical response to observed changes in TRCs when they were exposed to hypertonic solutions. Superfusion of the tongue with 1 M mannitol resulted in a brief, phasic CT response that spontaneously decreased to rinse level (55). The cause of this brief, phasic CT response is seen when TRCs shrink following exposure to hypertonic solution (Figure 3.1, A-B; blue line).

Stimulation with both strong and weak acids after preshrinking with 1 M mannitol produced on average a 50% decrease in the phasic part of the nerve response with little or no decrease in the tonic phase (57). The conclusion drawn from these results parallels the *in vitro* studies findings that volume changes can modulate pH. Indeed, CT recordings found that the phasic part of the response to acid stimulation is modulated by increasing osmolarity (57).

4.2.2 Actin Cytoskeleton & CT Nerve Responses

Modulating the volume of TRCs using hyperosmotic solutions does result in a decreased CT response. It has already been hypothesized and seen that the actin cytoskeleton plays a role in the associated TRC volume response to acid-stimuli. In CT experiments, cytochalasin-B was used to disrupt actin binding. Paralleling *in vitro* experimentation, treatment of the rat tongue with Cytochalasin-B produced an 80% decrease in CT phasic response to acid stimuli. Increasing the osmolarity of acid stimuli resulted in complete elimination of the phasic CT response.
Treatment of the tongue with phalloidin to stabilize F-actin concentration inside TRCs resulted in a response that was identical in nature to the response seen when the tongue was treated with cytochalasin-B. CT response after phalloidin treatment resulted in total inhibition of the phasic part of the CT response to acid stimuli. Treatment with cytochalasin-B followed by treatment with phalloidin demonstrated that the attenuated nerve response was completely reversible. These results support the conclusion that TRC volume change upon exposure to acid stimuli is responsible for the regulation of the phasic part of the CT response to acid stimuli.

4.3 Acid Sensing Cells

Initiation of a response to an acid stimulus is not a property possessed solely by TRCs. Other pH-sensitive chemosensory cells exist. Carotid and aortic bodies function as peripheral chemoreceptors. The ventrolateral medulla and nucleus of the solitary tract function as central chemoreceptors. Both these peripheral and central chemoreceptors primarily regulate respiratory activity in order to maintain arterial blood pO₂, pCO₂, and pH within their narrow range (19, 54).

The carotid bodies are a small cluster of chemoreceptive cells located at the bifurcation of the external and internal carotid arteries. They detect blood flow within the body. Decreasing blood flow in the carotid bodies is responsible for cellular hypoxia, hypercapnia, and decreased pH. These changes in cell function lead to an increase in receptor firing by the type-I cells in the carotid body (56, 6). Detection of an increase in pCO₂ the normal value of 40 mm Hg, or a decrease in pH, below 7.4 triggers a neural response caused by membrane depolarization (6).
4.4 Acid Stimuli and Chemosensitive Cell Response

This study has focused on the role of pH$_i$-associated volume as a possible mechanism for the phasic part of the CT nerve response to acid stimuli. Strong evidence has been presented that supports temporal cell volume induced by a changing pH$_i$ as the mechanism modulating phasic nerve response.

In their 2004 review, Putnam et al. (68) discuss the role of possible signals that could influence chemosensitive cells that modulate the neural response involved in the regulation of basic body functions such as ventilation. To date, scientists have been looking for one unifying theory that would explain how chemosensitive cells are able to signal the brain via their afferent neurons about changes in body function. Such a unifying theory that adequately explains physiological response to a variety of conditions does not exist at this time. Several candidates are considered highly likely to be the proximate signal that induces physiological response to homeostatic changes in functions that are regulated by acid-base chemistry. The three most popular are carbon dioxide (CO$_2$), pH (both intracellular and extracellular), and bicarbonate (HCO$_3^-$) (68).

Elevated levels of CO$_2$ in the blood cause an increase in respiration in an attempt to return blood pH levels to normal. Herein is the main argument upon which CO$_2$ as a signal for central chemosensitivity is based. Without question when blood CO$_2$ levels are increased and decreased there are changes in respiratory rate. However, simple correlation between two observed phenomena is not evidence that one directly causes the other. For instance, many studies could not plot ventilation as a unique function of pH$_o$, and thus assumed that another factor, i.e. CO$_2$, must be an independent signal (68). The
question that still must be answered is whether a change in CO₂ concentration, itself, is the signal by which a neural response is elicited from chemosensitive cells.

Two main problems present difficulties to CO₂ being the signal to which chemosensitive cells respond. First, all earlier theories considered only the inadequacy of pH₀ to explain respiratory response to various chemosensitive stimuli when proposing CO₂ to be a unique and independent signaling factor (68). Despite cellular pH being markedly affected by CO₂, no measurements of pH₀ in central chemosensitive neurons existed (67, 69). When considering the role of CO₂, the effect of other chemosensitive stimuli upon pHᵢ and thus the possibility that changes in CO₂ serve as an adequate stimulus, were rarely considered (68). Second, CO₂ signaling is tied very closely to its effects on pH₀ and pHᵢ. In fact, no one has proposed an appropriate cellular model whereby CO₂ could be an independent chemosensitive signal separate from its effects on pHᵢ and pH₀.

Despite these difficulties, CO₂ as an independent signal cannot be ruled out completely because a CO₂ receptor may exist. Evidence does exist for such a receptor that accounts for renal proximal tubule cells response to hypercapnia (92). Receptors for other gases, such as nitric oxide (62), carbon monoxide (91), and oxygen (87), have been reported. Recently, independent of its effects on increasing levels of pHᵢ, increased levels of molecular CO₂ have been reported to activate calcium channels in glomus cells and elevate cAMP (80, 81, 82). However, at this time, evidence for chemosensitive response from glomus cells due to activation of calcium channels by CO₂ does not exist. Perhaps
in the near future, researchers will be able to determine whether CO₂ can act as a signal for independent activation of chemosensitive neural response (68).

Extensive investigation into a potential role for HCO₃⁻ as a chemosensitive signal is lacking. Close interrelationship between HCO₃⁻ and pH complicate studying a role for HCO₃⁻ as an independent chemosensitive signal. Speculation has occurred for a possible role for HCO₃⁻ in glutamate uptake in astrocytes, but initial findings have indicated H⁺ instead of HCO₃⁻ as accompanying glutamate uptake. GABAₐ channels have been shown to be permeable to HCO₃⁻ (8, 36, 37). However, efflux of HCO₃⁻ as a result of GABAₐ activation results in an intracellular acidification. HCO₃⁻ has the ability to modulate a variety of cellular responses, free radicals, cAMP, and calcium. Due to its ability to activate cellular mechanisms, further investigation into its role in chemosensitive signaling should occur (68).

Almost 100 years ago scientists were proposing H⁺ ions as the signal for chemosensitive cells in the control of ventilation (90). Today, scientists are finding that pHᵢ is an important stimulus for many chemosensitive cells (51, 19). pHᵢ-induced increases and decreases in neuronal firing rate have been found in many regions of the brain through data gathered from BCECF fluorescence. Filosa et al. (24) studied CO₂-sensitive neurons and developed a new technique for simultaneously measuring Vₑ and pHᵢ. They were able to establish that a good correlation existed between changing pHᵢ and neuronal firing rate. They were also able to show that neuronal firing rate corresponded poorly to CO₂, changing HCO₃⁻, and the pHᵢ-pHₒ gradient. The evidence
suggests that a change in pH_i may be an adequate stimulus in chemosensitive neurons (68).

However, despite these recent findings that support pH_i being a major stimulus pathway, some problems do exist. First, a fall in pH_i in response to a weak acid is not unique to chemosensitive neurons. Multiple types of neurons in differing regions of the brain all showed a fall in pH_i when exposed to a weak acid stimulus. Not all these neurons were chemosensitive. Second, the firing rate of LC (locus coeruleus) neurons is not always a fixed function of pH_i. The phasic response to a change in pH_i corresponded well, but the tonic response to a change in pH_i did not (68). Third, not all stimuli used to produce pH_i acidification induced a change in neuronal firing rate (24).

Clearly changing pH_i is not the only mechanism involved in neuronal chemosensitivity. Changes in pH_i can modulate different cascade mechanisms within cells that, in turn, may affect neuronal response. In his review, Putnam et al. (68) clearly indicated that the best results for pH_i being a major stimulus pathway was the phasic part of the neural response. Based upon the in vitro and corresponding in vivo results presented earlier, the mechanism proposed by this study for modulation of phasic CT neural response fits in well with the current literature. This proposed mechanism adequately explains how the phasic part of the neuronal response can be modulated by partial blockage of normal physiological response when pH_i fluctuates.

4.5 Further Investigation

Further investigation is necessary in several areas. First, this study only approached the change in pH_i under normal or sodium-free conditions. Richter et al. (73)
have observed that in mice an increase in intracellular Ca\textsuperscript{2+} attenuates the pH\textsubscript{i} change. Experimenting with the effects of increasing extracellular Ca\textsuperscript{2+} on pH\textsubscript{i} and the associated volume change could further reveal how the CT phasic response is modulated.

Second, in vitro complete volume response was not inhibited in TRCs exposed to cytochalasin-B and phalloidin, but complete inhibition was seen during in vivo CT recordings. Is there a minimum pH\textsubscript{i}-associated volume response that must occur in the TRCs to elicit a phasic response? If so, what is that relative minimum volume change?

Third, data from these experiments in TRCs reveals high significance between pH\textsubscript{i} and initial cell volume changes. Different mechanisms for acid entry have been proposed for many animals (19). Will the same significant results be found in different animals? If these findings are true on a cellular level, will CT recordings confirm their physiological importance?

Fourth, does the proximate signal for bitter, salty, or sweet taste elicit this associated volume response? If so, is this mechanism (i.e. modulated in the same way) related to the one that operates for the sour phasic response discussed in this thesis?

Fifth, testing of these results in other chemosensitive neurons and their receptor cells is warranted to see if (1) initial linear change of both pH\textsubscript{i} and cell volume to acid stimuli occurs, (2) blocking the cytoskeleton modulates the volume response, and (3) blocking the cell volume response causes a modulation in the neural response.

4.6 Limitations of the Experimental Approach

The benefit of the experimental approach used in this study is that it provides a means of measuring both pH\textsubscript{i} and volume simultaneously allowing for changes in one
measurement to be viewed with respect to the other (61). Preservation of the polarity of the taste bud during *in vitro* experimentation allows for testing conditions to more accurately reflect physiological *in vivo* conditions. Consequently, application of a stimulant to the apical or basolateral membrane should result in TRC behavior similar to *in vivo* response.

The imaging technique used provides some advantages also. Specific regions of the taste bud can be isolated from a data recording standpoint without being physically separated from the surrounding tissue allowing for a better understanding of how the region responds in its natural environment. Also, different regions within the taste bud can be compared and contrasted to give a clearer picture of which regions within the taste bud show the greatest response to a stimulus.

Unfortunately, there are some drawbacks to the experimental procedure. Imaging of the taste bud only occurs within a single image plane. In reality, the taste bud exists in three dimensions and is shaped like a bulb. Since only a single image plan is analyzed, it is not possible to monitor the entire taste bud. Changes that occur in cells not within the image plane and not observed; therefore, the entire picture of how the bud responds to the stimulus is not obtained.

BCECF has limitations too that are in some ways enhanced by viewing the taste bud in a single image plane. When experimenting in conditions known or designed to cause cell shrinkage/swelling, it is difficult to not have some fluctuation in the single wavelength $F_{440}$ emission due to focus change. Focus changes during key changes in solution stimuli can lead to emission changes that do not accurately reflect cell behavior.
The only way to avoid this limitation is to stretch the epithelium tight to minimize its ability to move in and out of focus.

Using fluorescent microscopy to measure the single wavelength emission, $F_{440}$, does not allow for absolute changes in cell volume to be determined. For this or any other study using BCECF to measure volume, determining absolute volume changes based upon $F_{440}$ emission would require using a confocal microscope to take precise pictures of a cell at specific emission levels and then correlating those emission levels with changes in cell volume. Despite not being able to give an absolute volume change, accurate relative volume changes are easily obtained by establishing an accurate baseline emission at the beginning of the experiment. An accurate baseline reflects the 100% volume value of the cell. Changes in $F_{440}$ emission accurately correspond to relative changes in cell volume.

Despite these limitations to the experimental approach, the data from this study have been able to help establish a definitive cellular mechanism whereby the phasic part of the neural response to acid stimuli is modulated. With this new understanding of how TRCs work, perhaps this information can be used to understand other chemosensitive systems.
List of References
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Vita

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