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The Regulation of Epithelial Sodium Channels in Mammalian Taste Receptor Cells

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THE REGULATION OF EPITHELIAL SODIUM CHANNELS IN MAMMALIAN TASTE RECEPTOR CELLS

by

Arian F. Baquero

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

DOCTOR OF PHILOSOPHY

in

Biology

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UTAH STATE UNIVERSITY
Logan, Utah

2009
ABSTRACT

The Regulation of Epithelial Sodium Channels in Mammalian Taste Receptor Cells

by

Arian F. Baquero, Doctor of Philosophy

Utah State University, 2009

Nutrient recognition is one of the main physiological roles of the gustatory system. In mammals, it is well established that the taste of sodium salts is primarily mediated by sodium influx through the epithelial sodium channel. The epithelial sodium channel is a sodium-specific ion channel that is expressed across a wide range of transporting epithelia such as colon, kidney, and taste. In addition to its role as a salt taste receptor, sodium influx through the epithelial sodium channel is important systemically for maintaining sodium balance and blood pressure. Following our earlier work on the endocrine regulation of salt taste at the level of the epithelial sodium channel, we hypothesize that the epithelial sodium channel expressed in mouse taste receptor cells plays a central role in the restoration of salt and water balance. Using a multidisciplinary approach that includes patch clamp recording, functional sodium imaging,
molecular biology, Western blotting, and behavioral assays, we have begun to investigate different mechanisms of the epithelial sodium channel regulation in the taste system. In the present study, we have demonstrated a number of mechanisms that regulate the epithelial sodium channel by both ions and/or hormones in mouse taste cells. In general, three new mechanisms of the epithelial sodium channel regulation were identified: (1) regulation of the epithelial sodium channel by chloride ions, (2) regulation of the epithelial sodium channel by insulin, and (3) alterations of the epithelial sodium channel function in diabetic taste cells. To test the relevance of one or more of these regulatory mechanisms in the animals’ behavior, we used a variety of short-term behavioral assays. Interestingly, the results suggested that insulin regulates salt intake in rodents, which dovetails nicely with our functional and molecular findings.

Consistent with insulin’s physiological role in salt taste transduction, we investigated the modification of the epithelial sodium channel function during the onset of diabetes. Diabetic rodents displayed alterations in salt taste transduction via epithelial sodium channel from the gene level to the animals’ behavior. These results are an example of how regulatory cues, like hormones, act on specific transduction elements to modulate the peripheral gustatory system.
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Arian F. Baquero Gonzalez
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LIST OF ABREVIATIONS

TCRs: Taste receptor cells

ENaC: Epithelial sodium channel

PKD2L: Polycystic kidney disease 2-like

GPCRs : G protein-couple receptors

[Cl]i : Intracellular chloride concentration

NKCC: Na+/K+/2Cl⁻ cotransporter

CFTR: Cystic fibrosis transmembrane conductance regulator

AQP: Aquaporin channels

IR: Insulin receptor

IRS1: Insulin receptor substrate 1

IRS2: Insulin receptor substrate 2

PI3-K: Phosphatidylinositol 3-kinase

SGK: Serum- and glucocorticoid-regulated kinase

PDK1: Phosphatidylinositol-dependent protein kinase

NEDD4-2: Neural precursor cell expressed, developmentally down-regulated 4-2

PtdIns(4)P: Phosphatidylinositol 4-phosphate

PtdIns(3,4)P₂: Phosphatidylinositol 3,4-bisphosphate

PtdIns(4,5)P₂: Phosphatidylinositol 4,5-bisphosphate

PtdIns(3,4,5)P₃: Phosphatidylinositol 3,4,5-trisphosphate

PKB/Akt: Protein kinase B

STZ: Streptozotozin
Peripheral Gustatory System & Chemosensory Transduction

The taste system plays two main roles. One is to recognize nutritionally essential compounds (e.g. carbohydrates, salts, and amino acids) and the other is to avoid ingestion of harmful chemical compounds (e.g. alkaloids and acids). The ability to identify chemical stimuli in the peripheral gustatory system is carried out by taste receptors cells (TRCs), which are clustered in taste buds containing approximately 50-100 cells. Taste buds are located primarily in the tongue in three structures: fungiform, foliate, and circumvallate papillae but are also found in extralingual locations such as soft palate, epiglottis, nasoincisor cut, pharynx and larynx (Herness & Gilbertson, 1999).

Taste receptor cells synapse onto different nerve fibers depending on their location within the oral cavity. The primary nerve fibers entering the buds have origins in facial, glosopharyngeal, or vagus nerves (cranial nerves VII, IX, and X, respectively). Specifically, branches of the facial, chorda tympani and greater superficial petrosal nerves innervate the anterior tongue (e.g. fungiform and anterior foliate papillae) and palate. The glosopharyngeal nerve innervates the posterior tongue (e.g. posterior foliate and circumvallate papillae), and the superior laryngeal nerve innervates the epiglottal taste buds. Additionally, nerve fibers of trigeminal origin (cranial nerve V) surround taste buds and contribute to thermal and tactile (i.e. textural) information. Individual taste cells synapse on
fibers of VIIth, IXth, and Xth cranial nerves that connect directly to neurons in the nucleus of the solitary tract (NST). From here the gustatory information is carried to different nuclei and cortical areas of the brain (Boughter & Gilbertson, 1999; Herness & Gilbertson, 1999).

The identification of chemical compounds by taste receptor cells involves a variety of steps that culminate in the release of transmitter onto the afferent nerve fiber. Each taste bud contains a variety of taste cells which communicate to each other as well as the gustatory nerve fiber. TRCs are divided into Type I, II, III and IV by morphological characteristics and, in the recent years, a number of studies have extended this characterization at the functional level. Taste responses are initiated by the interaction between tastants and receptors or ion channels at the apical membrane of taste receptor cells. It is suggested that Type II appears to be responsible for most taste signaling. And Type III, on the other hand, are considered synaptic output cells (Roper, 2006). Although molecular studies have supported the difference on different cell types population in taste cells (DeFazio et al., 2006), I believe, this topic is still quite controversial because preliminary functional studies in our laboratory contradict these earlier findings on taste cell classification. Consistent with this idea, new models have emerged in the field of taste to explain how gustatory information from signal transduction is processed and carried out into nerve fibers. Several studies have primarily focused in neurotransmitter release and cell communication in the gustatory system. The identification of P2X$_2$ and P2X$_3$ receptors in taste cells
suggest that adenosine triphosphate (ATP) may serve as a neurotransmitter. Studies with P2X$_2$ and P2X$_3$ knockout mice support the role of ATP since these transgenic mice have a significant reduction on gustatory nerve and behavioral responses to sweet, bitter and umami compounds (Finger et al., 2005). Functional studies in isolate taste cells showed that Type II taste cells are able to release ATP (Roper, 2007). Thus, ATP release in response to sweet and bitter compounds is through pannexin 1 hemichannels (Huang et al., 2007). In this model, Type II taste cells release ATP in response to gustatory stimulation. ATP acts on both sensory afferent fibers and adjacent Type III taste cells indicating interaction among taste cells within the taste bud.

Several neurotransmitters have been proposed to be involved in synaptic transmission within the taste bud and onto the afferent nerve fibers. The mechanisms used by TRCs to relay signaling information to nerve fibers is not yet well understood. Two studies using biosensor cells expressing a specific neurotransmitter receptor have demonstrated that taste buds can release both serotonin (5-hydroxytryptamine; 5HT) and norepinephrine (NE) in response to gustatory stimulation (Huang, Maruyama, & Roper, 2008; Huang et al., 2005). Although it is suggested that NE and serotonin are co-released in Type III taste cells, further studies are needed in this area to clarify physiological roles of these neurotransmitters in the gustatory system. Together these results imply two different functional populations of taste cells, which transmit information onto afferents nerve fibers.
Taste receptor cells must recognize a vast array of different chemical structures, ranging from those that are small and ionic to compounds with complex tertiary structures (Gilbertson & Kinnamon, 1996). Not surprisingly, there are a number of transduction mechanisms that have been proposed to account for the ability of the gustatory system to recognize the five main classes of taste stimuli (salty, sour, sweet, bitter, and umami). Though varied and with few exceptions, the initial chemoreception of sapid molecules involves a direct interaction between the tastant and an ion channel or receptor.

Ion Channel-mediated Mechanisms of Taste Transduction

**Salt taste transduction**

Sodium chloride is the prototypical salty stimulus and in mammalian TRCs salt taste transduction is mediated primarily via sodium ion (Na\(^+\)) influx through the amiloride-sensitive epithelial sodium channel (ENaC). ENaC is formed by a heteromultimeric complex of three subunits (\(\alpha\), \(\beta\), and \(\gamma\)) in a stoichiometry of 2\(\alpha\):\(\beta\):\(\gamma\) or 3\(\alpha\):3\(\beta\):3\(\gamma\). ENaC has been characterized both in TRCs and other transporting epithelia and it shares a number of common features across tissue types. Similarities include; small conductance (~5 pS), Na\(^+\) ~ Li\(^+\) >> K\(^+\) ion selectivity, regulation by extracellular Na\(^+\) (self-inhibition), intracellular Na\(^+\) (feedback inhibition), and regulation by natriferic hormones. These functional similarities between ENaC in the taste system and other transporting epithelia
extend to the molecular level. Experiments in different rodent species have shown that ENaC channels expressed in TRCs have a high sequence homology with those channels expressed in other organs. Thus, in many regards, ENaC appears similar across organ types (Gilbertson, Damak, & Margolskee, 2000; Gilbertson, Boughter, Zhang, & Smith, 2001; Gilbertson & Zhang, 1998b; Yoshida et al., 2009).

In contrast to the well-defined role of ENaC in sodium salt taste, there appears to be an additional amiloride-insensitive pathway that contributes to salt taste transduction. Amiloride-insensitive salt taste is present in many species, including humans, though its relative importance and its underlying mechanism remain controversial. A vanilloid receptor-1 (VR-1) variant has been suggested as an amiloride-insensitive salt receptor. Vallinoid (VR-1) receptors are cation channels similar to members of the TRP channel family. Messenger RNA for VR-1 was identified in taste buds by RT-PCR and absence of amiloride-insensitive Na\(^+\) influx by chorda tympanic nerve in VR-1 knockouts support the interpretation of VR-1 as an amiloride-insensitive salt receptor (Lyall et al., 2004). However, behavioral studies using VR-1 transgenic mice did not reveal a deficit in salt taste (Ruiz, Gutknecht, Delay, & Kinnamon, 2006). Thus, there may be additional, yet unidentified mechanisms for salt taste transduction.
**Acid taste transduction**

Protons (H⁺) are the primary stimuli for acid (sour) sense in humans. Sour taste elicits a general repulsive behavior to avoid the ingestion of both spoiled food and acidic substances which may be harmful. A number of candidate receptors that may contribute to acid taste include the acid-sensing ion channels (ASICs). ASICs are considered members of ENaC/DEG channels family and four genes for ASICs (ASIC 1-4) have been identified. ASICs are activated by changes in extracellular pH. These channels are predicted to produce a cation conductance which increases [Ca²⁺]ᵢ, and neurotransmitter release in taste receptor cells (Ugawa et al., 1998). In addition, hyperpolarization-activated and cyclic nucleotide–gated cation channels (HCN) may activate a G protein that leads to a decrease in the release of [Ca²⁺]ᵢ in response to acid stimulation (Roper, 2007).

Recently, new potential candidate receptors for acid taste transduction have been proposed. Both polycystic kidney disease 2-like 1(PKD2L1) and polycystic kidney disease 2-like 3 (PKD1L3), which are members of the transient receptor potential (TRP) family, are expressed in taste cells (Huang et al., 2006; Ishimaru, 2009). Electrophysiological studies in HEK293 cells transfected with both PKD2L1 and PKD1L3 show evidence of inward current upon acid stimulation (Ishimaru et al., 2006). In some species, ENaC itself has been shown to be permeable to protons and this proton influx is capable of producing a depolarizing receptor potential (Gilbertson, Roper, & Kinnamon, 1993). Unlike the
other taste modalities, acid taste may arise due to activation of multiple receptor and channel types, perhaps owing to the diverse effects that protons (i.e. pH) have on cellular proteins (Medler, 2008).

Mechanisms Involving G Protein Coupled Receptors (GPCRS)

**Bitter taste transduction**

Bitter taste is innately unpleasant and produces an aversive response to avoid consumption of potentially harmful or toxic compounds such as quinine, urea, denatonium, certain amino acids, and potassium chloride. Research has proposed that the T2R family of G protein-coupled receptors is selective for bitter taste stimuli. In addition, there are approximately 30 GPCRs identified that are activated by a variety of bitter compounds (Mueller et al., 2005). Recent evidence, however, has challenged the idea that T2Rs represent the only bitter receptors, consistent with the notion of their being multiple transduction cascades for bitter taste. Following receptor activation, bitter taste transduction involves a series of steps that are activated by α-gustducin (α-transducin-like G protein are expressed in ~25-30% of 12 TRCs). α-gustducin is suggested to be important for bitter, sweet and umami taste transduction since α-gustducin knockout mice have significantly compromised ability to respond to these taste qualities. Moreover, α-gustducin protein mediates two responses: the first leads to a decrease in cyclic adenosine monophosphate (cAMP) levels via phosphodiesterase activation while
the second leads to activation of phospholipase Cβ2 (PLCβ2). PLCβ2 activation produces inositol triphosphate (IP₃), which binds and opens IP₃ receptor subtype3 (IP₃R3) in the endoplasmic reticulum. Ca²⁺ is then released from internal stores and activates a transient receptor potential channel subfamily M member 5 (TRPM5).

Generation and characterization of PLCβ2 and TRPM5 transgenic mice helped clarify the physiological role of these proteins in taste transduction. PLCβ2 knockout mice exhibit a reduction of bitter-taste perception but not a complete absence of bitter taste (Ishimaru, 2009; Medler, 2008; Roper, 2007). TRPM5 knockout mice, in comparison, showed alterations in the response to bitter, sweet, and umami compounds (Damak et al., 2006). Although great advances have been made in the elucidation of the transduction mechanisms of bitter stimuli in taste cells, more studies are needed in order to clarify the role of TRPM5 in taste transduction since TRPM5 can be directly opened by small and rapid increases of intracellular calcium (Zhang, Zhao, Margolskee, & Liman, 2007).

**Sweet taste transduction**

Sweet taste is produced by a variety of chemical compounds such as carbohydrates and specific amino acids. In addition, a number of synthetic “designer” sweeteners (e.g. saccharin and cyclamates) are recognized as sweet by humans. The recognition of sweet tastants is mediated by the T1R receptor
family in taste cells. T1Rs receptors are type-C G protein coupled receptors which function as heteromers. Recently, studies using heterologous expression and knock-out animals have shown that T1R2+3 heterodimers recognize both natural sugars and artificial sweeteners (Margolskee, 2002; Scott, 2004). Similar to the case for bitter transduction, molecular and electrophysiological studies of the gustatory system are consistent with the hypothesis that there are multiple mechanisms underlying sweet taste transduction. α-gustducin activation coupled to the activation of adenylyl cyclase and the production of cAMP are the initial events in one proposed pathway. The role of the increase in cAMP remains unclear but may activate cNMP-gated channels or protein kinase A, eventually leading to cell depolarization and neurotransmitter release. In addition, it has been reported that sucrose can also induce an increase in cyclic guanosine monophosphophate (cGMP) levels in circumvallate cells (Krizhanovsky, Agamy, & Naim, 2000). Alternative pathways which also involve α-gustducin activation lead to a variety of intracellular events which are similar to that delineated in bitter taste transduction involving G protein activation of PLC-β2, which then hydrolyzes phosphatidylinositol 4,5-bisphosphate PI(4,5)P2 into diacylglycerol (DAG) and inositol triphosphate (IP₃). IP₃ binds to the IP₃R3 causing the release of Ca²⁺ from intracellular Ca²⁺ stores. TRPM5, which is activated by the release of Ca²⁺, allows Na⁺ influx (Ishimaru, 2009; Medler, 2008; Roper, 2007) and the development of the receptor potential.
Amino acid taste transduction

The gustatory response to L-glutamate has been suggested to represent a unique fifth taste, called umami (Lindemann, Ogiwara, & Ninomiya, 2002). Glutamate, which is found in many high protein foods including meats, apparently acts on one of three possible receptors that have been identified to date. These receptors include members of the T1R receptor family and two variants of metabotropic glutamate receptors, mGluR4 and mGluR1. The taste-mGluR4, which is a G protein coupled receptor, leads to a decrease in cAMP. The ultimate physiological consequence of this decrease remains elusive. Similarly, mGluR1 has also been proposed as an additional putative umami receptor (San Gabriel, Uneyama, Yoshie, & Torii, 2005). Heterodimeric T1R1+3 receptors form a variety of amino acids receptors, some of which have been linked with umami taste and are predicted to activate similar pathways for other taste receptors, including T2Rs and the sweet receptor (T1R2 + T1R3). Studies in T1R1 or T1R3 null mice showed a decrease in both nerve and behavioral responses to amino acids (Roper, 2007). A recent paper has shown that umami taste transduction is complex and may involve other receptors in addition to T1Rs and the mGluRs (Maruyama, Pereira, Margolskee, Chaudhari, & Roper, 2006). These findings suggest that there are multiple receptors for amino acids and future investigation in umami taste is needed to more fully understand amino acid taste transduction (Yasuo, Kusuhara, Yasumatsu, & Ninomiya, 2008).
Salt Taste Transduction

In mammals, salt taste transduction is primarily mediated by Na\(^+\) ions. Salt taste in the peripheral gustatory system is composed of at least two different pathways for sodium movement. The best described mechanism is a Na\(^+\) specific influx through the apical amiloride-sensitive epithelial sodium channel (ENaC). The influx of Na\(^+\) ions directly depolarizes the taste cell, eventually leading to the release of neurotransmitters onto the afferent nerve fibers (Boughter & Gilbertson, 1999; Gilbertson & Kinnamon, 1996). Electrophysiological recording and molecular studies in the peripheral gustatory system showed that functional ENaC channels found in taste receptors cells are similar to those described in other Na\(^+\) transporting epithelia. These functional characteristics include: a small conductance (~ 5 pS) channel, inhibition by amiloride in the submicromolar range (EC\(_{50}\) ~0.1 μM), Na\(^+\) ~ Li\(^+\) >> K\(^+\) ion selectivity, regulation by extracellular Na\(^+\) (self-inhibition) and intracellular Na\(^+\) (feedback inhibition), and regulation by hormones (Gilbertson et al., 1993; Gilbertson & Zhang, 1998a, 1998b; Lin, Finger, Rossier, & Kinnamon, 1999).

In most transporting epithelia, ENaC is formed by a heteromultimeric complex of three subunits (α, β and γ) in a stoichiometry of either 2α:β:γ or 3α:3β:3γ. While amiloride-sensitive Na\(^+\) currents may be recorded by expression of α-ENaC subunits alone, coexpression with β and γ subunits ENaC increases these currents more than 100-fold (Canessa et al., 1994). RT-PCR,
immunocytochemical, and *in situ* hybridization studies have identified α-, β-, and γ-ENaC subunits in taste buds from all three lingual papillae (Kretz, Barbry, Bock, & Lindemann, 1999; Lindemann, Barbry, Kretz, & Bock, 1998). Additionally, differences in expression of specific ENaC subunits have been found between taste buds in the anterior and posterior rat tongue. Rat circumvallate taste buds exhibit little or no γ-ENaC expression; however, all three subunits are highly expressed in fungiform taste buds. These differences extend to the functional level because electrophysiological studies fail to demonstrate any amiloride-sensitive currents in rat vallate taste cells (Doolin & Gilbertson, 1996; Gilbertson & Zhang, 1998a; 1998b; Kretz et al., 1999). Interestingly, the relative expression of α-, β-, and γ-ENaC subunits are enhanced in taste cells from all three papillae by aldosterone (Lin et al., 1999).

ENaC expressed in mammalian TRCs appears to be similar in function across species since chorda tympani nerve recordings in mouse showed amiloride-sensitive responses to NaCl (Miyamoto, Fujiyama, Okada, & Sato, 1999). Recently, two reports have demonstrated the existence of functional amiloride-sensitive channels in mouse taste cells. First, Vandenbeuch and colleagues showed amiloride-sensitive currents in isolate fungiform taste cells (Vandenbeuch, Clapp, & Kinnamon, 2008). Second, molecular experiments using single cell RT-PCR confirm the expression of α- β- and γ- ENaC subunits in mouse taste epithelia (Yoshida et al., 2009). Although both studies reported the proportion of amiloride-sensitive cells in the various types of mouse taste
buds, their results are inconsistent with the ratio of amiloride-sensitive cells obtained in this dissertation work since I observed that approximately 60% of mouse taste cells, recorded in all experiments, were sensitive to amiloride. In addition, electrophysiological recording experiments in isolated taste cells during this dissertation work revealed that fungiform and circumvallate have functional amiloride-sensitive ENaC.

Mechanisms of ENaC Regulation

The regulation of sodium (Na⁺) transport via ENaC is essential for salt and water transport in various sodium transporting epithelia. Electrophysiological and molecular biological studies in the taste system and other transporting epithelia have shown that ENaC is regulated by natrieric hormones. Aldosterone (ALDO) and arginine-vasopressin (AVP) appear to increase ENaC mediated currents, while atrial natriuretic peptide and perhaps oxytocin reduce these Na⁺ currents. Hormones like ALDO and AVP can increase Na⁺ flux through ENaC by increasing the channel’s open probability and/or the decreasing the turnover of ENaC in the cell membrane (Gilbertson et al., 2000; Lin et al., 1999; Pearce & Kleyman, 2007). In addition to its role as a salt taste receptor, ENaC is important systemically for maintaining sodium balance, blood pressure, and extracellular fluid volume by regulating Na⁺ influx. Not surprisingly, studies in other epithelia suggest that ENaC regulation may be affected by several pathological diseases.
such as diabetes, which may alter salt and water balance in the body (Ecelbarger & Tiwari, 2006; Song, Knepper, Verbalis, & Ecelbarger, 2003; Tiwari, Riazi, & Ecelbarger, 2007). Current research has shown the importance of another hormone, insulin, in ENaC regulation. Cloning and expression of ENaC in A6 cells has suggested an intracellular signaling pathway of ENaC regulation mediated by insulin (Tong, Gamper, Medina, Shapiro, & Stockand, 2004). Moreover, heterologous expression studies in MDCK cells have shown that ENaC is also regulated by inorganic ions such as Na\(^+\) and Cl\(^-\) (Gilbertson & Zhang, 1998b; Kunzelmann, 2003; Xie & Schafer, 2004). However, little or nothing about these ENaC regulatory mechanisms is known in the taste system or how they may alter salt taste.

**Ion-mediated mechanisms of ENaC regulation**

*Sodium Feedback and Self-inhibition of Epithelial Sodium Channel*

The regulation of ENaC is important to control salt and water balance. In kidney transporting epithelia it is well understood that several hormones regulate ENaC in order to conserve or secrete Na\(^+\). Thus, ENaC regulation is necessary to protect the body against changes in either salt content or fluid volume. In addition, epithelial cells have two different mechanisms to control Na\(^+\) and volume. *Feedback regulation* is described as the reduction of Na\(^+\) permeability into the epithelial cell to prevent or limit Na\(^+\) overload. The other concept of *self-
inhibition is that extracellular Na\(^+\) itself can modify the activity of ENaC independent of changes in intracellular ion composition (Garty & Palmer, 1997). Both feedback regulation and self-inhibition features are present in amiloride-sensitive ENaC expressed in taste epithelia (Gilbertson & Zhang, 1998b).

Current evidence in the peripheral gustatory system suggests regulation of ENaC by self-inhibition may play an important role in adaptation of TRCs to Na\(^+\) salts. This feature of apparent saturation of Na\(^+\) transport could be explained by the inverse relationship between the extracellular Na\(^+\) concentration and permeability with a direct interaction of extracellular Na\(^+\) ions with ENaC. Thus, a number of Na\(^+\) transporting cells use self-inhibition to conserve resources during chronic sodium loads. In addition, the ability of epithelial cells to control and regulate Na\(^+\) entry via ENaC is a common mechanism across transporting epithelia (Abriel & Horisberger, 1999). Feedback inhibition, on the other hand, suggests that Na\(^+\) saturation due to direct or indirect inhibition of ENaC increases changes of intracellular Na\(^+\) leading to a rise in Ca\(^{2+}\) and a subsequent activation of G proteins (Komwatana, Dinudom, Young, & Cook, 1996a, 1996b). Unlike the self-inhibition mechanism, feedback inhibition is much slower, and in some cases this process may take several minutes to achieve any effects on Na\(^+\) permeability. A third potential feature of Na\(^+\) saturation is the reduction in driving force of Na\(^+\) due to significant increase in intracellular Na\(^+\) concentration. Because TRCs are constantly bathed with saliva, which in rats contains 60 mM
Na⁺, both mechanisms of feedback and self-inhibition could be important to limit the movement of Na⁺ through ENaC in the taste system (Gilbertson et al., 2000).

The role of intracellular Cl⁻ in ENaC regulation

Physiological studies in kidney transporting epithelia have suggested that intracellular Cl⁻ ions could act as a regulatory signal for Na⁺ movement via ENaC. Both ENaC open probability and ENaC membrane expression can be decreased by modifications in intracellular chloride concentration \([\text{Cl}^-]_i\). Thus, the ability of Cl⁻ ions to inhibit ENaC depends upon the magnitude and direction of Cl⁻ movement through apical Cl⁻ channels like, cystic fibrosis transmembrane conductance regulator (CFTR), CIC family channels, and Na⁺/K⁺/2Cl⁻ cotransporter (Veizis & Cotton, 2007). Typically, in epithelial cells, \([\text{Cl}^-]_i\) is determined by the relationship between Cl⁻ cotransporters and CIC channels in apical and basolateral membranes. Cotransporters, such as NKCC, actively accumulate Cl⁻ across the basolateral membrane to maintain \([\text{Cl}^-]_i\) above its electrochemical equilibrium. In contrast, the opposite occurs when Cl⁻ channels are activated (e.g. by cAMP), which result in a fall of \([\text{Cl}^-]_i\) (Kunzelmann, 2003; Letz & Korbmacher, 1997).

The effects of cytosolic chloride are important in the regulation of NaCl reabsorption. Several studies in MDCK and A6 cells have demonstrated a relationship between \([\text{Cl}^-]_i\) and Na⁺ transport (Fujimoto, Niisato, Sugimoto, & Marunaka, 2005; Miyazaki, Shiozaki, Niisato, & Marunaka, 2007; Niisato, Eaton,
& Marunaka, 2004; Xie & Schafer, 2004). In addition, ENaC expressed in salivary duct cells is inhibited by high [Cl\textsuperscript{-}], confirming that Na\textsuperscript{+} conductance is dramatically disturbed by changes in cytosolic Cl\textsuperscript{-} (Dinudom, Komwatana, Young, & Cook, 1995; Dinudom, Young, & Cook, 1993) The specific mechanism(s) of ENaC regulation by Cl\textsuperscript{-} ions is still controversial. Currently, there are two potential mechanisms of regulation: ENaC is regulated directly by the PDZ motif of CFTR channels (Reddy, Light, & Quinton, 1999) and ENaC function is reduced by cytosolic Cl\textsuperscript{-} ions. Thus, experiments in ENaC activity using patch clamping single channel recording show that changes in Cl\textsuperscript{-} concentration alters both ENaC conductance and open probability (Gu, 2008).

While the resting [Cl\textsuperscript{-}] concentration is unknown in taste cells, if similar to many neuron types, one would predict that salt (e.g. NaCl) stimulation could lead to an influx of Cl\textsuperscript{-} into the cell and a subsequent inhibition of ENaC activity. Comparable results have been reported in salivary glands in which inward Na\textsuperscript{+} current was decreased by stepwise increases of intracellular Cl\textsuperscript{-} (Dinudom et al., 1993). In addition, NKCC1 null mice showed a hyperosmotic saliva due to the inability of NaCl uptake (Evans et al., 2000). This may explain one of the mechanisms by which taste cells adapt to continued NaCl exposure.

**Hormonal regulation of ENaC**

Sodium (Na\textsuperscript{+}) movement through ENaC and the regulation of this process has been widely studied in kidney epithelia. Current evidence suggests that
ENaC is Na\(^+\)-selective, non-voltage gated, non-inactivated ion channel in the ENaC/Deg superfamily. ENaC is localized in the luminal plasma membrane of numerous Na\(^+\) reabsorbing epithelia such as distal nephron, lungs, colon and taste. ENaC’s physiological roles are the regulation of extracellular fluids, volume of extracellular fluids and blood pressure. Consequently, ENaC activity and open probability is essential for local movement of electrolytes and water across epithelial barriers as well as control of total body electrolyte and water homeostasis. In contrast, dysfunction and aberrant regulation of ENaC leads to a variety of diseases, including hypertension and hypotension, that are associated with improper renal Na\(^+\) conservation and wasting (Pochynyuk, Tong, Staruschenko, & Stockand, 2007).

**Regulation of ENaC by mineralocorticoids**

The mineralocorticoid hormone aldosterone regulates Na\(^+\) balance through ENaC activation. In general, aldosterone increases Na\(^+\) reabsorption via ENaC by two different mechanisms: (1) increasing the open probability of apical ENaCs and (2) increasing the number of active channels in the membrane. Both of these mechanisms are mediated by the aldosterone-induced immediate early gene, SGK (serum- and glucocorticoid-regulated kinase). SGK is a protein with a half life of 30 min and it is expressed in several tissues including brain, lung, colon, pancreas and kidney (Loffing, Flores, & Staub, 2006; Tiwari et al., 2007). SGK plays a central role in the regulation of Na\(^+\) transport by increasing the
number of apical ENaC in transporting epithelia. The interaction between SGK and ENaC is by phosphorylation of the ubiquitin ligase NEDD4-2, which ubiquitinates ENaC. Thus, NEDD4-2 regulates removal and degradation of ENaC protein from the cell membrane. Accordingly, phosphorylation of NEDD4-2 by SGK1 leads to an enhancement of ENaC abundance in the cell membrane (Debonneville et al., 2001; Faletti, Perrotti, Taylor, & Blazer-Yost, 2002; Staub & Verrey, 2005; Zhou & Snyder, 2005). Additionally, SGK also increases ENaC open probability by a mechanism that is not yet clear (Arteaga & Canessa, 2005). Perhaps one of the most interesting features of SGK regulation is that its activity is controlled by other hormones as well.

In addition, enhancement of ENaC function by aldosterone is also mediated by the mineralocorticoid receptor. The mineralocorticoid receptor (MR) target gene regulates α-ENaC expression. This mineralocorticoid receptor-mediated gene expression of α-ENaC is a comparatively slow process and in some cases there are no changes in ENaC expression seen even after several hours of exposure to aldosterone. The peak expression of α-ENaC is achieved around 12 hours post exposure to aldosterone. However, new evidence suggest that the direct MR regulation of α-ENaC gene transcription also involves SGK (Pearce & Kleyman, 2007).

In taste tissue, rats treated with aldosterone exhibited greater amiloride-sensitive responses to NaCl than non-treated littermates (Herness, 1992). The relative expression of the β- and γ-ENaC subunits is enhanced in TRCs from all
lingual papillae by aldosterone, leading to an increase in the amplitude of the amiloride-sensitive Na⁺ currents (Gilbertson, 1998; Gilbertson et al., 2000). Although the mechanism of aldosterone transduction in the taste system is not yet clear, molecular experiments in our laboratory have shown that TRCs express all the potential candidates necessary for this pathway.

*Regulation of ENaC by insulin*

A common feature of ENaC is that it is regulated by one or more hormones that help maintain salt and water balance in transporting epithelia. Several recent studies have discovered that insulin plays a central role in the regulation of salt transport by targeting ENaC. Both ENaC open probability and membrane expression of ENaC in the cell membrane can be increased by insulin stimulation acting through insulin/IGF-1 receptors. In this model, insulin binds its cognate receptor in the cell membrane, and produces a subsequent phosphorylation of insulin receptor substrate (IRS), which results in activation of PI3-kinase (PI3-K). PI3-K forms PtdIns(3,4,5)P₃ in the inner leaflet of the plasma membrane therefore these changes in the lipid composition provide a mode for direct activation of ENaC (Blazer-Yost, Vahle, Byars, & Bacallao, 2004). Thus, ENaC function is regulated by the direct effects of PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃.

In addition PtdIns(4,5)P₂ is necessary to generate PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ by PI3-Kinase. PtdIns(4,5)P₂, on the other hand, is suggested to
regulate a variety of ion channels including inward rectifying K⁺ channels (Zhang, He, Yan, Mirshahi, & Logothetis, 1999), TRPM8 (Rohacs, Lopes, Michailidis, & Logothetis, 2005) and voltage-gated Ca²⁺ channels (Wu, Bauer, Zhen, Xie, & Yang, 2002). In addition, β- and γ-ENaC subunits contain specific binding sites for PtdIns(4,5)P₂ in the N terminus (Pochynyuk et al., 2007). Electrophysiological experiments in A6 and M1 collecting duct cells have suggested that PtdIns(4,5)P₂ levels are critical for normal ENaC activity; since an increase in PtdIns(4,5)P₂ membrane levels will dramatically increase ENaC open probability. In contrast, ENaC function is decreased when PtdIns(4,5)P₂ levels are depleted (Kunzelmann et al., 2005; Ma, Saxena, & Warnock, 2002; Tong & Stockand, 2005).

An alternative pathway of ENaC regulation by PtdIns(3,4,5)P₃ is via the phosphatidylinositol-dependent protein kinase (PDK1). PDK1, in turn, activates serum and glucocorticoid–regulated kinase (SGK) which leads to both ENaC activation and insertion of the channel in the apical membrane. The enhancement of ENaC/SGK-mediated Na⁺ transport occurs via one of two mechanisms: (1) increasing the open probability of apical ENaCs and (2) increasing the number of active channels in the membrane. Additionally, both insulin and aldosterone signaling pathways converge at SGK to regulate ENaC activity. The ability of insulin to activate SGK shares a number of similarities with ENaC activation by aldosterone; these similar characteristics include phosphorylation of the ubiquitin ligase NeD4-2. Thus, insulin’s physiological
role on SGK is additive to that of aldosterone because insulin increases phosphorylation of SGK while aldosterone increases SGK expression (Faletti et al., 2002; Tiwari, Nordquist, Halagappa, & Ecelbarger, 2007).

The regulation of SGK by insulin is important for ENaC trafficking into the membrane (Blazer-Yost et al., 2004; Vallon et al., 2005). ENaC trafficking refers to the level of ENaC movement to the cell surface as well as endocytosis and degradation of ENaC. In general, insulin pretreated mice showed an increase in protein abundance of α- and γ-ENaC and SGK (Tiwari et al., 2007). In addition, electrophysiological studies showed that SGK can enhance Na⁺ reabsorption via ENaC (Lee, Dinudom, Sanchez-Perez, Kumar, & Cook, 2007). Together we hypothesized that a similar regulatory mechanism, in which insulin enhances ENaC activity, may occur in the gustatory system.

Diabetes

Diabetes mellitus is an endocrine disease in which the body does not produce or properly use insulin. In general, there are two types of diabetes: Type I (insulin dependent) which results from the body’s failure to produce insulin or Type 2 (non-insulin dependent) a condition in which the body doesn’t properly use insulin. This absence or decrease in insulin activity triggers hepatic glucose production and reduces glucose uptake, resulting in hyperglycemia. Associated with hyperglycemia are a variety of counterregulatory mechanisms, which are
activated to stabilize the diabetic decompensation. These metabolic abnormalities can be divided into either early phase compensatory mechanism or long term complications in which blood vessels, other organs and kidneys are involved. Perhaps the major portion of morbidity and mortality arising from diabetes is due to progressive kidney disease and diabetic nephropathy (Hills, Squires, & Bland, 2008; Peti-Peterdi, Kang, & Toma, 2008; Vidotti, Arnoni, Maquigussa, & Boim, 2008).

The pathogenesis of Type I diabetes is due to the destruction of insulin-producing β cells in the pancreatic islets. In humans, Type I diabetes has been associated with natriuresis and diuresis, as well as activation of vasopressin and the renin-angiotensin-aldosterone system (RAAS). Several studies have examined the effects of diabetes on sodium transporters and channels using Type I diabetic rodents. These animal models have emerged to study diabetes and its complications such as salt and water disturbances. One of the most common diabetic animal model involves the generation of Type I diabetes by treatment with streptozotocin (STZ), which directly destroys pancreatic β cells. Most of our current knowledge in pathophysiology from diabetes has come from diabetic STZ rodents.

Early stage diabetes mellitus is characterized by polyuria, glucosuria, glomerular hyperfiltration and osmotic diuresis. Osmotic diuresis results from unabsorbed glucose in the kidney tubule lumen, which leads to volume depletion and dehydration (Kim, Sands, & Klein, 2004; Vidotti et al., 2008). Thus,
dehydration is the main underlying complication in uncontrolled diabetes because it can lead to hyperglycemic hyperosmolar state and diabetic ketoacidosis (Chiasson et al., 2003). The development of dehydration in diabetes is the result of increased losses of electrolytes and urinary output. This severe imbalance of water and electrolytes triggers a series of compensatory mechanisms in the kidney in order to limit salt and water losses. However, patients and animals with uncontrolled diabetes rarely present evidence of volume depletion suggesting that the kidneys may adapt to compensate osmotic diuresis to prevent hypovolemic shock (Vidotti et al., 2008).

One mechanism of adaptation occurs via regulation of protein abundance of sodium transporters and channels, which line the renal transporting epithelia. Additionally, studies in Type I untreated diabetes have shown an enhancement of protein expression of all 3 (α, β, and γ) ENaC subunits, NKCC, and NCC (Bickel, Knepper, Verbalis, & Ecelbarger, 2002; Kim et al., 2004; Song et al., 2003). These changes in the protein expression in the kidney are accompanied by an increase in water intake of the diabetic animal (Kanarek & Ho, 1984). Thus, these results primarily demonstrate relative short-term effects of diabetes (4-14 days) in rats which are associated with significantly increases in the abundance of ENaC subunits (Song et al., 2003). In contrast, α-ENaC expression is significantly reduced in long term untreated diabetes (8 weeks). This reduction in α-ENaC protein abundance was attenuated by insulin treatment (Vidotti et al., 2008). Together these findings constitute a compensatory mechanism in which
adjustment of ENaC protein abundance may be important in order to prevent systemic volume depletion.

Taste impairment has been reported in patients with diabetes mellitus. Diabetes can decrease the individual’s ability to recognize and detect sweet, salty and bitter solutions (Hardy, Brennand, & Wyse, 1981). In addition, humans and animals with diabetes usually develop nerve damage known as diabetic neuropathies. This kind of neuropathy caused by diabetes generally creates sensory deficits including tactile and chemical senses (Lamster, Lalla, Borgnakke, & Taylor, 2008). Recently, a study in Type I (STZ) diabetic rats showed that long term diabetes is associated with a deficit in the quantity of cirumvallate taste cells in the diabetic animal compared to the control group (Pai, Ko, & Chou, 2007). In contrast, α-gustducin which is important for sweet, bitter, and umami is more immunoreactive in diabetic cells (Zhou, Liu, Feng, Han, & Liu, 2009). Given the previous results, this dissertation work is designed to determine the different mechanisms of adaptation that may occur in the gustatory system to help restore salt/water balance during the onset of diabetes mellitus.

I studied different mechanisms of ENaC regulation in mammalian taste receptor cells and how this might affect their ability to respond to NaCl stimuli. In the second chapter of the present dissertation study, I have investigated the physiological role of Cl⁻ channels in salt taste transduction. Consistent with other transporting epithelia, TRCs expressed a wide variety of CIC, CFTR and NKCC1 channels. These molecular results suggested that taste cells have all the
components to regulate \( \text{Na}^+ \) influx via ENaC. Using different electrophysiological techniques I evaluated the effects of Cl\(^-\) ions on \( \text{Na}^+ \) transport via ENaC in taste cells. In addition, I will address in more detail each of the experiments that I performed to determine the functional relationship among Cl\(^-\) ions and ENaC in Chapter 2.

Another goal of this dissertation work was to determine how \( \text{Na}^+ \) uptake via ENaC is regulated by insulin in the peripheral gustatory system. In Chapter 3, I evaluated the importance of insulin in TRCs using a combination of whole cell patch clamp recording and ratiometric functional \( \text{Na}^+ \) imaging. The results suggest insulin stimulates \( \text{Na}^+ \) currents through amiloride-sensitive channels. Therefore we tested if insulin activation of ENaC occurs through PI3-Kinase signaling cascade. In agreement with early reports in other transporting epithelia, insulin-ENaC-mediated responses in the peripheral gustatory system are carried out by PI3-Kinases and its phospholipid products (Staruschenko, Pochynyuk, Vandewalle, Bugaj, & Stockand, 2007; Wang et al., 2008). Then, I report other potential candidates that may influence the taste system. I found that PtdIns(4,5)P\(_2\) synthesis is vital for insulin/ENaC mediated responses. Moreover, using a SGK\(^{-/-}\) null mice I demonstrated the relevance of SGK in ENaC activation by insulin. Interestingly I was able to reveal a novel insulin pathway which regulates salt intake and ENaC activity in the peripheral gustatory system.

In Chapter 4, I report variations of insulin responses in diabetic taste cells and how the onset of diabetes influences salt taste via ENaC in Type I diabetes. I
used a multidisciplinary approach including functional Na\(^+\) imaging, qRT-PCR and behavioral assays to determine differences in ENaC activity between TRCs in diabetic and non-diabetic mice. The results showed unique changes in salt taste transduction via ENaC. I hypothesized that these finding are an example of how the gustatory system responds to nutritional challenges. As a hallmark of the current dissertation work, the findings in Chapter 4 are the first to show functional alteration of ENaC activity in a mammalian model of diabetes. In summary, this dissertation project explores the two major mechanisms of ENaC regulation in the taste system. The findings of each chapter allow me to elucidate new models for salt taste transduction. For instance, ENaC regulation by intracellular Cl\(^-\) ions was a milestone to the understanding of other regulatory mechanisms in the taste system which may have influence on the animals' salt and water balance. Chapter 3 was carried out with similar experimental approach allowing me to relate both ion regulation and hormonal regulation of ENaC in TRCs. In Chapter 4, I was able to employ new functional imaging techniques to determine insulin effects of Na\(^+\) influx via ENaC in diabetic taste cells without modifying [Cl\(^-\)].

REFERENCES


CHAPTER 2

REGULATION OF EPITHELIAL SODIUM CHANNEL BY INTRACELLULAR CHLORIDE AND CHARACTERIZATION OF CHLORIDE CHANNELS IN MAMMALIAN TASTE RECEPTOR CELLS

Abstract

The transduction of sodium salts (e.g. salty taste) has been shown to involve permeation of sodium ions through epithelial sodium channels (ENaC) in the gustatory system. Though numerous studies have demonstrated complex regulation of ENaC in other sodium transporting epithelia, there have been few studies that have focused on the regulation of ENaC in the taste system. To understand the regulatory capacity of the gustatory system, we have begun to investigate the ability of ENaC to be regulated by intracellular Cl⁻ ions. To identify expression and function of various transport proteins for Cl⁻ in the gustatory system, we have used RT-PCR and functional Cl⁻ imaging. Taste cells express a variety of Cl⁻ transport proteins including several members of the CIC family of Cl⁻ channels, the cystic fibrosis transmembrane conductance regulator (CFTR) and the sodium-potassium-chloride cotransporter, NKCC.

¹ Coauthored by Arian F. Baquero and Timothy A. Gilbertson
Functional Cl⁻ imaging recording on mouse taste cells revealed that Cl⁻ influx though these Cl⁻ transporting proteins changed the intracellular chloride concentration. To explore the role of cytosolic Cl⁻ in ENaC function, we performed patch clamp recording on isolated mouse taste cells. Fungiform TRCs showed that both the magnitude of ENaC currents and their sensitivity to the diuretic amiloride is inversely correlated with intracellular Cl concentration. In solutions high in extracellular Cl⁻, inhibition of Cl⁻ transport with pharmacological chloride channels antagonist leads to increases in ENaC-mediated sodium influx in taste cells. These findings suggest that ENaC is regulated by cytosolic Cl⁻ in the gustatory system. This regulatory mechanism may be important in the process of TRC adaptation to conserve cellular resources during chronic NaCl exposure.

Introduction

Sodium chloride is the prototypical salty stimulus and, in mammals, salt taste transduction is primarily mediated by Na⁺ ions. The best described mechanism for salt taste transduction involves Na⁺ specific influx through the apical amiloride-sensitive epithelial sodium channels (ENaC). The influx of Na⁺ ions directly depolarizes the taste cell, eventually leading to the release of neurotransmitter onto the afferent nerve fibers (Gilbertson & Kinnamon, 1996). Electrophysiological recordings and molecular studies in the peripheral gustatory system demonstrated that functional ENaC channels found in taste receptors
cells (TRCs) are similar to those described in other Na⁺ transporting epithelia. These functional characteristics include that these channels have a small conductance (~ 5 pS), are inhibited by amiloride in the submicromolar range (EC₅₀ ~0.1 μM), display Na⁺ ~ Li⁺ >> K⁺ ion selectivity, regulated by extracellular Na⁺ (self-inhibition) and intracellular Na⁺ (feedback inhibition), and regulated by a number of hormones, including aldosterone, and vasopressin (Doolin & Gilbertson, 1996; Lin, Finger, Rossier, & Kinnamon, 1999). Moreover, heterologous expression studies in MDKC cells have shown that ENaC it is also regulated by inorganic ions such as chloride (Xie & Schafer, 2004).

Recent, studies in kidney transporting epithelial have shown that a number of chloride transporting proteins including the ClC family of Cl⁻ channels, the Na⁺/K⁺/2Cl⁻ (NKCC) cotransporter, and the cystic fibrosis transmembrane conductance regulator (CFTR), are important in the inhibition of sodium Na⁺ influx through ENaC. Moreover, these effects extend to pathophysiological mechanisms since there is evidence that Cl⁻ transporting proteins play an essential role in salt and water retention in diabetic nephropathy (Bickel, Knepper, Verbalis, & Ecelbarger, 2002; Niisato, Eaton, & Marunaka, 2004; Schreiber, Boucherot, Murle, Sun, & Kunzelmann, 2004). Because of the similarities between salt/water transport pathways in lingual and kidney epithelia we initially focused our efforts to understanding the correlation between Na⁺ influx via ENaC and cell volume regulation in TRCs. Most transporting epithelia involved with NaCl absorption are exposed to extreme changes in tonicity and
alterations in solution tonicity lead to perturbations in cell volume by rapidly rising or lowering intracellular chloride [Cl\(^{-}\)]. Therefore, sodium and potassium are transported to maintain electroneutrality, following by movement of water, which is driven by osmotic gradients (Kahle et al., 2008; Niisato et al., 2004; Xie & Schafer, 2004).

Recently, we showed that changes in solution osmolarity lead to an increase in taste cell capacitance and a concomitant activation of a Cl\(^{-}\) conductance (Gilbertson, 2002; Watson et al., 2007). This Cl\(^{-}\) conductance exhibited all the pharmacological and permeability properties of ubiquitous swelling-activated Cl\(^{-}\) current (Jentsch, Friedrich, Schriever, & Yamada, 1999). Additionally, we have shown the presence of several aquaporin (AQP) channels and provided functional evidence in TRCs that hypoosmotic-induced currents are reduced by tetraethylammonium (TEA), an AQP inhibitor (Watson et al., 2007). Consequently, this hypoosmotic-activated current, termed I\(_{\text{HYPO-T}}\) in TRCs might play a role in regulatory volume decrease (RVD) (Gilbertson, 2002). Given these results, TRCs have all the cellular machinery necessary to respond to changes in the extracellular osmotic environment.

Despite the apparent importance of Cl\(^{-}\) flux in taste cells, little is known about the chloride transporting proteins in this system. Typically, in epithelial cells, [Cl\(^{-}\)]\(_{i}\) is determined by the relationship between Cl\(^{-}\) cotransporters and ClIC channels in apical and basolateral membranes. Cotransporters, such as NKCC, actively accumulate Cl\(^{-}\) across the basolateral membrane to maintain [Cl\(^{-}\)]\(_{i}\) above
its electrochemical equilibrium. The opposite occurs when Cl\textsuperscript{−} channels are activated (e.g. by cAMP), which results in a fall of [Cl\textsuperscript{−}]. Coexpression of Cl\textsuperscript{−} channels with ENaC in *Xenopus laevis* oocytes inhibits Na\textsuperscript{+} transport, and ENaC inhibition was mainly characterized by alterations in [Cl\textsuperscript{−}] (Kunzelmann, 2003).

Niisato et al. (2004) Thus, it would not be surprising to find that salt (e.g. NaCl) stimulation could lead to an influx of Cl\textsuperscript{−} into the cell and a subsequent inhibition of ENaC activity. This may represent one of the mechanisms by which taste cells adapt to continued NaCl exposure.

In the present study, we have explored ENaC regulation by Cl\textsuperscript{−} ions in taste cells. Using whole cell patch clamp recording and trans-lingual current recording, we have characterized the effect of cytosolic Cl\textsuperscript{−} on ENaC currents in TRCs. The results support the interpretation that increases in [Cl\textsuperscript{−}], inhibit amiloride-sensitive Na\textsuperscript{+} transport in taste cells. Using reverse-transcription polymerase chain reaction (RT-PCR), we have attempted to identify chloride channels and cotransporters (ClC family, CFTR, and NKCCs) in taste cells. Consistent with other salt- and water- transporting epithelia, taste cells contain several ClC channels, CFTR and NKCC, which are almost identical to those found in the kidney or lung. The importance of these chloride transporting proteins in the regulation of [Cl\textsuperscript{−}], is well understood. For example, activation of CFTR in mouse colon can increase [Cl\textsuperscript{−}], and therefore inhibit Na\textsuperscript{+} absorption (Schreiber, Konig, Sun, Markovich, & Kunzelmann, 2003). In addition, *Xenopus oocytes* coexpressing ENaC, CFTR and ClC, increase [Cl\textsuperscript{−}], after exposure to...
high extracellular Cl⁻ (Bachhuber et al., 2005). Because of the similarity in expression of Cl⁻ transporting proteins, we hypothesized that a similar change in [Cl⁻]ᵢ might occur in the taste system. To test this, we performed a series of experiments to determine the effect of Cl⁻ movement on ENaC-mediated currents using functional Cl⁻ imaging and whole cell patch clamp recording. We present evidence showing that taste cells are capable of transporting Cl⁻ ions across the membrane and modify the [Cl⁻]ᵢ. We conclude that a reduction in [Cl⁻] by blocking ClC channels and NKCC leads to an increase in Na⁺ influx via ENaC. Taken together, it appears Cl⁻ movement through chloride transporting proteins regulates Na⁺ transport through ENaC across taste cell membranes, which ultimately may affect the ability of the organism to recognize sodium salts in the gustatory system.

Methods

All experiments were performed on adult (2–4 months) male C57BL6 mice that were maintained on a 12-h:12 h day/night cycle with normal chow and water provided ad libitum. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Utah State University and were performed in accordance with American Veterinary Medical Association guidelines.
Taste bud isolation

Individual fungiform and circumvallate taste buds were isolated from mice tongues using techniques previously described (Béhé, DeSimone, Avenet, & Lindemann, 1990; Doolin & Gilbertson, 1996). Briefly, tongues were removed and immediately immersed in Tyrode’s solution containing (in mM): 140 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 N-2-hydroxyethylpiperazine- N’-2-ethanesulphonic acid (HEPES), 10 glucose, and 10 Na⁺ pyruvate (osmolarity; 310 mOsm). The pH was adjusted to 7.4 with NaOH. The anterior portion of the tongue containing the fungiform papilla was injected between the muscle layer and the lingual epithelium with approximately 0.15 ml of physiological saline (Tyrode's) containing a mixture of collagenase I (1.1 mg/ml; Roche Applied Science, Indianapolis, IN), dispase (2.4 mg/ml; Roche Applied Science), and trypsin inhibitor (1 mg/ml; type I-S; Sigma Chemical Corp., St. Louis, MO).

Approximately 0.1 ml of the same enzyme solution was also used to inject the area surrounding the circumvallate papilla. The injected tongue was incubated in Tyrode's solution and aerated with O₂ for 45 min. The lingual epithelium was then peeled from the underlying tissue and pinned out in Tyrode's in a Sylgard-lined petri dish with the mucosal side facing down. The epithelium was then incubated for 7 min with the same enzyme cocktail. Following the incubation, the epithelium was incubated in a Ca²⁺-Mg²⁺– free Tyrode's containing 2 mM BAPTA (Invitrogen, Eugene, OR) in place of CaCl₂ and MgCl₂ for 5 min. Amiloride (10 µM; Sigma Chemical Corp) was added to all solutions to help protect against
enzymatic degradation of ENaC (Gilbertson & Fontenot, 1998). Taste buds were removed from the epithelium using a large bore (~150-200 µm) pipette and plated either onto a charged microscope slide in a Tyrodes-containing Sylgard ring for patch clamp recording or onto a Cell-Tak (BD Science, Franklin Lakes, NJ) coated coverslip in a laminar flow perfusion chamber for functional imaging. For isolation of single taste cells, this procedure was slightly modified by an additional incubation in Ca\(^{2+}\)-Mg\(^{2+}\)-free Tyrode's for 10 min before the second enzyme treatment.

Patch clamp recording

Normal extracellular solution (Tyrode's) containing (in mM) 140 NaCl, 5 KCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, 10 glucose, and 10 Na\(^+\) pyruvate (osmolarity; 310 mOsm). The pH was adjusted to 7.4 with NaOH. Amiloride (0.01, 0.05, 0.1, 0.5, 1, and 10 µM) was made up in normal Tyrode's. A 100 mM bumetanide stock solution was prepared in dimethyl sulfoxide (DMSO), 10 µM bumetanide was prepared by dilution in Tyrode's from the stock solution. 10 µM 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) was prepared from stock solution of 50 mM by diluting in Tyrode. All solutions were made immediately before use.

Two types of intracellular solution were used in this work. Pipettes were filled either with high intracellular chloride solution (in mM): 140 KCl; 1 CaCl\(_2\); 2 MgCl\(_2\); 10 HEPES; 11 EGTA; and 3 ATP. The pH and osmolarity was adjusted to 7.2; ~310 mOsm or a low intracellular chloride solution (in mM): 140 K-gluconate;
1 CaCl₂; 2 MgCl₂; 10 HEPES; 11 EGTA; and 3 ATP. The pH and osmolarity was adjusted to 7.2; ~310 mOsm with KOH, respectively.

Individual fungiform taste receptor cells (TRCs) were recorded using conventional whole cell patch clamp. Patch pipettes were pulled to a resistance of 5–8 MΩ when filled with intracellular solution. Series resistance and capacitance were compensated optimally before recording. Currents were recorded in the presence and absence of test solutions in response to voltage ramps (-110 to +50 mV at 0.33 V/s). Current data were recorded and command potentials delivered using pClamp software (v. 10). This software was interfaced with an AxoPatch 200B amplifier and Digidata 1322A data acquisition system (Molecular Devices, Sunnyvale, CA). Current-voltage relationships (I-V curve) were used to determine whether test solutions significantly altered I-V curves in TRCs. To isolate this treatment-induced response, the I-V curves for the test solution were subtracted from those of the control solution.

RT-PCR

First-strand cDNA was synthesized using the iScript Kit (Bio-Rad, Hercules, CA). The maximum volume of taste RNA or 50 ng of kidney RNA was used for the reaction with the total volume being 20 µl. Reactions were also set up in which the reverse transcriptase enzyme was omitted as a control to detect DNA contamination. After first-strand synthesis, 1 µl of cDNA was added to a PCR reaction mixture containing final concentrations of 50 mM KCl, 10 mM Tris-
HCl (pH 8.3), 2.0 mM Mg$^{2+}$, 200 µM dNTPs, ~500 nM forward and reverse primers and 1.25 U Taq polymerase. PCR products were amplified using an initial 5 min denaturation step followed by 40 cycles of a 3-step PCR (30 sec denaturation at 95°C, 30 sec annealing at optimal temperature & 45 sec extension at 72°C), and concluding with a 7 min final extension step. Amplified sequences were visualized by electrophoresis in 2% agarose gels using 1X TAE buffer (40 mM tris-acetate & 1 mM EDTA). Primer sequences, accession numbers, and corresponding nucleotide sequences are shown in Table 2.1. Purification of PCR products for sequencing was performed using the QIAquick PCR purification kit (Qiagen, Valencia, CA). Sequences were determined by the dye-terminator method using an ABI Model 3100 Automatic Sequencer (Foster City, CA). Partial sequences for each product were examined using the BLAST 2.0 search engine (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Transepithelial current recording

Modified Krebs-Heinseleit (KH) buffer contained (in mM): 118 NaCl, 25 NaHCO$_3$, 1.3 NaH$_2$PO$_4$, 6 KCl, 2 CaCl$_2$, 2 MgCl$_2$, and 5.6 glucose. To record effects of Cl$^-$ ions in Na$^+$ movement in lingual epithelium, Cl$^-$ was replaced with NO$_3$ in KH Buffer. Intermediate 60 mM Cl$^-$ KH buffer contained (in mM): 70 NaNO$_3$, 48 NaCl, 25 NaHCO$_3$, 1.3 NaH$_2$PO$_4$, 6 KCl, 2 CaCl$_2$, 1.2 MgCl$_2$, and 5.6 glucose. Free Cl$^-$ KH buffer contained (in mM): 118 NaNO$_3$, 25 NaHCO$_3$, 1.3 NaH$_2$PO$_4$, 6 KNO$_3$, 2 Ca(NO$_3$)$_2$ and 5.6 glucose. All 3 types of KH buffer are
aerated with 95% O₂/5% CO₂ continuously, which produced a stable pH of 7.4. Mucosal solutions consisted of 10-500 mM NaNO₃ dissolved in distilled water.

Lingual epithelia were isolated using methods previously described (Gilbertson & Zhang, 1998a, 1998b). Briefly, isolated lingual epithelia containing fungiform papilla were mounted in a bipartitioned Ussing chamber (World Precision Instruments, Inc., Sarasota, FL), each side of which had a volume of 0.5 ml and an opening with an area of 0.126 cm². Epithelia were supported by a nylon mesh filter covering the opening to the serosal chamber that prevented damage to the tissue. The chamber was assembled and both chambers were filled with KH buffer and allowed to equilibrate for 30 min. Solutions were maintained at 35°C in a thermal bath and were applied by gravity flow to the two chambers through 8-to-1 tubing connectors (Small Parts, Miami Lakes, FL). This arrangement allowed solution change without disturbance of the Ussing chamber and eliminated the possibility of introducing bubbles. Solutions were perfused continually through the mucosal and serosal chambers for the duration of the experiment at a rate of ~5 ml/min (complete change in <10 s).

Transepithelial currents were recorded by a dual voltage clamp (DVC-1000; WPI, New Haven, CT) connected to the Ussing chamber by Ag/AgCl electrodes. Each side of the chamber had a current and voltage electrode in series with 0.9% NaCl agar bridges. Fluid resistance was compensated before the introduction of an epithelium with symmetrical KH buffer. Short-circuit current
(I\textsubscript{SC}) was recorded on a four-channel data acquisition system (Lab-Trax-4/16, WPI, New Haven, CT) at a frequency of 40 kHz and displayed on computer driven software (Data Trax 2 WPI, New Haven, CT). Transepithelial resistance was monitored by \(-10\) mV pulses delivered by a stimulator (Master 8; A.M.P.I. Jerusalem, Israel) connected to the voltage clamp. All experiments were conducted with the tissue voltage clamped to 0 mV. By convention (Mierson, Olson, & Tietz, 1996), positive I\textsubscript{SC} indicates the net movement of cations from the apical to the basolateral side of the tissue.

Functional chloride imaging

Cl\textsuperscript{-}-free Tyrode's containing (in mM): 140 NaNO\textsubscript{3}, 5 KNO\textsubscript{3}, 1 Ca(NO\textsubscript{3})\textsubscript{2}, 10 HEPES 10 glucose and 10 Na\textsuperscript{+} pyruvate. The pH was adjusted with KOH. Cl\textsuperscript{-} Tyrode's at (10, 30, 60, and 90 mM) was made up by replacing NaNO\textsubscript{3} with NaCl at each concentration, respectively.

Fluorescence imaging of taste receptor cells was carried out on cells loaded with a Cl\textsuperscript{-} sensitive dye, \textit{N}-[(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE; Invitrogen, Eugene, OR). Single taste cells were isolated as described above and plated onto a Cell-Tak (BD Science) coated coverslip in a laminar flow perfusion chamber (RC-25F, Warner Instruments, Hamden, CT). Then, TRCs were loaded with 15 mM MQAE in hypotonic (220 mOsm) Cl\textsuperscript{-}-free Tyrode's for 45 min. Cells were then incubated in isotonic Cl\textsuperscript{-}-free Tyrode's for 45 min allowing taste cells to recover from the hypotonic shock. Changes in [Cl\textsubscript{i}].
were measured by MQAE quenching which is a qualitative tool for measuring concentration changes, not absolute Cl\textsuperscript{−} concentrations (Bachmann et al., 2003). Data collection and analyses were performed using an InCyt High Speed I/P\textsuperscript{TM} imaging system (Intracellular Imaging Inc, Cincinnati, OH). Briefly, images were acquired with an integrating CCD camera through a 40x objective lens mounted on an inverted Nikon TS-100 microscope. Excitation wavelength of 350 ± 10 nm was emitted by a Benthan FGS 150 fast switching monochromator (Intracellular Imaging Inc.) with an emission wavelength ~470 nm. Images were captured every 3 s using the InCyt Im1 software (Intracellular Imaging Inc). Results were graphed using F/F\textsubscript{0}; F= amount of MQAE fluorescence quenched by Cl\textsuperscript{−} ions. F\textsubscript{0} = amount of MQAE fluorescence in Cl\textsuperscript{−} free Tyrode, with the initial value normalized to 100%.

Results

Regulation of ENaC currents by intracellular chloride

To determine the effects of intracellular Cl\textsuperscript{−} concentration on Na\textsuperscript{+} movement through ENaC in mouse taste receptor cells we recorded from >60 taste receptor cells using whole cell patch clamp. In all cases, we recorded whole cell currents in response to a command of voltage ramp from -110 to +50 mV in the presence and absence of amiloride at different concentrations. Conservatively, we classified as an amiloride-sensitive cell only those cells that
showed a reversible >15% decrease in current measured at -80 mV (Gilbertson & Fontenot, 1998). In addition, amiloride sensitivity was evident as a decrease in the slope caused by an inhibition of ENaC (Fig. 2.1A). Initially, in order to determine the correlation between amiloride-sensitive Na⁺ transport and [Cl⁻], in taste receptor cells, we used either a high (140 mM) or a low (10 mM) intracellular cellular Cl⁻ solution. Amiloride-sensitive Na⁺ currents (ASSC) were significantly larger in low intracellular chloride (Fig. 2.1). This is an effect seen at all concentrations of amiloride (0.1, 0.5, 1 and 10 µM; Fig. 2.1B). Our data suggest a possible regulatory mechanism of ENaC by chloride ions since the amount of amiloride-sensitive Na⁺ transport was increased in low intracellular chloride (Fig. 2.1B).

The dose response curves for the effect of amiloride on Na⁺ currents in high and low chloride showed significant differences in the ability of amiloride to inhibit Na⁺ influx (Fig. 2.1C). Interestingly, amiloride was effective at much lower concentrations (0.01 and 0.05 µM) in taste cells recorded using low chloride intracellular (10 mM). Paired Student’s t-test statistical analysis revealed significant differences between high and low chloride at amiloride (0.1µM; p<0.05) and (1 µM; p<0.05). This analysis revealed that Na⁺ transport through ENaC was greater in taste cells containing low intracellular chloride. Taken together, the magnitude of ASSC in the peripheral gustatory system appears to be significantly dependent upon [Cl⁻].
Cl⁻-dependence of Na⁺ transport

As an independent verification of the effects we were seeing using patch clamp recording, we used transepithelial voltage clamp recording in intact mouse lingual epithelia containing fungiform taste buds to monitor Na⁺ movement. We measured $I_{SC}$ during the stimulation with mucosal NaNO₃ while perfusing of the serosal surface with, 60mM Cl⁻, 140mM Cl⁻ and nominally Cl⁻-free KH buffers. Stepwise increases in NaNO₃ concentration from 10 to 500 mM in the mucosal chamber caused increases in $I_{SC}$ in all epithelia tested in this study. In general, NaNO₃-induced $I_{SC}$ was larger in KH Cl⁻ free buffer, though there was no underlying difference between Na⁺-$I_{SC}$ changes in 60 mM and 140 mM Cl⁻ (Fig. 2.2).

The concentration-response curves for NaNO₃-induced changes in $I_{SC}$ indicated that sodium transport was greater in Cl⁻ free than 60 mM and 140 mM Cl⁻ (Fig. 2.3). One-way ANOVA revealed significant difference between $I_{SC}$ with serosal Cl⁻-free solutions ($p<0.05$). There was no significant difference among 60 mM and 140 mM Cl⁻. This analysis revealed that sodium transport was altered in fungiform-containing epithelia by chloride ions. Clearly, sodium (Na⁺) transport in the gustatory epithelia is dependent upon the concentration of Cl⁻ ions.

Expression of chloride channels in mice

Given our electrophysiological data, the next stop was to explore the expression of chloride transporting proteins in the gustatory system. We used
reverse transcription-polymerase chain reaction (RT-PCR) to determine the expression of chloride channels (ClC), Na⁺/K⁺/2Cl⁻ cotransporters (NKCC), and cystic fibrosis transmembrane regulator (CFTR) in mouse taste buds. We isolated RNA from each of the 3 lingual papillae and probed with specific primers for each of 8 subtypes of ClC, NKCCs and CFTR expression Table 2.1. PCR products representing ClC2, ClC3, ClC4, ClC5 and ClC7 messages could be found in fungiform, foliate, and circumvallate taste buds in a minimum of three independent experiments (Fig. 2.4). All PCR products were sequenced using a PE Biosystem 377 automated DNA sequencer, and mouse taste bud ClC2 showed 99% homology with the published sequence for mouse kidney ClC2, while mouse taste ClC3, ClC4, ClC5 and ClC7 were 100% homologous with these channel genes from mouse kidney. PCR products representing NKCC1 were found in all taste bud types (Fig. 2.4F). However, PCR products amplified using CFTR primers produced bands only in foliate and circumvallate taste buds (Fig. 2.4G). These results were verified in three separate sets of experiments. To further confirm their identity, PCR products representing NKCC1 and CFTR were sequenced, both were found to be 100% homologous with NKCC1 and CFTR from mouse lung. RT-PCR assays for ClC6, ClCKa, and ClCKb, produced no bands representing the appropriately sized PCR products and this was found consistently in any of all 3 lingual papilla, although bands in the positive control (e.g., kidney) were found (Figs. 2.4 H-J).
Chloride transport in mouse taste receptor cells

In the present study, we have shown a number of chloride transporters are expressed in mouse TRCs Table 2.2. Since our electrophysiological data showed a relationship between Na$^+$ currents through ENaC and [Cl$^-$], we next explored the functional role of Cl$^-$ transport in taste cells. Using MQAE, we measured changes [Cl$^-$] in taste cells from fungiform and circumvallate papillae. Taste cells loaded with the Cl$^-$ sensitive dye, MQAE, were analyzed for changes in fluorescence that occurs when intracellular chloride is replaced with NO$_3$. MQAE fluorescence is quenched by Cl$^-$ however, it is not affected by NO$_3$ (Hoffman & Geibel, 2005; Verkman, Weyer, Brown, & Ausiello, 1989). As shown in Figure 2.5, MQAE fluorescence intensity was rapidly quenched in fungiform taste cells by switching from a nominally Cl$^-$-free to a high Cl$^-$ (140 mM) solution. Overall, increases in [Cl$^-$] of extracellular solutions (30, 60, 90, and 140 mM) caused a concentration-dependent decrease in fluorescence intensity in all taste cells (Figs. 2.5 and 2.6). These changes in MQAE fluorescence were reversible as demonstrated by changes from high Cl$^-$ (90 mM) to nominally Cl$^-$-free solutions (Fig. 2.6).

Similar results were obtained in circumvallate cells in terms of MQAE fluorescence changes by [Cl$^-$]. Most circumvallate cells exhibited decreases in fluorescence intensity by stepwise increases of Cl$^-$ (90 and 140 mM) in the extracellular solution (Figs. 2.7 and 2.8). These fluorescence changes were reversible as extracellular Cl$^-$ was changed from high (140 mM) to nominally Cl$^-$-
free solutions (Fig. 2.8). Both fungiform and circumvallate cells showed decrease of MQAE fluorescence by application of extracellular Cl⁻ suggesting that there is functional chloride transport in taste cells.

The concentration-response curve for Cl⁻-induced changes of MQAE fluorescence showed a difference between fungiform and circumvallate at concentrations < 90 mM (Fig. 2.9). In all experiments, there was a trend indicating that Cl⁻ influx was greater in fungiform than circumvallate cells at low Cl⁻ (10, 30, and 60 mM) concentrations. Statistical analysis by paired Student's t-test comparing fungiform and circumvallate revealed significant differences between MQAE fluorescence with Cl⁻ (10 mM: p<0.01; 30 mM: p<0.01; 60 mM: p<0.01). In addition, there is no apparent difference in Cl⁻ movement between 30 mM and 60 mM [Cl⁻] in fungiform papillae.

Effects of cytosolic chloride on Na⁺ transport in taste cells

It has been suggested that in most transporting epithelia that actively secrete Cl⁻ ions, Cl⁻ is continuously accumulated across the basolateral membrane. Depending on the cytosolic chloride concentration, Cl⁻ ions either enter the cells and lead to hyperpolarization or leave the cells and depolarize it (Delpire, 2000; Xie & Schafer, 2004). Based upon our cell-based and molecular (RT-PCR) results, we examined the effect of Cl⁻ movement thorough ClC channels and NKCC1 on amiloride-sensitive Na⁺ movement via ENaC. Using
whole cell patch clamp recording, we recorded ASSC in the presence and absence of NPPB (10 µM, a general CIC channel blocker) and bumetanide (10 µM, NKCC1 blocker) in fungiform taste cells. Amiloride-sensitive responses were observed in 14 of 47 (29.8%) TRCs analyzed in this experiment. As shown in Figure 2.10, there was an increase in the magnitude of amiloride-sensitive Na⁺ current during treatment with chloride transport antagonists. Overall, the magnitude of amiloride sensitive currents (at -80 mV) was increased when both NKCC1 and CIC channels were blocked. In the course of these studies, it became apparent that there were three different types of responses found when using antagonists of Cl⁻ transport. One, most commonly, amiloride-sensitive Na⁺ currents were enhanced when Cl⁻ ion movement was impaired by chloride channels blockers (Fig. 2.10B). Two, in some cells, amiloride-sensitive currents were only recorded in the presence of chloride antagonists (Figs. 2.11A and 2.11B). Three, a subset of taste cells showed large ASSCs that were unaffected when NKCC1 and CIC channels were inhibited (Figs. 2.11C and D). Clearly, our findings are consistent with the idea that Na⁺ transport into taste cells via ENaC inhibition is inversely proportional to the cytoplasmic Cl⁻ concentration.

Discussion

The regulation of Na⁺ transport via ENaC is important for salt and water balance in various transporting epithelia. Because of the similarities between salt
and water transport in the taste and kidney epithelia, we initially focused on identifying ENaC regulatory mechanisms and how they may alter salt transport and, potentially, salt taste. In the present study, we have demonstrated a link between intracellular Cl⁻ and Na⁺ transport in taste cells. Our electrophysiological assays provided clear evidence that TRCs exhibit an enhancement of Na⁺ currents through the apical amiloride sensitive channels (i.e. ENaC) with low intracellular Cl⁻ (10 mM). In addition, the relative inhibition by amiloride was greater in low Cl⁻ (EC₅₀: 0.08 µM) than it was in high intracellular Cl⁻ (EC₅₀: 0.74 µM). Consistent with these data, we only observed amiloride inhibition of Na⁺ currents at 0.01 and 0.05 µM in recordings with low Cl⁻ (10 mM; Fig. 2.1). Similar to our results in mammalian taste cells, Avenet and Lindemann reported that amiloride-sensitive Na⁺ currents were decreased in frog taste cells by high intracellular Cl⁻ (Avenet & Lindemann, 1988). Clearly, in most amiloride-sensitive taste cells Na⁺ influx via ENaC is inhibited by high [Cl⁻]. Taken together, these increases in [Cl⁻], during activation of chloride channels might cause a decrease in Na⁺ conductance. Interestingly, all previous functional studies of ENaC in mammalian TRCs were conducted with high intracellular Cl⁻ (Doolin & Gilbertson, 1996; Gilbertson & Fontenot, 1998; Gilbertson, Roper, & Kinnamon, 1993), which may partially account for the relatively small currents recorded in these experiments.

Using voltage clamped lingual epithelia containing fungiform taste buds mounted in an Ussing chamber; we have recorded total Na⁺-dependent changes
in $I_{SC}$ by altering only the chloride [Cl\(^-\)] composition of the serosal side of the chamber through changes in NO\(_3\) concentration. In all epithelia, the magnitude of Na\(^+\) movement was considerably larger in nominally Cl\(^-\)-free solutions. There was a direct correlation between cytosolic [Cl\(^-\)] and Na\(^+\)-dependent $I_{SC}$ because modification of Cl\(^-\) concentration in the serosal side of the lingual epithelium decreased transepithelial Na\(^+\) movement (Fig. 2.2). In addition, there was no significant difference in Na\(^+\)-dependent $I_{SC}$ between 60 and 140 mM [Cl\(^-\)] (Fig. 2.3). These responses suggest that the magnitude of Na\(^+\)-dependent $I_{SC}$ is relatively constant at [Cl\(^-\)] above 60 mM. Similar results have been shown in mouse mandibular glands in which inward Na\(^+\) current was decreased by stepwise increases of intracellular Cl\(^-\) in the recording pipette and the half-maximal inhibitory effect of intracellular [Cl\(^-\)] was determined to be approximately 50 mM (Dinudom, Young, & Cook, 1993). These results are consistent with the interpretation that chloride transporting proteins are involved in sodium movement in mouse taste cells. Since Cl\(^-\) channels are located in the basolateral membrane of the kidney epithelia and participate in NaCl reabsorption (Veizis & Cotton, 2007), and taste cells express a similar complement of Cl\(^-\) transport proteins, we hypothesized that TRCs are able to transport Cl\(^-\) ions which, in turn, alters Na\(^+\) movement through ENaC.

The importance of chloride channels and cotransporters is well established in transporting epithelia. Under physiological conditions, extracellular isoosmolarity is required to maintain appropriate cellular volume. Most cells when
exposed to a non-isosmotic stimulus have mechanisms to compensate for the osmotic difference and increase or decrease their cell volume accordingly. Cell volume perturbations can rapidly increase or decrease $[\text{Cl}^-]_i$. These acute responses are achieved by changes in the balance between Cl$^-$ influx and efflux (Devuyst & Guggino, 2002). For instance, hypotonic stimuli cause initial cell swelling followed by regulatory volume decrease (RVD) to attempt to restore the cell to its original volume. This response is mediated by volume-sensitive K$^+$ and Cl$^-$ channels and K-Cl cotransporters (KCCs) leading to a substantial decrease in the intracellular $[\text{Cl}^-]_i$. Thus, KCCs are also responsible for electroneutral potassium and chloride transport during RVD. This large reduction in cytosolic Cl$^-$ leads to an inactivation and modification of gene expression of chloride cotransporters (i.e. NKCC1) to maintain the chloride electrochemical equilibrium (Kahle et al., 2008; Niisato et al., 2004). Furthermore, the oral cavity is exposed to a variety of solution tonicities, ranging from extremely hyposmotic (distilled water) to high-salt solutions that may be an order of magnitude greater than normal salivary ion concentrations (Feldman & Barnett, 1995). It has been established that taste receptors cells change size and function in response to different osmotic stimuli (Gilbertson, 2002; Lyall, Heck, DeSimone, & Feldman, 1999). Presently, much less is known about the expression of these chloride transporting proteins in the gustatory system.

In the present study, we looked at the expression of chloride transporting proteins in mammalian taste cells. Using RT-PCR, we demonstrated the
presence of ClC2, ClC3, ClC4, ClC5, ClC7, and NKCC1 in all three lingual papillae. Interestingly, we only observed CFTR channel expression in the posterior mouse tongue (Fig. 2.4; Table. 2.2). Base upon our electrophysiological experiments, we hypothesized that these channels provide a molecular route for Cl ions in TRCs. Next, we attempted to investigate the functional role of these proteins in taste cells. We assessed Cl movement and changes in [Cl]i in taste cells using functional imaging with a Cl sensitive dye (MQAE). In fungiform cells, Cl-dependent decreases in MQAE fluorescence were consistent with the interpretation that chloride transport proteins expressed in the plasma membrane are capable of transporting Cl ions across the membrane, leading to increases in cytosolic [Cl] (Figs. 2.5 and 2.7). The amount MQAE quenching was relative to the extracellular Cl which suggests the ability of taste cells to equilibrate to a new stable [Cl] based upon the extracellular environment (Figs. 2.6 and 2.8). Dose response curves between fungiform and circumvallate cells revealed significance difference in Cl transport at 10 mM, 30 mM, and 60 mM (Fig. 2.9).

Our functional assays and molecular biological experiments give insight into specific interactions between ENaC and Cl transporting proteins in the gustatory system. We hypothesized that these Cl transporting proteins might contribute to the mechanisms of salt taste transduction. The present study elucidated the ability of bumetanide and NPPB, effective blockers of NKCC and CIC channels respectively, to enhance the magnitude of amiloride-sensitive currents in taste cells (Fig. 2.10). Based upon both of our previous studies
(Gilbertson, 2002; Watson et al., 2007) and the identification of several ClC channels in the present work, we revealed that uptake and secretion of Cl− in the taste cells may play a regulatory role in salt taste and, ultimately, salt intake.

In conclusion, we have demonstrated a mechanism of ENaC regulation by Cl− ions in taste cells. Thus, ENaC activity can be altered by changes in [Cl−]. Clearly, our results provide important information for the understanding of salt taste transduction. However, it will be interesting to evaluate the potential interaction between ENaC and chloride transporting proteins in both hypoosmotic and hyperosmotic responses in the gustatory system.

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<td>160-178 567-587</td>
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<tr>
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<td>NM_011930</td>
<td>5’ ACG AAT CAA CCA CAC GGC TT 3’  5’ CAG AAT CAC GCC GGA TAC CT 3’</td>
<td>414-434 766-786</td>
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<tr>
<td>ClC-Ka</td>
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<td>866-885 1034-1052</td>
</tr>
<tr>
<td>ClC-Kb</td>
<td>NM_019701</td>
<td>5’ CTC CGT CTG GGA TTA CTG GA 3’  5’ CAT CAG TGC CCA GGA GTT GT 3’</td>
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<tr>
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<tr>
<td>NKCC1</td>
<td>NM_009194</td>
<td>5’ GCC CAG GAT CGC CCA CTA 3’  5’ CAT GCA GCG GAC TAA TAC AC 3’</td>
<td>771-789 976-996</td>
</tr>
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TABLE 2.2. Expression of chloride channels in fungiform, foliate, and circumvallate taste buds.

<table>
<thead>
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<th>Channel</th>
<th>Fungiform</th>
<th>Foliate</th>
<th>Circumvallate</th>
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<td>ClC-2</td>
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<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>ClC-3</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
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<td>✓</td>
<td>✓</td>
</tr>
<tr>
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<td>✓</td>
<td>✓</td>
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</tr>
<tr>
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<td>✗</td>
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<td>ClC-7</td>
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<td>✓</td>
<td>✓</td>
</tr>
<tr>
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<td>✗</td>
<td>✗</td>
</tr>
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<td>ClC-Kb</td>
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<td>✗</td>
</tr>
<tr>
<td>CFTR</td>
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<td>✓</td>
</tr>
<tr>
<td>NKCC1</td>
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</tbody>
</table>

✓ RT-PCR indicated positive expression of chloride channel.

✗ Not expressed in taste buds by RT-PCR
Fig. 2.1. Effects of intracellular Cl⁻ on amiloride-sensitive taste cells. (A), current-voltage curve for a mouse taste cell in whole cell patch clamp recording during the application of amiloride (0.1 µM) using a low Intracellular Cl⁻ solution (10 mM). (B), bar graph showing the magnitude of amiloride-sensitive Na⁺ currents with either high (140 mM) or low (10 mM) intracellular Cl⁻ concentrations at different amiloride concentrations (0.1, 0.5, 1, and 10 µM; data are mean ± SD; n=5).
**Fig. 2.1 continued.** (C), amiloride dose-response curves in high (140 mM) or low (10 mM) chloride. Amiloride was applied by bath perfusion at 0.01, 0.05, 0.1, 0.5, 1 and Solid lines are the best fit with a logistic relation with EC$_{50}$'s of 0.74 µM for high Cl$^-$ and 0.08 µM for low Cl$^-$. Each amiloride concentration was compared using an independent Student’s t-test ($p<0.05$).
**Fig. 2.2.** Effects of serosal chloride concentration on short-circuit currents ($I_{SC}$) induced by 500 mM NaNO$_3$. Fungiform-containing mouse epithelia, like this one, showed changes in Na$^+$-$I_{SC}$ by stepwise increases in serosal KH buffer Cl$^-$ concentrations (in mM) in the absence or presence of 500 mM NaNO$_3$ in the mucosal chamber. Initial mucosal solution was 10 mM NaNO$_3$ and KH Cl$^-$ free buffer was in the serosal chamber. Increases in $I_{SC}$ are reflected upward and transient deflections are current responses to brief 10 mV hyperpolarizations of the epithelia to monitor transepithelial resistance. (Inset) representation of an isolated lingual epithelia containing fungiform papilla mounted into a bipartitioned Ussing chamber.
Fig. 2.3. Comparison of steady-state sodium transport in the gustatory epithelia of the mouse with different chloride concentration in the serosal chamber. Values of relative $I_{SC}$ (normalized to 500 mM NaCl/0 [Cl$^-$]) are shown ± SEM and each point represents 7 individual epithelia. Curves are best fits using standard concentration-response relationship.
Fig. 2.4. RT-PCR reveals the presence of several CIC channels, NKCC1 and CFTR in mRNA from the 3 types of taste buds. Primers for CIC-2 (A), CIC-3 (B), CIC-4 (C), CIC-5 (D), CIC-7 (E), NKCC1 (F) and CFTR (G) amplify stained PCR products of expected sizes (ClC-2 321 bp; ClC-3 384 bp; ClC-4 486 bp; ClC-5 606 bp; ClC-7 187 bp; NKCC1 225 bp; CFTR 367 bp), but ClC-6 (H), CIC-Ka (I), CIC-Kb (J) in most cases were negative (expected product: ClC-6 428 bp; ClC-
Ka 418 bp; ClC-Kb 373bp). Positive controls (mouse kidney or lung RNA) are shown for ClC-2, ClC-3, ClC-4, ClC-5, ClC-7, NKCC1, CFTR, ClC-6, ClC-Ka, and ClC-Kb with each set of primers. Negative control (-) lanes represent those in which cDNA was omitted from the PCR reaction.
**Fig. 2.5.** Fluorescence imaging with the dye, MQAE, of Cl⁻ in fungiform taste receptor cells. The changes in the distribution of intracellular chloride are evident upon changes in extracellular chloride. Fungiform cells showed high fluorescence intensity in Cl⁻ free solution however upon exchange of extracellular chloride concentration, the relative fluorescence of the cytoplasm is markedly decreased due to Cl⁻ entry into the cell. All solutions in the experiment were the same, with equal osmolalities, except for the change of anions. The changes in fluorescence were stable for at least 3 min. It should be emphasized that the fluorescence of the dye is decreased (quenched) by Cl⁻. Arrows denotes the same fungiform taste receptor cell during the functional imaging recording.
Fig. 2.6. Changes in fluorescence intensity induced by increases in extracellular chloride concentration (in mM) in MQAE preloaded fungiform taste cells. Fungiform taste cells showed a decrease of the relative fluorescence due to Cl⁻ ions entry into the taste cells. In addition, fluorescence changes were reversible as extracellular chloride concentration was reduced to 0. ▼, Tyrode's solution was replaced with a different Cl⁻ concentration.
Fig. 2.7. Fluorescence imaging with the dye, MQAE, of Cl⁻ in circumvallate taste receptor cells. Circumvallate taste cells, like this one, showed relative decreases in cytoplasmatic fluorescence intensity as extracellular chloride concentration is increased. Other details are identical to those given in Fig. 2.5. Arrows denote the same circumvallate taste receptor cell during the same experiment.
Fig. 2.8. Changes in fluorescence intensity induced by increases in extracellular chloride concentration (in mM) in MQAE preloaded circumvallate taste cells. Other details are identical to those given in Fig. 2.7.
Fig. 2.9. Concentration-response curve for Cl⁻-induced changes in MQAE fluorescence intensity in fungiform and circumvallate taste cells. Data are shown mean ± SEM. Curves are the best fits with a logistic relation. Asterisks denote significant difference in Cl⁻ influx between circumvallate and fungiform taste cells (p< 0.01, Simple Effects ANOVA).
Fig. 2.10. Effects of chloride transport inhibitors on amiloride-sensitive cells. (A), Current-voltage curves for a mouse taste cell using whole cell patch clamp recording during application of amiloride (1 µM). (B), I-V curves from the same mouse taste cell in the presence of a Cl⁻ channel blocker (NPPB, 10 µM) and an inhibitor of NKCC1, bumetanide (10 µM) during application of amiloride (1 µM). Taste cells, like this one, show an increase in amiloride-sensitive Na⁺ channel activity by blocking chloride transport. Tyrodes minus amiloride (Tyrodes-amiloride 1 µM) trace reflects the net current elicited by the amiloride-sensitive Na⁺ channel. (C), Magnitude of amiloride-sensitive Na⁺ current before and after the treatment with chloride antagonists: bumetanide (10 µM) & NPPB (10 µM) at a single concentration of amiloride (1 µM). Each bar represents the (mean ± SEM) from 8 cells.
Fig. 2.11. Taste cells can be characterized by differences in amiloride-sensitive Na$^+$ currents. (A), I-V curves from a mouse taste cell during the application of amiloride (1 µM). There is no evidence of amiloride-sensitive Na$^+$ channel activity. (B), the same cell in (A) showing amiloride-sensitive Na$^+$ currents after treatment with chloride antagonist (bumetanide 10µM & NPPB 10µM). Amiloride-sensitive currents in the absence (C) or presence (D) of chloride transport inhibitors. Taste cells, like this type, show no changes in the amiloride-sensitive Na$^+$ channel activity.
CHAPTER 3
INSULIN ACTIVATES EPITHELIAL SODIUM CHANNELS VIA PHOSPHOINOSITIDE 3-KINASE IN MAMMALIAN TASTE RECEPTOR CELLS²

Abstract

Diabetes is a profound disease that results in a severe lack of regulation of systemic salt and water balance. From our earlier work on the endocrine regulation of salt taste at the level of the epithelial sodium channel (ENaC), we have begun to investigate the ability of insulin to alter ENaC function with patch clamp recording on isolated mouse taste cells (TRCs). In fungiform and vallate TRCs that exhibit functional ENaC currents (e.g. amiloride- or benzamil-sensitive Na⁺ influx), insulin (5-20 nM) caused a significant increase in Na⁺ influx at -80 mV (EC₅₀ = 6.1 nM). The insulin-enhanced currents were inhibited by amiloride (30 µM). Similarly, in ratiometric Na⁺ imaging using the Na-sensitive dye SBFI, increasing extracellular Na⁺ from 0 to 140 mM elicited an increase in Na⁺ influx in a subset of TRCs. Insulin treatment (20 nM) enhanced Na⁺ movement in TRCs consistent with its action in electrophysiological assays.

² Coauthored by Arian F. Baquero and Timothy A. Gilbertson.
The ability of insulin to regulate ENaC function is dependent upon the enzyme PI3-kinase since treatment with the inhibitor LY294002 (10 µM) abolished insulin-induced changes in ENaC currents. To test the role of insulin in the regulation of salt taste, we have characterized behavioral responses to NaCl using a mouse model of acute hyperinsulinemia. Insulin-treated mice show significant avoidance for NaCl at lower concentrations than the control group using short-term taste assays. Interestingly, these differences between groups were abolished when amiloride (100 µM) was added into NaCl solutions, suggesting that insulin was regulating ENaC. Our results are consistent with a role for insulin in maintaining functional expression of ENaC in mouse TRCs and may be an example of the ability of the gustatory system to respond to nutritional challenges.

Introduction

Taste receptor cells (TRC) must recognize a vast array of different chemical structures, ranging from those that are small and ionic to compounds with complex tertiary structures. This chemosensory ability allow animals to distinguish compounds may be either harmful to the organism or, alternatively, necessary for nutritional needs. In mammals, it is well established that salt taste transduction is primarily mediated by sodium (Na\(^+\)) ions through the apical amiloride-sensitive epithelial sodium channel (ENaC). The influx of Na\(^+\) ions directly depolarizes the taste cell, eventually leading to the release of neurotransmitter onto the afferent nerve fibers (Gilbertson, Damak, &
ENaC has been characterized both in TRCs and other transporting epithelia and it shares a number of common features across tissue types. Similarities include small conductance (~5 pS), Na\(^+\)~ Li\(^+\) >> K\(^+\) ion selectivity, regulation by extracellular Na\(^+\) (self-inhibition) and intracellular Na\(^+\) (feedback inhibition) and regulation by hormones. These functional similarities between ENaC in the taste system and other transporting epithelia extend to the molecular level. Experiments in different rodent species have shown that ENaC channels expressed in TRCs has a high sequence homology with those channels expressed in other organs. Thus, in many regards, ENaC appears similar across organ types (Gilbertson & Fontenot, 1998; Gilbertson & Zhang, 1998; Kretz, Barbry, Bock, & Lindemann, 1999; Lindemann, Barbry, Kretz, & Bock, 1998; Shigemura et al., 2008).

The regulation of ENaC is essential for salt and water balance in various Na\(^+\) transporting epithelia. Current evidence in taste cells and other transporting epithelia has demonstrated that ENaC expression and/or function may be altered by a number of hormones (e.g. aldosterone, vasopressin, atrial natriuretic peptide). Aldosterone (ALDO) and arginine\(^8\)-vasopressin (AVP) appear to increase ENaC mediated currents, while atrial natriuretic peptide and perhaps oxytocin, reduces these Na\(^+\) currents. Hormones like ALDO and AVP can increase Na\(^+\) influx through ENaC by increasing the channel’s open probability and/or the decreasing the turnover of ENaC in the cell membrane increasing functional ENaC expression (Gilbertson, Roper, & Kinnamon, 1993; Herness,
Additionally, the importance of ENaC regulation is well understood for sodium balance, blood pressure, and extracellular fluid volume (Vallon, Blantz, & Thomson, 2005).

Recently, research in kidney epithelia has suggested that insulin contributes to Na\(^+\) movement via ENaC. In general, both ENaC open probability and membrane expression of ENaC in the cell membrane can be increased by insulin stimulation acting through insulin/IGF-1 receptors. Insulin-mediated Na\(^+\) reabsorption is believed to occur by the activation of phosphoinositide 3-kinase OH (PI3-kinase) and its phospholipids products (Record, Froelich, Vlahos, & Blazer-Yost, 1998; Tong, Gamper, Medina, Shapiro, & Stockand, 2004). The relevance of PI3-kinase signaling pathway is extended to other tissues such as insulin effects on cell volume in the liver which are mediated by PI3-kinase and related molecules (Schliess & Haussinger, 2003). Additionally, insulin-stimulated Na\(^+\) reabsorption shares a number of similarities with ENaC activation by other hormones (Tong, Booth, Worrell, & Stockand, 2004).

Activation of PI3-kinase by insulin leads to phosphorylation of two downstream signaling cascades. (1) PI3-kinase signaling pathway leads to the phosphorylation of ENaC and/or (2) activation of phosphoinositide-dependent kinase 1 (PDK1). Thus, synthesis of PtdIns(3,4)P\(_2\)/PtdIns(3,4,5)P\(_3\) by PI3-kinase directly affects ENaC activity and open probability (Kunzelmann et al., 2005; Staruschenko, Pochynyuk, Vandewalle, Bugaj, & Stockand, 2007; Tong et al.,
In addition, PtdIns(3,4,5)P$_3$ phosphorylates PDK1 and then activates serum- and glucocorticoid-regulated kinase-1 (SGK1). SGK1 is considered a key regulator of Na$^+$ reabsorption via ENaC in kidney epithelia since several hormones such as insulin, ALDO and AVP regulate ENaC through this pathway (Faletti, Perrotti, Taylor, & Blazer-Yost, 2002; Vallon et al., 2005). SGK1 function may increase either the open probability of apical ENaCs or the number of active channels in the membrane, although the mechanism of ENaC trafficking by insulin-SGK1 mediated responses is not well understood (Hills, Squires, & Bland, 2008; Lee, Dinudom, Sanchez-Perez, Kumar, & Cook, 2007; Loffing, Flores, & Staub, 2006). SGK is critical for insulin action since SGK$^{-/-}$ transgenic mice showed alterations in insulin sensitivity with a high salt diet (Boini et al., 2006).

PtdIns(4,5)P$_2$ is phosphorylated by PI3-kinase to generate PtdIns(3,4)P$_2$/PtdIns(3,4,5)P$_3$. PtdIns(4,5)P$_2$, on the other hand, is implicated in the regulation of a number of ion channel including inward rectifying K$^+$ channels (Zhang, He, Yan, Mirshahi, & Logothetis, 1999), TRMP8 (Rohacs, Lopes, Michailidis, & Logothetis, 2005) and voltage-gated Ca$^{2+}$ channels (Wu, Bauer, Zhen, Xie, & Yang, 2002). In addition, ENaC $\beta$ and $\gamma$ subunits contain specific binding sites for PtdIns(4,5)P$_2$ in the N terminus (Pochynyuk, Tong, Staruschenko, & Stockand, 2007). Electrophysiological experiments in kidney A6 and M1 collecting duct cells have suggested that PtdIns(4,5)P$_2$ levels are critical for normal ENaC activity; since an increase in PtdIns(4,5)P$_2$ membrane levels will dramatically increase
ENaC open probability. In contrast, ENaC function is decreased when PtdIns(4,5)P$_2$ levels are depleted (Kunzelmann et al., 2005; Ma, Saxena, & Warnock, 2002; Tong & Stockand, 2005).

Little is known about the importance of insulin in the peripheral gustatory system. In the present study, we use a combination of whole cell patch clamp recording and ratiometric functional Na$^+$ imaging to determine whether insulin influences Na$^+$ transport via ENaC in isolated taste cells from the fungiform and circumvallate papillae. Our results showed that insulin stimulates Na$^+$ currents through amiloride-sensitive channels (i.e. ENaC) in these cell-based assays. In agreement with earlier reports in other transporting epithelia, insulin-ENaC-mediated responses in the peripheral gustatory system occurred via PI3-kinase-mediated pathways and its phospholipid products (Staruschenko et al., 2007; Wang et al., 2008). We next examined the physiological role of PI4-kinase, which is essential for PtdIns(4,5)P$_2$ synthesis, in the taste system. We found that PI4-kinase related products such as PtdIns(3,4)P$_2$ and PtdIns(4,5)P$_2$ are vital for insulin/ENaC-mediated responses. Moreover, using SGK$^{-/-}$ null mice we showed that SGK1 plays a significant role in ENaC activation by insulin. These findings support the interpretation that insulin stimulates amiloride-mediated Na$^+$ transport in taste cells.

To identify the molecular underpinnings of these cellular responses, we used reverse transcription polymerase chain reaction (RT-PCR) to identify components of this signaling pathway including the insulin receptor (IR), insulin
receptor substrate 1 (IRS1) and insulin receptor substrate 2 (IRS2) in taste cells, Consistent with other salt- and water- transporting epithelia, taste cells express IR, IRS1 and IRS2, similar to those found in kidney and liver. Following our functional and molecular studies we attempted to investigate the role of insulin in the regulation of salt taste. Using a mouse model of acute hyperinsulinemia we characterized behavioral responses to NaCl. Our results showed that insulin-treated mice have a greater avoidance of NaCl than the control group. The results showed a novel insulin pathway which regulates salt intake and ENaC activity in the peripheral gustatory system.

Methods

All experiments were performed on adult (2–4 months) male C57BL/6 mice that were maintained on a 12 h light/12 h dark cycle with normal chow and water provided ad libitum. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Utah State University and were performed in accordance with American Veterinary Medical Association guidelines.

Isolation of taste receptor cells

Individual taste buds were isolated from mouse tongues following protocols used in early reports (Béhé, DeSimone, Avenet, & Lindemann, 1990; Doolin & Gilbertson, 1996). Briefly, tongues were removed and immediately
immersed in Tyrode's solution containing 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM N-2-hydroxyethylpiperazine- N’-2-ethanesulphonic acid (HEPES), 10 mM glucose, and 10 mM Na⁺ pyruvate (osmolarity; 310 mOsm). The pH was adjusted to 7.4 with NaOH. The anterior portion of the tongue containing the fungiform papilla was injected between the muscle layer and the lingual epithelium with approximately 0.15 ml of physiological saline (Tyrode's) containing a mixture of collagenase A (1.1 mg/ml; Roche Applied Science, Indianapolis, IN), dispase II (2.4 mg/ml; Roche Applied Science), and trypsin inhibitor (1 mg/ml; type I-S; Sigma Chemical Corp., St Louis, MO). Between 0.1ml of the same enzyme solution was also used to inject the area surrounding the circumvallate papilla. The injected tongue was incubated in Tyrode's solution and bubbled O₂ for 45 min. The lingual epithelium was then peeled from the underlying tissue and pinned out in Tyrode's in a Sylgard-lined petri dish with the mucosal side facing down. The epithelium was then incubated for 7 min with the same enzyme cocktail. Following the incubation, the epithelium was incubated in a Ca²⁺-Mg²⁺– free Tyrode's containing 2 mM BAPTA (Invitrogen, Eugene, OR) in place of CaCl₂ and MgCl₂ for 5 min. Amiloride (10 µM Sigma Chemical Corp.) was added to all solutions to help protect against enzymatic degradation of epithelial sodium channels (ENaC) (Gilbertson & Fontenot, 1998). Taste buds were removed from the epithelium using a large bore (~150-200 µm) pipette and plated either onto a charged microscope slide in a Tyrode-containing Sylgard ring for patch clamp recording or onto a Cell-Tak-coated coverslip in a laminar flow
perfusion chamber for functional imaging. For isolation of single taste cells, this procedure was slightly modified by an additional incubation in Ca\textsuperscript{2+}-Mg\textsuperscript{2+}- free Tyrode's for 10 min before the second enzyme treatment.

Patch clamp solutions and recording conditions

Extracellular saline solution (Tyrode's) containing (in mM): 140 NaCl, 5 KCl, 1 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 HEPES, 10 glucose, and 10 Na\textsuperscript{+} pyruvate (osmolarity; 310 mOsm). The pH was adjusted to 7.40 with NaOH. Na\textsuperscript{+}-free solution was made by replacing NaCl with equimolar N-methyl-D-glucamine (NMDG; a large impermeant cation) in Tyrode's solution. Amiloride (30 μM) solutions were made up in normal Tyrode. For insulin concentrations (1, 5, 10 and 20 nM), insulin from porcine pancreas (Sigma) was dissolved in ddH\textsubscript{2}O at a concentration of 10 mM and then diluted to each concentration, respectively. To establish conditions whereby Na\textsuperscript{+} was the only ion contributing to the whole cell current through ENaC, cells were held at -80 mV and intracellular and extracellular solutions were used that set $E_{Cl}$ and $E_K$ at ~-80 mV. In this experiment, recording pipettes were filled with a solution containing (in mM): 140 K-gluconate; 1 CaCl\textsubscript{2}; 2 MgCl\textsubscript{2}; 10 HEPES; 11 EGTA; and 3 ATP. The pH and osmolarity were adjusted to 7.2 and ~310 mOsm, respectively, with KOH. This low intracellular Cl\textsuperscript{-} (10 mM) helped to eliminate most of the inward Cl\textsuperscript{-} current, which facilitates the analysis of insulin activated ENaC current and set the chloride equilibrium potential ($E_{Cl}$) near ~-80 mV.
Individual fungiform TRCs were recorded using conventional whole cell patch clamp. Patch pipettes were pulled to a resistance of 5–8 MΩ when filled with intracellular solution. Series resistance and capacitance were compensated optimally before recording. Currents were recorded in the presence and absence of test solutions in a continuous (gap-free) recording mode. The holding potential in all experiments were -80 mV. Current data were recorded and command potentials delivered using pClamp software (v.10). This software was interfaced with an AxoPatch 200B amplifier and Digidata 1322A data acquisition system (Molecular Devices, Sunnyvale, CA). Current-time relationships (I-T curve) were used to determine whether test solutions significantly altered amiloride-sensitive currents in TRCs.

Functional Sodium (Na⁺) Imaging

Functional imaging of taste receptor cells was carried out on cells loaded with a Na⁺ sensitive dye, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (SBFI-AM; Invitrogen, Eugene, OR). Single taste cells were isolated as described above and plated onto laminar flow perfusion chamber (RC-25F Warner Instruments, Hamden, CT). TRCs were loaded with ~4 μM SBFI-AM (Invitrogen) in Hanks' buffer salt solution with HEPES, sodium pyruvate, 1% pluronic acid F-127 (Invitrogen) and 2% fetal bovine serum (FBS) for 60 min. The cells were perfused with Na⁺-free Tyrode's (in mM): 140 NMDG, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, 10 glucose, and 10 Na⁺ pyruvate, adjusted to pH 7.4 with HCl.
Increases in intracellular Na$^+$ were recorded in Tyrode's solution, with and without insulin 20 nM and/or amiloride 30 μM. PI3-kinase inhibitors LY294002 (10 μM) and its inactivate analog LY303511 (10 μM) were prepared from a stock solution of 30 mM (CalBiochem San Diego, CA). Wortmannin (0.05 and 1μM) was prepared from a 2 mM stock solution (Sigma). All inhibitors were diluted in Tyrode's solution and made fresh daily before use. Data collection and analyses were recorded by InCyt High Speed I/M$^{TM}$ imaging system (Intracellular Imaging Inc., Cincinnati, OH). Briefly, images were acquired with a monochrome integrating CCD camera through a 40x oil-immersion objective lens of an inverted Nikon TE-2000s microscope. Excitation wavelengths of 340 nm and 380 nm were emitted by a Benthan FGS 150 fast changing monochromator (Intracellular Imaging Inc.) with an emission wavelength ~510 nm. Images obtained were captured every 3 s by InCyt Im2 software (Intracellular Imaging Inc). The SBFI ratio (340/380) was used to determine whether test solutions significantly altered Na$^+$ influx on TRCs. Data analyses were carried out by establishing the area under the curve of SBFI ratio in the presence and/or absence of both amiloride and/or insulin using Origin software (Ver. 7 Northampton, MA).

RT-PCR

First-strand cDNA was synthesized using the iScript RT Kit (Bio-rad, Hercules, CA). The maximum volume of taste RNA or 50 ng of kidney RNA was
used for the reaction with the total volume being 20 µl. Reactions were also set up in which the reverse transcriptase enzyme was omitted as a control to detect genomic DNA contamination. After first-strand synthesis, 1 µl of cDNA was added to a PCR reaction mixture containing final concentrations of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM Mg²⁺, 200 µM dNTPs, ~500 nM forward and reverse primers and 1.25 U Taq polymerase. PCR products were amplified using an initial 5 min denaturation step followed by 40 cycles of a 3-step PCR (30 sec denaturation at 95°C, 30 sec annealing at optimal temperature & 45 sec extension at 72°C), and concluding with a 7 min final extension step. Amplified sequences were visualized by electrophoresis in 2% agarose gels using 1X TAE buffer (40 mM tris-acetate & 1 mM EDTA). Primer sequences, accession numbers, expected product sizes and corresponding nucleotide sequences are shown in Table 3.1. Purification of PCR products for sequencing was performed using the QIAquick PCR purification kit (Qiagen, Valencia, CA). Sequences were determined by the dye-terminator method using an ABI Model 3100 Automatic Sequencer (Foster City, CA). Partial sequences for each product were examined using the BLAST 2.0 search engine (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Western blotting

To verify the presence of protein kinase B PKB/Akt in taste tissue with corresponding molecular weight, taste tissue containing circumvallate papillae
collected from four adult mice and non-taste lingual tissue were homogenized in a buffer containing 50 mM KCl, 1% NP-40, 25 mM HEPES-pH 7.8, 10 µg/ml leupeptin, 20 µg/ml aprotonin, 125 µM dithiothreitol (DTT), 1 mM phenylmethysulphonyl fluoride (PMSF) and 1 mM orthovanadate. Homogenates were sonicated, then centrifuged at 4°C, 15,000 rpm for 30 min to obtain total protein. 50 µg of total protein from each sample was separated on 10% SDS-PAGE and electrophoretically transferred to polyvinylidene fluoride (PVDF, Pierce, Rockford, IL) membrane for 3 hours at 80 V using transfer buffer (24 mM TRIS, 194 mM glycine and 20% methanol). The membrane was blocked for 2 hour at room temperature with 5% milk buffer (5% nonfat milk TRIS buffered saline with Tween20) then incubated overnight at 4°C with phospho-specific primary antibody in 1% milk buffer (1% nonfat milk in TBS-T). After washing 4 times with TBS-T, the membrane was incubated with the appropriate secondary antibody for 1 h at room temperature and incubated with ECL detection kit (Pierce) and exposed to X-ray film for 20 Sec. Antibody for pPKB/Akt was obtained from Cell Signaling (Danvers, MA) and β-actin antibody (ab6276) used as control was purchased from Abcam (Cambridge, UK).

Animal model of acute hyperinsulinemia

Male, 2-4 month old C57BL/6 mice from Charles River Laboratories (Wilmington, MA) were administered either vehicle or insulin (Novolin; Novo Nordisk Inc Princeton, NJ) at 0.75 U/kg of body weight in sterile 0.9% NaCl as a
single intraperitoneal injection. Both insulin-treated (n=24) or vehicle treated (n=24) mice received an injection 15 minutes prior to the behavioral testing. At the end of the behavioral assay blood glucose (mg/dl) was measured with a glucometer (BD Bioscience San Diego, CA) using blood obtained from the saphenous vein using techniques previously described (Hem, Smith, & Solberg, 1998).

Behavioral experiments

*Lickometer training*

All behavioral experiments were performed using a computer-controlled stimulus delivery and a lick monitoring station ("Davis Rig"; model MS-180, Dilog instruments, Tallahassee, FL). Mice were trained and tested under overnight water access restriction. Training consisted of 2 days: First, the mice were allowed to one presentation of distilled water for 15 minutes and a 15 minute time limit to first lick. The second day of training consisted of thirty 10-15 second presentations of distilled water with a 12 second intertrial interval and 150 second time limit to first lick.

*Testing procedure*

Following the training phase, mice were subjected to behavioral testing for three consecutive days. Each day all mice received either an insulin or vehicle injection 15 minutes prior to the trial. All experiments began at the same time.
every morning and each mouse from either insulin-treated or vehicle-injected
group was presented with two 5 second presentations of each concentration in
an ascending order from the lowest to the highest concentration followed by a 2
second rinse with distilled water. The intertrial interval was 10 seconds with a 150
second time limit for the first lick. Seven NaCl concentrations (30, 150, 270, 330,
450, 600, and 1000 mM) were tested to determine relative preference for NaCl
solutions using lick rates as the dependent variable. For experiments using orally
administered amiloride. A 10 mM amiloride stock was prepared in ddH₂O, 100
µM amiloride was made by dilution in each of the seven NaCl concentrations
including ddH₂O used for water stimulus and rinses. All NaCl solutions were
prepared fresh every day before testing. At the end of the behavioral
experiments, concentration-response curves plotting lick rate versus stimulus
concentration were generated and analyzed between test and control mice.

Results

Insulin increases amiloride-sensitive Na⁺
currents in mouse taste cells

To determine whether insulin stimulates sodium (Na⁺) transport in the
gustatory system, we recorded insulin effects in Na⁺ transport in taste cells using
conventional whole cell patch clamp recording. In all experiments cells were held
at -80 mV in a continuous (gap-free) recording mode with the pipette containing
140 mM K-gluconate and Tyrode's as the extracellular solution. This intracellular
solution helped to eliminate any inward Cl⁻ or K⁺ current since the ECl and Ek were approximately -80 mV. In most TRCs, insulin increased inward Na⁺ currents, consistent with previous studies in other transporting epithelia that showed the ability of insulin to enhance Na⁺ movement is through the activation of ENaC (Record et al., 1998; Tiwari, Riazi, & Ecelbarger, 2007). To determine if the insulin-enhanced responses we recorded were attributable to ENaC, we recorded insulin-induced responses in the presence or absence of amiloride (30 µM; an antagonist of ENaC). As Fig. 3.1 shows insulin dramatically increases inward Na⁺ current in taste cells and the enhancing effects of insulin were sensitive to amiloride suggesting that insulin activates ENaC in the taste system. In contrast, insulin had no effect on taste cell conductance when Na⁺ was replaced with NMDG (Fig. 3.1).

To investigate whether insulin-stimulation of inward Na⁺ currents occurred in a concentration dependent manner, we characterized insulin responses in TRCs using whole cell patch clamp. In all fungiform cells, the enhancement of inward Na⁺ currents correlated with insulin concentration (Fig. 3.2). The concentration-dependent changes in Na⁺ transport illustrated that the magnitude of Na⁺ current depends upon insulin and these responses occurs within seconds. Interestingly, insulin (10-20 nM) has a maximum effect on Na⁺ influx in the taste system and concentrations of insulin >20 nM did not show any difference in the magnitude of inward Na⁺ currents (data not shown). Concentration-response curves for insulin in the gustatory system reveal an EC₅₀ ~ 6.11nM (Fig. 3.2D).
Clearly, the magnitude of sodium transport in taste epithelia appears to be regulated by insulin.

To confirm the ability of insulin to enhance Na\(^+\) movement in the gustatory system, we performed a series of experiments using functional sodium imaging in isolated taste cells. Using a Na\(^+\) sensitive dye SBFI we measured ratiometric changes of intracellular Na\(^+\) in taste cells from fungiform and circumvallate papillae. Taste cells loaded with SBFI were analyzed by obtaining the area under the curve of changes in 340/380 ratio for each condition in a manner analogous to the patch clamp experiments. Conservatively, we classified as amiloride-sensitive cells only those cells that show a reversible decrease in SBFI ratio. In all cases, fungiform or circumvallate cells were perfused with Na\(^+\)-free solution and 140 mM NaCl was used as the prototypical salt stimulus. As shown in Fig.3.3, 140 mM NaCl evokes Na\(^+\) responses (AUC: 30.5 ± 2.9) in fungiform taste cells and these NaCl-induced responses were inhibited by amiloride (30 µM; AUC: 23.3 ± 3.4). Repeated measurements ANOVA showed statistical significant difference between NaCl and amiloride treatments (p <0.01; Fig. 3.3B). Following from our electrophysiological results we investigated whether insulin could stimulate Na\(^+\) movement into taste cells. Na\(^+\) movement in fungiform taste cells was dramatically increased from (AUC: 30.5 ± 2.9) to (AUC: 85.1 ± 13.6) when insulin 20 nM was added into the solution (Figs. 3.3A and 3.2B). Insulin-induced Na\(^+\) transport in TRCs is mostly through ENaC since Na\(^+\) responses were dramatically decreased by amiloride (30 µM; AUC: 45.2 ± 7). Statistical analysis
with repeated measurements ANOVA revealed a significant differences between 
Na⁺ responses with insulin (p< 0.01) while amiloride+insulin-mediated responses 
were significantly different from those with insulin treatment alone (p< 0.01).

The ability of insulin to regulate Na⁺ movement via ENaC is extended to 
other areas of the tongue. Circumvallate cells loaded with SBFI evoke similar Na⁺ 
responses (AUC: 27.3 ± 4.3; Fig. 3.4). These responses were inhibited by 
amiloride (30 µM; AUC: 16.9 ± 3). Similar to the anterior taste buds, the posterior 
part of the mouse tongue exhibited amiloride sensitivity. Insulin treatment in 
circumvallate cells stimulated Na⁺ movement approximately two-fold (AUC: 27.3 
± 4.3 to 54.3 ± 5.1). Amiloride (30 µM) diminished the insulin-mediated Na⁺ 
enhancement back to near control levels (AUC: 24.0 ± 2) in the posterior tongue, 
consistent with the action of insulin on ENaC-mediated Na⁺ movement. Repeated 
measurements ANOVA analysis revealed significant difference between NaCl 
and amiloride (30 µM; p<0.01). Insulin treatment showed significant differences 
compared to NaCl (p<0.01) and Na⁺ responses between insulin and 
insulin+amiloride treated cells were significantly different (p<0.01). Importantly, 
the insulin-mediated enhancement of Na⁺ influx in taste cells was rapid and 
reversible. However, we observed a difference in the amount of Na⁺ movement 
mediated by insulin between fungiform and circumvallate (Figs. 3.3B and 3.4B).
Insulin mediated Na\(^+\) transport occurs via PI3-kinase

Recent studies in kidney epithelia have suggested that PI3-kinase is involved in the activation of ENaC by insulin (Record et al., 1998; Staruschenko et al., 2007; Tong et al., 2004). However the role of PI3-kinase in the signaling pathway in taste transduction is unknown. Therefore, we tested the sensitivity of insulin-induced Na\(^+\) transport to applications of PI3-kinase inhibitors. LY294002 (10 µM), a specific pharmacological blocker of PI3-kinase, can decrease insulin's effects on Na\(^+\) responses in SBFI loaded mouse taste cells (Fig. 3.5). Insulin enhancement of Na\(^+\) movement dramatically decreases with LY294002 treatment from (0.89 ± 0.09) to (-0.1 ± 0.09). Paired Student's t-test statistical analysis revealed differences between insulin (20 nM) and insulin (20 nM)+LY294002 (10 µM) (p<0.01; Fig. 3.5B).

To determine whether PI3-kinase was involved in insulin-mediated responses in the posterior tongue, SBFI loaded circumvallate taste cells were treated with insulin (20 nM) and LY294002 (10 µM). As shown in Fig. 3.6, LY294002 blocked insulin's effects on relative Na\(^+\) influx from (1.36 ± 0.2) to (0.22 ± 0.18) and the difference in Na\(^+\) response between insulin and insulin+LY294002 was statistically significant (p<0.01). In contrast, LY303511, an inactive analog of LY294002, had no effect on insulin stimulation of relative Na\(^+\) transport (from 2.87 ± 0.44 to 3.37 ± 0.45; Fig. 3.7). Therefore, we observed reversible effects of LY294002 on insulin-stimulated Na\(^+\) movement within 10 min following the initial presence of the inhibitor (data not shown). Together, our
observations showed that PI3-kinase is critical for insulin-enhanced Na\(^+\) transport in the taste system.

The ability of insulin to enhance ENaC function is mediated by phosphatidylinositides

A number of phosphatidylinositides have been suggested to participate in ENaC activation. Several of these phosphatidylinositides are products of the PI4-kinase signaling pathway such as PtdIns(4,5)P\(_2\). Other phosphatidylinositides like PtdIns(3,4)P\(_2\)/PtdIns(3,4,5)P\(_3\) are synthesized by PI3-kinase, which is directly activated by insulin (Ma, Chou, Wei, & Eaton, 2007; Markadieu, Blero, Boom, Erneux, & Beauwens, 2004; Pochynyuk, Staruschenko, Tong, Medina, & Stockand, 2005; Pochynyuk, Tong, Staruschenko, Ma, & Stockand, 2006; Pochynyuk et al., 2007; Tong et al., 2004). Based on our imaging results in which PI3-kinase blockers abolished acute insulin-enhanced Na\(^+\) responses, we next wanted to investigate the role of phosphatidylinositides in insulin/ENaC-mediated responses by inhibiting the PI4-kinase signaling pathway. Using wortmannin (1 \(\mu\)M), which is an inhibitor of type III PI4-kinase and PI-related kinases at higher (\(\mu\)M) concentrations (Balla, 2001), we performed functional Na\(^+\) imaging in TRCs. The area under the curve obtained from SBFI ratio in the presence and absence of the test solution was analyzed by the relative amount of insulin enhancement (using the 140 mM Na\(^+\) response as baseline). Insulin-induced effects on amiloride-sensitive cells were abolished by wortmannin (1 \(\mu\)M) treatment (Fig.
Inhibition of PI4-kinase decreased insulin-mediated Na\(^+\) responses from 
(0.96 ± 0.19) to (-0.5 ± 0.07). Consistent with this response, amiloride sensitivity 
is absent in these cells which suggest that PI4-kinase phospholipids products 
PtdIns(4)P/ PtdIns(4,5)P\(_2\) are vital to maintain Na\(^+\) influx via ENaC.

Since PI3-kinase is a downstream signaling pathway of PI4-kinase and 
PtdIns(4)P/ PtdIns(4,5)P\(_2\) pools are breakdown by PI3-kinases to produce 
PtdIns(3,4)P\(_2\)/PtdIns-(3,4,5)P\(_3\). To determine the significance of PtdIns(3,4)P\(_2\) 
and PtdIns(3,4,5)P\(_3\) on insulin/ENaC-mediated responses in TRCs, we use two 
pharmacological PI3-kinase blockers, wortmannin (50 nM) and LY294002 (10 
µM). Insulin evoked changes in taste cells were inhibited by both wortmannin 
from (1.37 ± 0.23) to (-0.11 ± 0.07) and LY294002 from (0.89 ± 0.09) to (-0.01 ± 
0.05; Fig. 3.9). In contrast to what we found for PI4-kinase, inhibition of PI3-
kinase does not affect 140 mM Na\(^+\) responses in the absence of insulin.
Amiloride (30 µM) only reduces 140 mM Na\(^+\) responses, which suggests that 
there are functional ENaC channels in the plasma membrane but that PIP\(_2\) and 
PIP\(_3\) are necessary to increase insulin-mediated Na\(^+\) movement through ENaC.
Clearly, our findings strongly suggest that ENaC function is regulated by 
phosphatidylinositol synthesis in the gustatory system and both PI3-kinase and 
PI4-kinases are capable of enhancing and maintaining ENaC channel activity, 
respectively.
Physiological role of Serum and Glucocorticoid-regulated Kinase (SGK) in insulin/ENaC-mediated responses

Having established that PI3-kinase is crucial for ENaC activation by insulin in TRCs, we performed experiments to explore other mechanisms of insulin action. PtdIns(3,4,5)P₃, which is a product of PI3-kinase activation, is capable of interacting with the PH domain of PDK1 and PKB/Akt and then activating SGK (Loffing et al., 2006; Markadieu et al., 2004; Pearce & Kleyman, 2007). To investigate whether insulin could enhance Na⁺ movement via SGK in the gustatory system, functional Na⁺ imaging assays in the presence and absence of insulin (20 nM) were performed in taste cells from transgenic SGK⁻/⁻ mice (Fejes-Toth, Frindt, Naray-Fejes-Toth, & Palmer, 2008). Insulin had no effect on Na⁺ transport in taste cells from SGK⁻/⁻ mice (Figs. 3.10B and 3.10D). In contrast, taste cells from SGK⁺/⁺ evoke greater Na⁺ responses to insulin (Figs. 3.10A and 3.10C). Amiloride sensitivity was, however, found in taste cells from both SGK⁻/⁻ and SGK⁺/⁺ mice. Student t-test analysis of the area under the curve showed a significant difference (p<0.01) between 140 mM Na⁺ and amiloride (30 µM) treatment for both SGK⁻/⁻ and SGK⁺/⁺ TRCs (Figs. 3.10C and 3.10D).

Interestingly, insulin-induced Na⁺ influx is severely impaired in SGK⁻/⁻ compare to SGK⁺/⁺ littermates. The magnitude of the insulin-stimulated Na⁺ transport was found to be less in SGK⁻/⁻ (26.2 ± 3) than SGK⁺/⁺ (48.9 ± 11.7). Our observations imply that both SGK⁻/⁻ and SGK⁺/⁺ taste cells have functional ENaC channels. However, functional ENaC activity seems dramatically reduced in SGK⁻/⁻ taste
cells. Consequently, our findings suggest that SGK is essential to maintain normal ENaC function and the absence of SGK protein in the null mice severely ameliorates insulin’s effects in the gustatory system.

Given the importance of SGK in maintaining basal ENaC activity, we also examined PKB/Akt expression in taste epithelia following a recent study in Fischer rat thyroid cells which suggested that Akt and SGK, which are downstream effectors of PI3-kinase signaling cascade, might contribute insulin/ENaC-mediated responses (Lee et al., 2007). To determine whether Akt was present in TRCs, western blotts of protein from circumvallate taste buds probed with anti-pAkt antibody confirmed the expression of pAkt only in taste epithelia (Fig. 3.11). Consistent with both functional data and protein expression, it appears taste cells contain all downstream mediators of the insulin/PI-3-kinase signaling pathway.

Expression of Insulin Receptor (IR), Insulin Receptor Substrate 1 (IRS1), and Insulin Receptor Substrate 2 (IRS2) in taste cells

In most transporting epithelia, insulin reabsorption of NaCl is initiated when insulin binds to the basolateral IR. Autophosphorylation of IR set of a number of downstream events including phosphorylation of IRS and PI3-kinase (Blazer-Yost, Vahle, Byars, & Bacallao, 2004; Tiwari et al., 2007). To verify the expression of IR, IRS1 and IRS2 in mouse taste buds, a series of RT-PCR assays were performed using total RNA isolated from fungiform, foliate, and
circumvallate taste buds. Expression of IR, IRS1, and IRS2 was found in all three lingual taste bud types in a minimum of three independent experiments (Fig. 3.12). All PCR products were sequenced using a PE Biosystem 377 automated DNA sequencer. The sequence for mouse taste bud IR, IRS1 and IRS2 were at least 99% homologous with sequences from GenBank.

Insulin regulates salt intake in mice

We next performed a series of brief access behavioral tests to determine if insulin enhancement of Na\(^+\) movement via ENaC in mouse taste cells were correlated with any differences in behavioral responsiveness to NaCl. Since we hypothesized that insulin could act primarily as regulator of salt appetite in the taste system, we tested the ability of insulin to alter NaCl preference in a hyperinsulemic animal model. For all experiments 24 C57BL/6 mice were trained for three days and then divided into two groups. Each group received either i.p. injections of insulin (0.75 U/kg) or vehicle. Both insulin-treated and vehicle-treated groups were injected 15 minutes prior to the behavioral test and preference for seven different NaCl concentrations was tested. To evaluate the efficiency of the treatment, blood glucose (mg/dl) was measured in each of the animals tested. In all cases, insulin-treated mice had lower blood glucose levels (47.7 ± 5.6) compared to the vehicle injected group (155.6 ± 3.8; p<0.01 paired Student’s t-test; Fig. 3.13B).
In addition, insulin-treated mice exhibited significant avoidance of NaCl at lower concentrations than the control group (Fig. 3.13A). These changes in NaCl preference in hyperinsulemic mice are more evident at concentrations between 150 mM to 600 mM NaCl. Simple effects ANOVA analysis reveals highly significant differences (p<0.01) between insulin-treated and control group at the following NaCl concentrations: 150, 270, 330, 450 and 600 mM. Noticeably, insulin treatment appeared to have an effect in NaCl preference in all of the mice tested in these experiments.

To elucidate the functional role of ENaC in alterations of NaCl sensitivity by insulin treatment, we added amiloride (100 µM), a diuretic by means of its antagonism of ENaC, to all NaCl solutions (Eylam & Spector, 2002). Additionally, amiloride was also added into the ddH2O used for water stimulus and rinses to eliminate any possible taste cue from amiloride during the short-term taste assays. In contrast, behavioral effects of insulin in salt preference were abolished by amiloride (Fig. 3.14A). Thus, NaCl taste is diminished in both insulin-treated and control groups in the presence of amiloride (100 µM). The pharmacological effect of amiloride on ENaC channels attenuated any difference in NaCl sensitivity between hyperinsulinemic and control group at concentrations below 330 mM NaCl. However, we observed differences between insulin-treated and control mice in the presence of amiloride (100 µM) at 600 mM NaCl (p=0.01). Blood glucose levels differed between the control group (160.8 ± 5.6) and the insulin-treated group (55.3 ± 4.1; p<0.01 paired Student’s t-test; Fig. 3.14B).
Taken together, our results from behavioral assays confirm the physiological importance of insulin/ENaC-mediated responses to regulate salt intake in the peripheral gustatory system.

Discussion

The importance of insulin in maintaining homeostasis is well established. For instance, diabetes mellitus is characterized both for its deficiency of insulin and its serious metabolic abnormalities. Associated with insulin deficiency are a wide number of counter regulatory mechanisms, which are activated to stabilize the diabetic decompensation. Insulin deficiency triggers hepatic glucose production and reduces glucose uptake, resulting in hyperglycemia and ketogenesis. Both hyperglycemia and hyperketonemia induce osmotic diuresis. Osmotic diuresis results from unabsorbed glucose in the kidney tubule lumen, which leads to volume depletion and dehydration (Chiasson et al., 2003; Kim, Sands, & Klein, 2004). Consequently, insulin plays a physiological role in Na\(^+\) reabsorption in the proximal tubule, the thick ascending limb and the collecting duct of the kidney. The ability of insulin to increase Na\(^+\) influx is through ENaC (Tiwari, Nordquist, Halagappa, & Ecelbarger, 2007; Tiwari et al., 2007). In the present study, we have identified a novel mechanism of salt taste regulation by insulin in mammalian taste buds. Using whole cell patch clamp recording we recorded insulin responses in isolate taste cells. In most cells insulin increased
Na\(^+\) transport in both fungiform and circumvallate cells and since this insulin-induced enhancement of inward Na\(^+\) current was abolished by amiloride, it appears to be linked with ENaC (Fig. 3.1). Because of the similarities between taste and kidney epithelia, we hypothesize that insulin plays a vital physiological role in salt taste transduction by virtue of its ability to modulate ENaC, the primary salt taste transducer in rodents.

Nature of insulin response is via PI3-kinase in taste receptor cells

Insulin-mediated Na\(^+\) reabsorption is believed to occur by the activation of PI3-kinase and its phospholipids products. Both ENaC activity in A6 cells and ENaC heterologously expressed in *Xenopus oocytes* are stimulated by PtdIns(3,4,5)P\(_3\). Thus, PI3-kinase signaling via production of PtdIns(3,4)P\(_2\) /PtdIns(3,4,5)P\(_3\) directly affects ENaC activity and open probability (Ma & Eaton, 2005; Record et al., 1998; Staruschenko et al., 2007; Tong et al., 2004). In agreement with these results we found that the PI3-kinase signaling pathway is necessary for insulin mediated Na\(^+\) influx through ENaC in TRCs. Using functional Na\(^+\) imaging and pharmacological blockers of PI3-kinase, LY294002 (10 \(\mu\)M) and wortmannin (50 nM), we showed that increases in Na\(^+\) movement via amiloride-sensitive Na\(^+\) channels required PI3-kinase in taste cells. Since PI3-kinase blockers dramatically decreased insulin's effects on Na\(^+\) influx in TRCs (Fig. 3.9) we hypothesized that PI3-kinase phospholipids products PtdIns(3,4)P\(_2\)
/PtdIns(3,4,5)P₃ are critical for insulin signaling transduction in the taste system. In this regard, it was clear the direct correlation between PtdIns(3,4)P₂/PtdIns(3,4,5)P₃ and Na⁺ transport because LY303511, which is an inactive analog of LY294002, did not have any effects on insulin enhancement of Na⁺ movement (Fig. 3.7). Additionally, we did not observe any evidence of inward currents at -80 mV in fungiform amiloride-sensitive taste cells when insulin+LY294002 (10 µM) were applied (data not show).

Insulin-stimulated Na⁺ reabsorption shares a number of similarities with ENaC activation by other hormones (i.e. ALDO); these similar characteristics include phosphorylation of ENaC subunits by PI3-kinase phospholipids products (Blazer-Yost & Nofziger, 2004; Helms et al., 2005). Although we have provided the first evidence about the role of PI3-kinase in insulin signaling in taste cells, further experiments need to be performed to elucidate the characteristics of PtdIns(3,4)P₂/PtdIns(3,4,5)P₃ interaction with ENaC.

Involvement of phosphoinositides in ENaC function

Recent studies have showed that ENaC activity can be increased directly by phosphatidylinositides (Pochynyuk et al., 2007; Tong et al., 2004). These phosphatidylinositides bind ENaC and increase its open probability. In addition, three hormone systems (insulin, ALDO and AVP) can regulate ENaC individually or synergistically in kidney epithelium. Hormonal stimulation requires the activation of enzymes (i.e. protein kinases) to produce phosphoinositides. These
related kinases are key mediators in several cellular functions including peptide stimulation of ion channel activity. Consistent with this idea, PI3-kinase is a common intermediary for hormonal actions including those activated by insulin. Activation of PI3-kinase by IRS can trigger production of PtdIns(3,4)P$_2$ /PtdIns(3,4,5)P$_3$, however PtdIns(4,5)P$_2$ levels are essential for PI3-kinase enzyme action. Although the existence of hormonal-sensitive or insensitive pools of phosphoinositides remains controversial, there is enough evidence to suggest that PtdIns(4)P could be generated by a hormone-sensitive type III PI4-kinase (Nakanishi, Catt, & Balla, 1995). In general, PtdIns(4,5)P$_2$ is produced by two pathways: (1) PI4-kinases phosphorylates PtdIns(4)P and then a sequential phosphorylation by type I PIP kinases. (2) minor pathway in which PtdIns(4,5)P$_2$ is produced from PtdIns(5)P (Balla, 2006). The importance of PtdIns(4,5)P$_2$ as a second messenger has been shown in the regulation of several ion channels. Reports from Kir, KCNQ and Trp channels conclude that PtdIns(4,5)P$_2$ is required for ion channel activation and/or function (Staruschenko et al., 2007). In addition, electrophysiological studies on ENaC have suggested that PtdIns(4,5)P$_2$ has a physiological role in ENaC function. However these results are contradictory because PtdIns(4,5)P$_2$ can either increase or decrease ENaC activity. It is believed that PtdIns(4,5)P$_2$ is vital in maintaining ENaC function as loss of ENaC activity in inside-out patches could be recovered with the addition of PtdIns(4,5)P$_2$ (Yue, Edinger, Bao, Johnson, & Eaton, 2000; Yue, Malik, Yue, & Eaton, 2002). Pochynyuk and colleagues recently determined that the N terminal
of β- and γ- ENaC subunit are the binding sites for PtdIns(4,5)P₂ (Pochynyuk et al., 2007). We revealed the presence of hormonal-sensitive PI4-kinases in taste cells. Using wortmannin, which is a pharmacological blocker of PI4-kinases at high concentrations (i.e. 1 µM), we inhibited the synthesis of PI(4,5)P₂ and others downstream of intracellular signaling that require conversion of PI(4,5)P₂.

Treatment with wortmannin (1 µM) significantly reduced the ability of insulin to enhance Na⁺ transport in taste cells (Fig. 3.8). These results suggested that PtdIns(4)P₂/ PtdIns(4,5)P₂ are likely involved in Na⁺ transport. Even though our results showed a reduction in 140 mM NaCl responses we could not draw any meaningful conclusions about role of PI4-kinase products since wortmannin also blocks PI3-kinases and PI-related kinases at this concentration (Balla, 2001).

One possibility to overcome this issue was to block PI3-kinase with LY294002 (10 µM), a specific blocker of PI3-kinase, and wortmannin (50 nM), which is considered a pharmacological blocker of PI3-kinase at this concentration. As shown Figs. 3.9A and 3.9B, both LY294002 and wortmannin (at 50 nM) inhibited insulin’s effect on Na⁺ influx, yet did not affect 140 mM NaCl responses. In addition, there was evidence of amiloride-sensitive ENaC activity with the presence of both blockers. Taken together, our results are consistent with the interpretations that PtdIns(3,4)P₂/PtdIns(3,4,5)P₃ increases ENaC open probability in taste cells. Acute effects of insulin are mostly through the activation of PI3-kinase signaling cascade. Thus the present study is the first to use pharmacological blockers to show in native cells the physiological role of
PtdIns(4,5)P₂ on ENaC function. However, future experiments will need to be done to demonstrate the direct interaction, if any, between ENaC and phosphatidylinositolides in taste cells.

Role of SGK1 in activation of ENaC by insulin

Insulin stimulation of ENaC-mediated Na⁺ transport occurs via PI3-kinase signaling pathway cascade by one of two mechanisms: (1) increasing the open probability of apical ENaCs and/or (2) increasing the number of active channels in the membrane. Insulin-induced PI3-kinase signaling cascade leads to phosphorylation of PDK1 and SGK1 activation. SGK1 is a protein with a half life of ~30 min and it is expressed in several tissues including brain, lung, colon, pancreas and kidney (Loffing et al., 2006; Tiwari et al., 2007). One of the best described mechanisms of SGK1 function is stimulation of Na⁺ transport by increasing the number of apical ENaC in transporting epithelia. The interaction between SGK and ENaC occurs via phosphorylation of the ubiquitin ligase NEDD4-2, which ubiquitinates ENaC. Thus, NEDD4-2 removes and degrades ENaC protein from the cell membrane. The consequence of phosphorylation of NEDD4-2 by SGK1 leads to enhanced ENaC abundance in the cell membrane (Debonneville et al., 2001; Faletti et al., 2002; Staub & Verrey, 2005; Zhou & Snyder, 2005). In addition, studies in kidney epithelia have showed that insulin promotes both activity and translocation of ENaC into the cell membrane (Arteaga & Canessa, 2005; Blazer-Yost et al., 2004; Tiwari et al., 2007).
The present study attempted to demonstrate the physiological role of SGK in insulin-mediated signaling in isolated mammalian taste cells. Using functional Na\(^+\) imaging we characterized Na\(^+\) transport via ENaC in TRCs from SGK\(^{-/-}\) mice. Isolated taste cells from SGK\(^{-/-}\) mice evoked alterations in the magnitude of insulin-mediated Na\(^+\) movement (Figs. 3.10B and 3.10D). In contrast, SGK\(^{+/+}\) taste cells showed normal Na\(^+\) enhancement by insulin (Figs. 3.10A and 3.10D). Even though both SGK\(^{+/+}\) and SGK\(^{-/-}\) cells exhibited amiloride sensitivity, the amount of amiloride inhibition was reduced in TRCs from SGK\(^{-/-}\) mice. These results illustrate the inability of insulin to increase Na\(^+\) influx in SGK\(^{-/-}\) taste cells. Moreover, the present observations are also consistent with a reduction in functional ENaC expression in SGK\(^{-/-}\) taste cells. It could be possible that absence of SGK1 protein allow a greater degradation of ENaC in the peripheral taste system. Similar results have been shown in kidney epithelia in which insulin-mediated Na\(^+\) retention was abolished in SGK\(^{-/-}\) mice (Huang et al., 2006). In addition, SGK\(^{-/-}\) transgenic mice have shown impairment in the ability to maintain blood pressure when exposed to salt depletion (Fejes-Toth et al., 2008; Huang et al., 2006). More studies are needed to be done to investigate the link between SGK and insulin-mediated salt appetite.

PKB/Akt contains a PH domain, which is important for Akt activation by PI3-kinase. A recent finding in Fisher rat thymus cells (FRT) has shown that activation of SGK as well as Akt can increase ENaC activity. Both of these kinases are downstream insulin signaling effectors of PI3-kinase/PDK1.
Therefore, activation of Akt is suggested to increase both activity and ENaC expression (Lee et al., 2007). We identified Akt protein expression in taste cells with Western blotting (Fig. 3.11). While we have no functional data indicating a role for Akt, its presence in TRCs is consistent with the idea that it may play a regulatory role in the activation of ENaC by insulin. Presently, virtually nothing is known about the functional properties of Akt in the taste system; however Akt−/− transgenic mice show alterations in growth and glucose metabolism (Dummler et al., 2006).

Circulating insulin wields its effects by binding the insulin receptor (IR) on target tissues. IR activation by insulin binding creates a conformational change which induces tyrosine kinase activity on the cytoplasmic surface of the cell membrane resulting in both autophosphorylation of the receptor and phosphorylation of other proteins. The immediate effectors of these tyrosine receptors are members of the insulin substrate (IRS) family. Phosphorylation of IRS results in the activation of signaling molecules such as PI3-kinase (Miura et al., 2004; Thirone, Huang, & Klip, 2006; Wing, 2008). RT-PCR assays were consistent with the expression of IR, IRS1 and IRS2 in mouse taste buds. Together, our data provide strong evidence for a signaling pathway for insulin to regulate Na⁺ movement via ENaC in mouse TRCs.
Implications of insulin in salt chemoreception

Our results showed insulin-mediated enhancement of Na⁺ influx through ENaC in mice. From these data, we hypothesize that insulin plays a physiological role in the regulation of salt taste. To test this, in the present study we characterized insulin’s effects on behavioral responses to NaCl using a mouse model of acute hyperinsulinemia. The results showed that insulin-treated mice displayed a strong avoidance for NaCl solutions at the following concentrations 150 mM, 270 mM, 330 mM, 450 mM and 600 mM (Fig. 3.13). Consequently, Na⁺ detection performance was dramatically altered in hyperinsulinemic mice compared to control group. In the present study, NaCl concentrations were presented in ascending order because we found this to be most effective for maintaining the behavioral momentum of the animal. Given this, it could be possible that the ability of each mouse to discriminate NaCl concentrations during the trial may be reduced as they got to the higher concentrations. We find this to be unlikely since we performed another experiment with a separate group of 5 vehicle-injected mice. In this case, NaCl concentrations were presented in a random order. Since no differences in NaCl lick ratio were found between the two experiments (data not shown) we concluded that at least with the current testing conditions, there is no evidence of stimulus order presenting a confound in our analyses.

Our results provide clear evidence that insulin-mediated effects on Na⁺ movement extend to the animal’s behavior. This link between the endocrine
system and chemoreception of salt would be predicted on the assumption that circulating insulin activates insulin receptor in taste cells and its activation would lead to a number of intracellular downstream effectors which enhance ENaC activity. Using amiloride to block ENaC express in taste cells we attempted determine the specificity of insulin's effect to ENaC. If insulin was targeting Na⁺ movement through ENaC, it would be expected that NaCl preference will be similar in both hyperinsulinemic and control groups. Clearly, the presence of amiloride in all solutions was very effective at suppressing insulin's effects on NaCl responses at concentrations below 330 mM (Fig. 3.14). Although the results showed significant difference at 600 mM between hyperinsulinemic and control, the magnitude of NaCl avoidance was much less with the presence of amiloride. This data suggested that insulin activates ENaC channels expressed across the plasma membrane of taste cells and therefore these animals experience a change in their NaCl taste sensitivity. Taken together, our behavioral findings are the first evidence that showed an interaction between insulin and the detection of NaCl salts. However, more studies are needed in this area to understand in greater detail the effects of insulin in the animal's taste behavior.

In conclusion, the present study has demonstrated a new insulin signaling pathway for maintaining functional ENaC expression in the taste system. Thus, insulin leads to PI3-kinase activation which either increases ENaC activity with its phospholipids products or increases the number of apical ENaC by the activation
of SGK. Our results are consistent with the emerging idea that the gustatory system is capable of responding to nutritional challenges and may play a central role in the restoration of nutritional balance.

References


TABLE 3.1. Nucleotide sequences for the primers in the RT-PCR assays.

<table>
<thead>
<tr>
<th>Target</th>
<th>GenBank Accession No.</th>
<th>Sense primer/Antisense primer</th>
<th>Corresponding nucleotide sequence</th>
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<tr>
<td>IR</td>
<td>NM_010568</td>
<td>5’ CTT TGG GAA ATC ACT AGC TTG 3’&lt;br&gt;5’ CCT TGT TCT CCT CGC TGT AG 3’</td>
<td>3694-3715&lt;br&gt;3929-3949</td>
</tr>
<tr>
<td>IRS-1</td>
<td>NM_010570</td>
<td>5’ CGC AAC TGC CGA AGA TTC 3’&lt;br&gt;5’ CCT ATT CTG CCC AAC TCA ACT 3’</td>
<td>3311-3329&lt;br&gt;3781-3802</td>
</tr>
<tr>
<td>IRS-2</td>
<td>NM_001081212</td>
<td>5’ GCG GAG ACA ATG ACC AGT ATG T 3’&lt;br&gt;5’ TCT TGG GCT CTG TGG GTA GA 3’</td>
<td>2423-2445&lt;br&gt;2699-2719</td>
</tr>
</tbody>
</table>
Fig. 3.1. Effects of insulin on amiloride-sensitive cells. Steady-state current in a mouse taste cell during whole cell patch clamp recording. Continuous recording from a fungiform taste cell during the application of insulin (10 nM), insulin with amiloride (30 μM), and insulin in Na⁺ free Tyrodes. Taste cells, like this one, showed an evident increase in sodium (Na⁺) inward current by the application of insulin. Solid line indicates timing of normal physiological saline. Inset illustration shows ion distributions and patch clamp conditions used to isolate Na⁺ currents in these electrophysiological experiments.
Fig. 3.2. Concentration-dependent effects of insulin on amiloride-sensitive currents in mouse taste cells. Steady-state currents from mouse taste cells during whole cell patch clamp recording. (A-C), steady-state currents from different taste cells during the application of insulin at 5, 10, & 20 nM. The bracket in A-C is used to illustrate the increase in magnitude compared with the 5 nM insulin effect. (D), Insulin dose-response curve. Insulin was applied by bath perfusion at 1, 5, 10, and 20 nM. Data points are the mean ± S.E.M. of the current enhancement as a function of insulin concentration. Solid line is the statistically weighted best fit with a logistic relation with EC$_{50}$ of 6.11 nM.
Fig. 3.3. Functional sodium imaging confirms insulin enhancement of ENaC. Fungiform taste cells were preloaded with SBFI and Na⁺ mobilization was measured. (A), SBFI ratio elicited by 140 mM NaCl delivered to fungiform taste cells in the presence or absence of insulin (20 nM) and subsequent application of amiloride (30 μM). (B), summary graph comparing the area under the curve of 140 mM NaCl responses before and after insulin (20 nM) application, as well as comparing the effects of amiloride (30 μM) treatment. Data are shown mean ± SEM. a & c denote significant reduction in SBFI ratio; b, denotes significant increase in SBFI ratio compared with 140mM NaCl (p< 0.01, ANOVA).
Fig. 3.4. Insulin increases sodium (Na⁺) influx in circumvallate mouse taste cells. (A), circumvallate taste cells evoked greater responses to 140 mM Na⁺ in the presence of insulin. These insulin-mediated Na⁺ responses were generally inhibited by amiloride (30 µM). (B), summary graph comparing insulin effects in the posterior mouse tongue before and after amiloride (30 µM) application. Data are shown mean ± SEM. a & c denote significant reduction in SBFI ratio; b, denotes significant increase in SBFI ratio compared with 140 mM NaCl (p< 0.01, ANOVA).
**Fig. 3.5.** Insulin enhancement of ENaC-mediated Na\(^+\) transport occurs via PI3-kinase signaling pathway. (A), effects of PI3-K inhibitor LY294002 (10 µM) on insulin-induced Na\(^+\) responses in mouse fungiform taste cells using functional Na\(^+\) imaging with SBFI. (B), summary graph of insulin enhancement in Na\(^+\) influx in the absence and presence of LY294002 (10 µM). Data are shown mean ± SEM. Asterisk denotes significant reduction in insulin mediated Na\(^+\) influx by the PI3-K inhibitor (p< 0.01, Student's t-test).
Fig. 3.6. PI3-kinase antagonist (LY294002) inhibits insulin effects on Na\(^+\) movement in mouse circumvallate taste cells. (A), functional Na\(^+\) imaging recording from circumvallate TRCs showing changes in SBFI ratio to 140 mM NaCl in the absence and presence of insulin (20 nM) and before and after acute treatment with LY294002 (10 µM). (B), Summary graph of insulin effects in Na\(^+\) influx before and after acute treatment with LY294002 (10 µM). Data are shown mean ± SEM. Asterisk denotes significant reduction in insulin mediated Na\(^+\) influx by the PI3-K inhibitor (p< 0.01, Student’s t-test).
Fig. 3.7. LY303511, an inactive analogue of LY294002, does not reduce insulin-mediated enhancement of Na⁺ influx in mouse taste cells. (A), functional imaging recording from SBFI preloaded fungiform cells showing insulin stimulation in Na⁺ movement in the absence and presence of LY303511 (10 µM). (B), summary graph of insulin effects in Na⁺ influx before and after LY303511 treatment. Data are shown mean ± SEM.
Fig 3.8. Insulin-induced effects on amiloride-sensitive cells were abolished by an inhibitor of the enzyme PI4-kinase. Insulin enhancement on Na⁺ influx were recorded with functional Na⁺ imaging in the absence and presence of wortmannin (1 µM) treatment for 1 h and before and after amiloride (30 µM). All results obtained were compared to preceding 140 mM Na⁺ response. Values shown are means ± SEM of between 8 and 10 cells per point.
Fig. 3.9. Effects of two PI3-kinase blockers on insulin-induced changes in amiloride-sensitive cells. (A), insulin-mediated responses in the absence and presence of pretreatment with wortmannin (50 nM) for 1 h and subsequently, amiloride (30 µM). (B), insulin-mediated responses in the absence and presence of acute treatment with LY294002 (10 µM) and subsequently, amiloride (30 µM). Data are shown mean ± SEM.
Fig. 3.10. The importance of SGK in insulin/ENaC mediated responses in taste cells. (A), functional Na⁺ imaging with SBFI from a SGK (+/+) wild type mice in the presence of insulin (20 nM) and subsequently amiloride (30 µM). (B), Insulin effects on Na⁺ influx in SGK (-/-) mice in the absence and presence of amiloride (30 µM) treatment. (C), Summary graph showing insulin-mediated effects in Na⁺ transport in SGK (+/+ ) wild type animals. (D), Area under the curve graph from SGK (-/-) mice showing a dramatic reduction in the magnitude of insulin-mediated Na⁺ responses. Data are shown mean ± SEM. Asterisks denote significant reduction in insulin-mediated Na⁺ influx by amiloride (30 µM; p< 0.01, Student’s t-test).
**Fig. 3.11.** Expression of Akt protein in TRCs. Western blot showing an anti-p-Ser-AKT antibody labeled a ~65 KD band indicating the presence of Akt protein in mouse circumvallate taste buds (circumvallate). Negative control was non-taste lingual tissue (Non-taste).
Fig. 3.12. RT-PCR reveals the presence of insulin receptor (IR), insulin receptor substrate 1 (IRS-1), insulin receptor substrate 2 (IRS-2). Primers for IR, IRS-1, and IRS-2 amplify stained PCR products of expected sizes (IR 255bp, IRS-1 491bp, and IRS-2 296bp). Positive controls (mouse kidney or liver RNA) are shown for IR, IRS-1, and IRS-2 with each set of primers. Negative control (-) lanes represent those in which cDNA was omitted from the PCR reaction.
Fig. 3.13. Insulin-treated mice reveal differences in NaCl preference. (A), lick ratio of NaCl/water (mean ± SEM) measured in short term taste assays using the Davis Rig in two groups of 22 mice. Insulin-treated mice significantly avoid NaCl solutions between 150 mM to 600 mM. Asterisks denote significant difference in NaCl preference compared to control mice (p< 0.01, Simple Effects ANOVA). (B), Values shown are mean ± SEM for blood glucose levels (mg/dl) in each group. Asterisk denotes significant reduction in blood glucose levels by insulin treatment compared to control (p< 0.01, Student’s t-test).
Fig. 3.14. Behavioral effects of insulin on salt preference appear to be via ENaC channels. (A) Values of lick ratio of NaCl/water are shown (mean ± SEM) and each point represents 12 mice for each group. Avoidance to NaCl solutions as reduced in both control and insulin-treated mice due to the blocking of ENaC channel by amiloride (100 µM), which was present in all solutions. Asterisks denote significant difference in NaCl preference compared to control mice (p< 0.01, Simple Effects ANOVA). (B) Graph of mean ± SEM for blood glucose levels (mg/dl) in each group. Asterisk denotes significant reduction in blood glucose levels by insulin treatment compared to control (p< 0.01, Student’s t-test).
CHAPTER 4
ALTERATIONS OF EPITHELIAL SODIUM CHANNEL FUNCTION
IN DIABETIC TASTE RECEPTOR CELLS

Abstract

Untreated diabetes is a profound disease that is reflected in a severe impairment of systemic salt and water balance. Based on our earlier work demonstrating the insulin regulation of epithelial sodium channels (ENaC), we hypothesize that ENaC in mouse taste receptor cells (TRCs) plays a central role in the restoration of salt and water intake by virtue of insulin’s effect on the gustatory system. To investigate whether ENaC function is altered during the onset of diabetes, we performed functional ratiometric Na⁺ imaging in isolated taste cells from a mouse model of Type I (insulin-dependent) diabetes. Taste cells from diabetic mice exhibit Na⁺ responses and amiloride sensitivity similar to non–diabetic littermates. However, insulin enhancement of Na⁺ influx via ENaC was abolished in diabetic taste cells. In contrast, taste cells from diabetic mice, especially those in the posterior mouse tongue, evoke greater responses to 140 mM NaCl (60±5.3 AUC) than those from non-diabetic mice (26.4±5.4).

3 Coauthored by Arian F. Baquero and Timothy A. Gilbertson.
To test for alterations in ENaC expression in TRCs littermates, we used qRT-PCR to quantify relative differences between diabetic and non-diabetic. All three ENaC subunits α-, β- and γ- were highly expressed in the diabetic mice compared to control. To evaluate the effects of the diabetic state in salt taste, we next characterized behavioral responses to NaCl using a brief access test. Diabetic mice showed avoidance of NaCl at significantly lower concentrations than the non-diabetic group. In contrast, diabetic animals showed no significant avoidance to these NaCl solutions (p<0.01) when amiloride (100 µM) was added to NaCl solutions, indicating a role for ENaC in this increased sensitivity. Our results are consistent with the hypothesis that ENaC alterations during diabetes may be an example of the ability of the gustatory system to respond to nutritional changes.

Introduction

Taste receptor cells (TRCs) recognize a wide variety of chemical stimuli. In mammals, taste has been classified in the five principal modalities of sweet, salt, umami, sour, and bitter. Some tastants (sweet, salty, and umami) can generate an appetitive drive which is believed to play a role in nutrient recognition for carbohydrates, proteins, and minerals, respectively. The other taste classes (bitter and sour) generate typically aversive responses which are important to the avoidance of potentially toxic compounds or spoiled foods.
Accordingly, there are a number of transduction mechanisms that have been proposed to account for the ability of the gustatory system to recognize taste stimuli. These mechanisms are divided into interactions with ion channels and activation of ionotropic and metabotropic receptors (Gilbertson, Damak, & Margolskee, 2000; Roper, 2007).

A classic example of an ion channel mediated mechanism of taste transduction is in the recognition of sodium chloride (NaCl). Salt taste is mediated primarily via sodium (Na⁺) influx through the amiloride-sensitive epithelial sodium channel (ENaC). ENaC is formed by a heteromultimeric complex of three subunits (α-, β-, and γ-) in a stoichiometry of 1α:1β:1γ (Staruschenko, Adams, Booth, & Stockand, 2005). ENaC has been characterized in TRCs and other transporting epithelia and it shares a number of common features across tissue types. Similarities include small conductance (~5 pS), Na⁺ ~ Li⁺ >> K⁺ ion selectivity, regulation by extracellular Na⁺ (self-inhibition) and intracellular Na⁺ (feedback inhibition), and regulation by hormones. It has been suggested that these functional similarities between ENaC in the taste system and other transporting epithelia extend to the molecular level. Thus, in many regards ENaC appears similar across organ types (Gilbertson & Zhang, 1998a, 1998b; Lin, Finger, Rossier, & Kinnamon, 1999).

The physiological role of ENaC in salt and water balance is well established. Recent studies in kidney transporting epithelia from untreated diabetic Type I and Type 2 animals have shown an enhancement of protein
abundance of all (α-, β-, and γ-) ENaC subunits (Bickel, Knepper, Verbalis, & Ecelbarger, 2002; Song, Knepper, Verbalis, & Ecelbarger, 2003). These changes in the protein expression in kidney epithelia are accompanied by compensatory mechanisms in the kidney in order to limit salt and water losses. Overall, diabetes mellitus is an endocrine disease characterized by both a deficiency of insulin and a severe impairment of systemic salt and water balance. Associated with the salt and water imbalance are a number of counterregulatory mechanisms such as polyuria, polydipsia and osmotic diuresis, which are activated to stabilized the diabetic decompensation (Chiasson et al., 2003; Vidotti, Arnoni, Maquigussa, & Boim, 2008). Osmotic diuresis is the main underlying complication of uncontrolled diabetes that results in an increase of electrolyte losses and urinary output. This severe imbalance of water and electrolytes triggers volume depletion and dehydration. Thus, dehydration is a critical complication of uncontrolled diabetes because it can lead to a hyperglycemic hyperosmolar state and diabetic ketoacidosis (Kim, Sands, & Klein, 2004; Song et al., 2003).

Behavioral studies are consistent with a potential relationship between diabetes and the gustatory system since diabetic rodents exhibit a loss of preference for saccharin and sucrose at high concentrations (Smith & Gannon, 1991). However, little is known about the functional adjustments of ENaC expressed in the taste system during the onset of diabetes. Much of our current understanding about salt transduction mechanisms in mammalian taste cells has
come from isolated taste cells using electrophysiological assays on normal chow-
type diets. In addition, studies in kidney epithelia in which rodents were fed with
diets containing different NaCl levels have suggested that ENaC protein
abundance is modified. For instance, dietary NaCl restriction induces both a
marked increase in α-ENaC subunit and a reduction in β- and γ-ENaC subunit
protein abundance in kidney (Knepper, Kim, & Masilamani, 2003). In contrast, a
high NaCl diet, which is associated with high blood pressure, exhibited the
opposite effect on ENaC expression. α-ENaC subunit protein expression was
lower with a high NaCl diet and β- and γ-ENaC subunit protein abundance was
enhanced by high NaCl (Song, Hu, Shi, Knepper, & Ecelbarger, 2004).
Brattleboro rats with diabetes insipidus (insufficient production of vasopressin)
displayed an increase in water intake and reduction in salt consumption (Yirmiya,
Holder, & Derdiarian, 1988). Because rats with diabetes insipidus have an
inability to concentrate urine, these changes in ingestive behavior may be
reflective of alterations in the salt-sensing taste pathways as well, where ENaC
appears to be the primary transduction channel for salty taste.

Recently, our laboratory described a novel interaction between salt taste
transduction pathway and its regulation by insulin in TRCs. In general, our
functional and molecular experiments showed that insulin enhances Na\textsuperscript{+}
transport in murine taste cells and does so by virtue of its ability to modulate
expression and/or function of ENaC. Specifically, the ability of insulin to stimulate
ENaC activity in taste cells was carried out through the PI3-kinase signaling
cascade (unpublished data). Interestingly, insulin's effects on the taste system are extended to the behavioral level since hyperinsulinemic mice showed stronger avoidance to NaCl solutions than control mice and these effects were attenuated by orally administered amiloride. Thus, all of the molecular components are present in TRCs allowing insulin to enable the animal to compensate for disturbances in salt and water balance.

In the present study, we investigated functional mechanisms of ENaC regulation during the onset of diabetes. Using functional Na⁺ imaging in isolated taste cells from a mouse model of Type I (insulin dependent) diabetes, we demonstrated that cells from diabetic mice exhibited Na⁺ responses and amiloride sensitivity that were qualitatively similar to non–diabetic littermates. Insulin enhancement of Na⁺ influx via ENaC, however, was abolished in diabetic taste cells. In contrast, taste cells from diabetic mice, especially those in the posterior mouse tongue, evoked more robust responses to 140 mM NaCl than those from non-diabetic mice. Based on these findings, we next investigated the relative expression of α- β- and γ- ENaC subunits in the diabetic and control groups. The results showed that one or more ENaC subunits are more highly expressed during the onset of diabetes, which dovetails nicely with differences in the magnitude of Na⁺ influx via ENaC observed in functional Na⁺ imaging studies. To determine the effects of diabetic state in salt taste, the two groups were compared for NaCl preference in behavioral assays. Diabetic mice showed significance avoidance for NaCl solutions. Together, these results suggested that
ENaC expressed in taste epithelia may play a role in helping to shape dietary preferences in order to restore salt and water balance.

Methods

All experiments were performed on adult (2–4 months) male C57BL/6 mice that were maintained on a 12h:12h day/night cycle with normal chow and water provided ad libitum. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Utah State University and were performed in accordance with American Veterinary Medical Association guidelines.

Induction of diabetes

Mice were individually housed and divided into weight- and age-matched diabetic and control groups. Diabetes was induced by two consecutive daily intraperitoneal (i.p.) injections of streptozotocin (STZ; MP Biomedicals, LLC Solon OH) at a dose of 50 mg/kg dissolved in Na⁺-citrate. Na⁺-citrate buffer was made fresh every day before use, and the pH was adjusted to 4.5 with HCl. The control group received daily i.p. injections of equivalent volume of Na⁺-citrate buffer alone. To determine the onset of diabetes, blood plasma glucose (mg/dl) levels were measured using a glucometer (BD Bioscience, San Jose, CA).
Isolation of single taste receptor cells

Taste buds were isolated from fungiform and circumvallate papillae of the mouse tongue according to previously described techniques (Béhé, DeSimone, Avenet, & Lindemann, 1990; Gilbertson, Roper, & Kinnamon, 1993). Briefly, tongues were removed and immediately immersed in Tyrode's solution containing 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM N-2-hydroxyethylpiperazine- N' -2-ethanesulphonic acid (HEPES), 10 mM glucose, and 10 mM Na⁺ pyruvate (osmolarity; 310 mOsm). The pH was adjusted to 7.4 with NaOH. The fungiform papillae located in the anterior portion of the tongue was injected between the muscle layer and the lingual epithelium with approximately 0.15 ml of physiological saline (Tyrode's) containing a mixture of collagenase I (1.1 mg/ml; Roche Applied Science, Indianapolis, IN), dispase (2.4 mg/ml; Roche Applied Science), and trypsin inhibitor (1 mg/ml; type I-S; Sigma Chemical Corp., St. Louis, MO). The circumvallate papillae was also injected with 0.05-0.1 ml of the same enzyme cocktail. The injected tongue was incubated in Tyrode's solution and bubbled in O₂ for 45 min. The lingual epithelium was then peeled from the underlying tissue and pinned out in Tyrode’s in a Sylgard-lined petri dish with the mucosal side facing down. To isolate single taste cells from fungiform and circumvallate taste buds, the lingual epithelium was incubated first in Ca²⁺-Mg²⁺- free Tyrode's containing 2 mM BAPTA (Invitrogen, Eugene, OR) in place of CaCl₂ and MgCl₂ for 10 min. Then the epithelium was rinsed twice with Tyrode's, followed by a second enzyme incubation, using the same enzyme
cocktail for 7 min. After the second enzyme treatment, the epithelium was incubated in a Ca$^{2+}$-Mg$^{2+}$– free Tyrode's for 5 min. Amiloride (10 µM; Sigma Chemical Corp.) was included into all solutions to protect the enzymatic degradation of ENaC (Gilbertson & Fontenot, 1998). Single taste cells were removed from the epithelium using a large bore (~150-200 µm) pipette and plated onto a Cell-Tak (BD Bioscience, Franklin Lakes, NJ) coated microscope coverslides fitted in a laminar flow perfusion chamber (RC-25F Warner Instruments, Hamden, CT) for functional imaging.

Functional sodium (Na$^+$) imaging

Single taste cells from fungiform and circumvallate papillae were isolated as described above and loaded with ~4 µM of N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid (SBFI-AM; Invitrogen, Eugene, OR) diluted in Hanks’ buffer salt solution containing HEPES, sodium pyruvate, 1% pluronic acid F-127 (Invitrogen) and 2% fetal bovine serum (FBS) for 60 min. The cells were perfused with nominal Na$^+$-free Tyrode’s containing (in mM): 140 NMDG, 5 KCl, 1 MgCl$_2$, 1 CaCl$_2$, 10 HEPES, 10 glucose, and 10 Na$^+$ pyruvate, adjusted to pH 7.4 with HCl. Increases in intracellular Na$^+$ were recorded in the presence and absence of physiological saline solution (Tyrode's) containing insulin (20 nM) and/or amiloride (30 µM). Data collection and analyses were performed on an InCyt High Speed I/M$^\text{TM}$ imaging system (Intracellular Imaging Inc., Cincinnati, OH). Images were acquired with a monochrome integrating CCD camera through a
40x oil-immersion objective lens of an inverted Nikon TE-2000s microscope. Excitation wavelengths of 340 nm and 380 nm were emitted by a Benthan FGS 150 fast changing monochromator (Intracellular Imaging Inc.) with an emission wavelength ~510 nm. Images obtained were captured every 3 s by InCyt Im2 software (Intracellular Imaging Inc). Analysis of the SBFI ratio (340 nm/380 nm) was used to determine whether test solutions significantly altered Na⁺ influx in TRCs. Data analyses were carried out by measuring the area under the curve (AUC) of the SBFI ratio in the presence or absence of both amiloride and/or insulin using Origin software (Ver. 7; OriginLab Corp., Northampton, MA).

Quantitative real-time PCR

Taste buds were isolated from the fungiform, cirumvallate and foliate papillae of either diabetic (STZ) or control (Na⁺-citrate) C57BL/6 mice as described above. Taste buds were collected individually and immediately placed into 1.5 ml microfuge tubes with 200 µl RNAlater (Ambion, Austin, TX) on ice. The taste buds were centrifuged at 5000 rpm (3300xg) for 7 min. The resulting pellet was resuspended in lysis buffer from the RNeasy Micro Kit (Qiagen, Valencia, CA), mixed rapidly on a vortex for 2 min, then passed through a prefiltration column (MiniPrefilter column #5188-2736; Agilent, Wilmington, DE) to remove genomic DNA. RNA was then extracted according to the instructions for the RNeasy Micro Kit including a 2 h DNase I treatment. For positive controls, RNA was extracted from approximately 100 mg of kidney tissue using Tri
Reagent (MRC, Inc., Cincinnati, OH) according to the manufacturer’s instructions and qualitatively assessed on a BioAnalyzer (Agilent model 2100, Palo Alto CA).

To quantify α-, β-, and γ-ENaC subunit mRNA levels in fungiform, circumvallate and foliate taste buds, we used a two-tube RT-PCR assay with the PCR step conducted in a real-time thermal cycler (SmartCycler™, Cepheid, Sunnyvale CA). First-strand cDNA synthesis was performed using the i-Script™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules CA). The maximum volume of taste RNA or 50 ng of kidney RNA was used for the reaction in a total volume of 100 µl. Two microliters of cDNA was used for each qPCR reaction. The HotMaster Taq DNA polymerase kit (5 Prime, Gaithersburg, MD) was used, with the following final concentrations: 1 X reaction buffer, 3.5 mM Mg2+, 200 µM dNTPs, primers and probe sets according with manufacturer instructions (Taqman® Gene Expression Assays; ABI, Foster City, CA) and 1.25 U HotMaster Taq. A 2-step PCR protocol was used for α-, β-, and γ-ENaC qPCR assays consisting of a 6 s denaturation step at 95 °C and 60 s annealing and extension at 60 °C.

Using our established procedure (Gilbertson, Liu, Kim, Burks, & Hansen, 2005; Lin, Burks, Hansen, Kinnamon, & Gilbertson, 2004; Liu, Hansen, Kim, & Gilbertson, 2005), we employed a TaqMan (ABI Foster City, CA) detection system in which the primer pairs for channel-specific sequences were multiplexed with the primer pairs for the housekeeping gene, GAPDH (accession no. NM_017008), 5’ TGCACCACCAACTGCTTAG 3’ (sense nucleotides 476-
496), 5’ GGATGCAGG- GATGATGTTC 3’ (antisense 636-654), 5’
ATCAGCCACAGCT-TTCCAGAGGG 3’ (probe 595-618) for direct comparison
of expression levels in the fungiform, circuvallate and foliates taste buds. The
GAPDH probe was labeled with ROX as the reporter fluorophore and BHQ-2 as
the quencher. All α-, β-, and γ-ENaC primers and probes sets were commercially
available Taqman® Gene Expression Assays from ABI.

For quantitative analysis, fluorescent signals in the samples were plotted
against the respective qPCR cycle number. The cycle at which the growth curve
crossed 15 fluorescent units was defined as the cycle threshold (CT). This user-
defined threshold was selected to occur during the log-linear phase of the growth
curve, which is inversely proportional to the starting amount of target in the
sample. Exact cycle thresholds were measured for the each α-, β-, and γ-ENaC
subunits as well as for the housekeeping gene, GAPDH. Delta CT (ΔCT) was
calculated by subtracting the GAPDH CT from the individual ENaC subunit CT.
Comparing ΔCT values allowed for determinator of relative transcript abundance
between different sets of pooled taste buds by normalizing ENaC α-, β-, and γ-
ENaC expression to a constitutively expressed gene. Therefore, the smaller the
ΔCT, the greater the ENaC subunit expression. As previously described (Lin et
al., 2004; Liu et al., 2005), for relative quantification of our samples, the
arithmetic formula $2^{-\Delta \Delta CT}$ was used and takes into account the amount of target,
normalized to an endogenous reference and relative to a calibrator. The α-, β-, or
γ- ENaC subunit with the highest expression (or the lowest ΔCT) for each set of
pooled taste buds was defined as the calibrator for that set. The calculation of \(\Delta\Delta C_T\) involved subtraction of the \(\Delta C_T\) for each channel from the \(\Delta C_T\) calibrator value. The relative amount of target expression was determined according to the following relation:

\[
\Delta C_T^1 = C_T^{\text{ENaC}} - C_T^{\text{GAPDH}}
\]

\[
\Delta C_T^2 = C_T^{\text{CAL}} - C_T^{\text{GAPDH}}
\]

\[
\Delta\Delta C_T = \Delta C_T^2 - \Delta C_T^1
\]

Relative expression = \(1/ (2^{\Delta\Delta C_T})\)

where \(C_T\) is the cycle threshold for the \(\alpha\), \(\beta\), and \(\gamma\)-ENaC or GAPDH determined empirically; \(C_T^{\text{CAL}}\) is the cycle threshold for the calibrator, the most highly expressed channel in each assay.

Behavioral assays

Behavioral studies were performed with a computer-controlled stimulus delivery and lick monitoring stations ("Davis Rig"; model MS-180, DiLog Instruments, Tallahassee, FL). Mice were water deprived overnight during training. Training consisted of one day with a 15-minute exposure to distilled water and a 15-minute time limit to first lick. The second day of training consisted of thirty 10-15 second presentations of distilled water with a 12 second intertrial interval and 150 second time limit to first lick. Following the training period, mice
were allowed free access to water for 24 h and then each animal receive either
STZ (50 mg/kg) or vehicle injection for 2 consecutive days at the same time. To
evaluate the onset of diabetes blood glucose levels were measured as described
in Chapter 2. Both diabetic and vehicle-treated were water restricted for 12 h
prior to the trial. Behavioral assays were performed for one day. Briefly, mice
were presented with two 5 second presentations of each concentration in random
order followed by a 2 second rinse with distilled water. The intertrial interval was
10 seconds with a 150 second time limit for the first lick. Seven NaCl
concentrations (30, 150, 270, 330, 450, 600, and 1000 mM) were tested and
licking responses were measured. In order to determine ENaC role in diabetes
amiloride (100 µM) was added to all NaCl solution including ddH₂O used for
rinses. At the conclusion of the experiment, concentration-response curves
plotting lick rate versus stimulus concentration were generated and analyzed
between test and control mice.

Results

Differences in sodium influx via ENaC in taste
receptor cells from diabetic mice

To determine the effects of Type I diabetes in ENaC activity and salt taste
transduction, we performed a series of functional imaging experiments evaluating
the responses of TRCs from diabetic (STZ-treated) mice to NaCl. We recorded
from >200 taste receptor cells using a Na⁺ sensitive dye (SBFI). Fungiform taste
cells loaded with SBFI were analyzed by ratiometric changes in fluorescence that occur when each testing solution was applied. To calculate the magnitude of each response we obtained the area under the curve (AUC) of changes in the 340/380 ratio for each testing solution. In all cases, we recorded ratiometric SBFI changes in response to 140 mM Na⁺ in the presence or absence of amiloride (30 µM). Conservatively, we classified amiloride sensitive cells as only those cells that showed a reversible decrease in SBFI ratio due to inhibition of ENaC by amiloride. Both diabetic (STZ-treated) and control (Na-citrate) TRCs evoked responses to 140 mM Na⁺ and amiloride inhibited the magnitude (i.e. AUC) of these responses. These data are summarized in Table 4.2.

Previously we found that insulin increases Na⁺ influx via ENaC in TRCs (cf. Chapter 3). Thus, insulin/ENaC-mediated responses may be functionally important in salt taste transduction (Fig. 4.1). To investigate if insulin effects on salt taste transduction may be altered during the onset of diabetes, we performed a series of experiments using a mouse model of Type I (STZ-induced) diabetes. Similar to our initial study, insulin enhanced Na⁺ transport from 36.8 ± 5.5 (AUC) to 56.3 ± 6.7 in fungiform taste cells when compared with the control group (Fig. 4.2). Additionally, insulin-mediated stimulation of Na⁺ influx in TRCs was effectively blocked by amiloride (30 µM) showing a reduction in NaCl responsiveness from 56.3 ± 6.7 (AUC) to 22 ± 2.8. This suggests insulin effects on salt taste transduction are largely, if not exclusively, through apical amiloride-sensitive ENaC (Fig. 4.2B). Repeated measures ANOVA analysis reveals
significant difference between 140 mM NaCl and 140 mM NaCl+amiloride (p< 0.001); insulin enhancement of NaCl responses was statistically significant (p<0.001) and amiloride significantly decreased insulin stimulated Na\(^+\) movement (p<0.001).

In contrast, insulin's enhancing effects on Na\(^+\) movement via ENaC are abolished in diabetic animals. Though functional ENaC channels are expressed in diabetic taste cells because Na\(^+\)-induced responses were reduced from 42.4 ± 2.3 (AUC) to 20.9 ± 1.3 by the presence of amiloride (30 µM; Fig. 4.3). Interestingly, we observed that Na\(^+\)-induced responses were reduced from 42.4 ± 2.3 (AUC) to 35.2 ± 2.2 in the presence of insulin. However, amiloride sensitivity remained after insulin treatment since insulin-mediated responses were blocked by amiloride (35.2 ± 2.2 to 18.4 ± 1.0). Our findings implied that regulation of ENaC by insulin is severely impaired in TRCs from diabetic mice. Moreover, repeated measures ANOVA analysis disclosed a significant difference between 140 mM Na\(^+\) and NaCl+amiloride (p<0.001); insulin treatment showed significant differences from 140 mM Na\(^+\) (p<0.001); and addition of amiloride significantly decreases Na\(^+\) transport via ENaC (p<0.001).

In these experiments, we did observe that a subset of cells from diabetic mice (~16%; n=100) evoked an increase in Na\(^+\) influx by insulin treatment from 45 ± 5.4 (AUC) to 63.7 ± 7.2 (Fig. 4.4). For diabetic fungiform TRCs that were sensitive to insulin, we observed inhibition of 140 mM Na\(^+\) responses from 45 ± 5.4 (AUC) to 22.7 ± 3.8 by amiloride and insulin-mediated Na\(^+\) transport was also
diminished from 63.7 ± 7.2 (AUC) to 35 ± 4.0 by amiloride (Fig 4.4B). Repeated measures ANOVA analysis showed statistical difference between 140 mM Na⁺ responses ± amiloride treatment (p<0.001) and insulin's effects on Na⁺ movement were also significant (p<0.001). Application of amiloride significantly reduced the effects of insulin on Na⁺ movement (p<0.001).

To look for differences in the magnitude of 140 mM Na⁺ ± insulin responses among diabetic and control TRCs, we performed simple effects ANOVA analysis. Analysis revealed the magnitude of 140 mM Na⁺ responses as significantly different between diabetic and control taste cells (p=0.046). Additionally, insulin’s effect on Na⁺ movement was also significantly different between diabetic and control (p<0.001). The proportion of amiloride-sensitive fungiform taste cells found in this study from both diabetic and control TRCs is summarized in Table 4.3.

Characterization of ENaC responses in the posterior tongue of diabetic mice

Following up on our initial findings in diabetic fungiform taste cells, we hypothesized that the loss of ENaC regulation by insulin associated with Type I (insulin-dependent) diabetes may also alter the regulation of Na⁺ transport in the posterior tongue. To determine whether insulin-mediated Na⁺ influx is deficient in diabetic circumvallate taste cells, we used functional Na⁺ imaging. Both diabetic and control circumvallate TRCs were preloaded with SBFI and were tested in the
presence or absence of 140 mM Na\(^+\) (i.e. salt stimulus) with or without insulin (20 nM) and/or amiloride (30 µM). As shown in Fig. 4.5, circumvallate TRCs from control mice showed both amiloride sensitivity as well as an increase in Na\(^+\) transport by insulin. Overall, 140 mM Na\(^+\) responses were blocked by amiloride, reducing the Na\(^+\) response from 26.4 \(\pm\) 5.4 (AUC) to 17.3 \(\pm\) 2.6. Insulin-stimulated Na\(^+\) influx was also decreased by amiloride, from 45.2 \(\pm\) 6.7 (AUC) to 24.6 \(\pm\) 2.7 (Fig. 4.5B). Repeated measures ANOVA analysis showed significant differences between 140 mM Na\(^+\) and Na\(^+\) + amiloride (p=0.039). Insulin-stimulated Na\(^+\) movement was significantly higher than 140 mM Na\(^+\) (p=0.004). Inhibitory effects of amiloride on insulin-stimulated Na\(^+\) transport was significantly different (p=0.002). Clearly, mouse circumvallate taste cells expressed functional ENaC and insulin enhances Na\(^+\) influx via ENaC.

In contrast, the ability of insulin to stimulate Na\(^+\) transport through ENaC is absent in mammalian taste cells from diabetic mice. Consistent with our observations in fungiform papillae of the diabetic mice, circumvallate TRCs from diabetic mice exhibited functional amiloride-sensitive Na\(^+\) transport since 140 mM Na\(^+\) responses evoked by diabetic circumvallate taste cells were inhibited by amiloride from 60.0 \(\pm\) 5.3 (AUC) to 19.1 \(\pm\) 1.8 (Fig. 4.6). Interestingly, insulin lacks any ability to enhance Na\(^+\) movement in circumvallate taste cells of diabetic mice as shown by the fact that the magnitude of 140 mM Na\(^+\) responses (60.0 \(\pm\) 5.3; AUC) is unchanged in the presence of insulin (51.8 \(\pm\) 6). In all experiments, diabetic circumvallate TRCs were also tested with insulin (20 nM) + amiloride (30
µM) and we found that amiloride similarly inhibited Na⁺ transport in control and insulin-treated cells (51.8 ± 6 to 20.2 ± 1.7; Fig. 4.6A and Fig. 4.6B). These results suggest that insulin-mediated Na⁺ influx observed in the control group is mostly through ENaC and it is absent in circumvallate TRCs from diabetic mice. Repeated measures ANOVA between 140 mM Na⁺ and 140 mM Na⁺ + amiloride (30 µM) showed significant differences (p<0.001). There was no significant difference in insulin treatment, however, the effects of amiloride on insulin treated cells was significantly different (p< 0.001; Fig. 4.6B).

To determine any difference in the magnitude of Na⁺ responses and/or insulin treatment responses among diabetic and control animals, we performed statistical analysis using simple effects ANOVA. The analysis revealed that the magnitude of 140 mM Na⁺ responses evoked by circumvallate TRCs were significantly higher in the diabetic group compared to controls (p<0.001). We found no significant difference with insulin responses between both groups. There were no significant differences in amiloride inhibition between diabetic and control TRCs before or after insulin treatment. The relative proportion of circumvallate taste cells containing functional amiloride-sensitive ENaC in both diabetic and control groups is summarized in Table 4.3.
Diabetic-induced changes in Na\(^+\) movement are linked to ENaC expression

Given our functional Na\(^+\) imaging data, we have hypothesized that ENaC expression may be altered in diabetic taste cells accounting for the difference in Na\(^+\) transport between control and diabetic mice. Simply, TRCs in both groups showed functional amiloride-sensitive ENaC but differ in the magnitude of Na\(^+\) influx, especially in circumvallate taste cells. In addition, insulin enhancement of Na\(^+\) influx via ENaC was abolished in TRCs from diabetic mice. We propose that the functional differences observed in imaging experiments may lie in the ratio of expression of \(\alpha\)-, \(\beta\)-, and \(\gamma\)-ENaC subunits from diabetic and non-diabetic animals. This would then suggest that the gustatory system adapts to metabolic disorders, perhaps in an effort to restore the body’s normal salt and water balance.

We used quantitative real-time PCR to look for differences in \(\alpha\)-, \(\beta\)- and \(\gamma\)-ENaC subunits from fungiform, circumvallate and foliate TRCs in control and diabetic mice. Using a TaqMan detection system and Taqman Gene Expression Assays (ABI) for \(\alpha\)-, \(\beta\)-, and \(\gamma\)-ENaC in reactions multiplexed with primers pairs for the housekeeping gene, GAPDH. We measured relative expression for each ENaC subunit in control and diabetic. As shown in Fig. 4.7 for fungiform taste cells, all 3 ENaC subunits were more highly expressed in diabetic than in control mice. Expression of \(\gamma\)-ENaC subunit was greater in circumvallate taste cells from diabetic group compared to the control group. Similar results were observed in
foliate TRCs in which γ-ENaC subunit was more highly expressed in the diabetic than in control group. In contrast, there was no apparent difference in expression of α- and β-ENaC subunits in the posterior mouse tongue between control and diabetic mice.

Differences NaCl preference in diabetic mice

Diabetes Mellitus is a disease that decreases taste acuity and impairs taste preference. Current evidence has shown that diabetic animals have variations in behavioral responses to sweet and other foods, including fat (Bellush & Rowland, 1985; Le Floch et al., 1989; Matsuo et al., 2003; Ninomiya et al., 1998; Smith & Gannon, 1991). In addition, it has been suggested that hypertensive patients with Type 2 diabetes (non-insulin dependent) might exhibit alterations in salt taste (Isezuo, Saidu, Anas, Tambuwal, & Bilbis, 2008). Based on our functional imaging and molecular results in which Na⁺ transport and α- β- and γ-ENaC expression differ between diabetic and non-diabetic mice, we next performed a series of brief access behavioral tests to determine the effects of diabetes in salt taste preference. We hypothesized that the peripheral gustatory system responds to nutritional challenges, such as diabetes, and alters taste-guided behavior.

We evaluated NaCl preference using computer-controlled lickometer in diabetic and control mice. C57BL/6 mice were trained and then divided into two groups, diabetic or control. Each mouse received two injections of either STZ (50
mg/kg) or vehicle and then blood glucose levels were measured to assess the development of diabetes. After blood glucose levels reached above 300 mg/dl in the STZ-treated group, we considered these values representative of a successful diabetic induction. Consequently, diabetic animals were separated among two subgroups: (1) Diabetic with moderate hyperglycemia (300-375 mg/dl) and (2) Diabetic with severe hyperglycemia (375-566 mg/dl; Fig. 4.8B). Diabetic mice with moderate hyperglycemia display significant avoidance to NaCl solutions at concentrations lower than 600 mM compared to non-diabetic littermates (Fig. 4.8A). Interestingly, diabetic mice with severe hyperglycemia exhibited completely different taste behavior compared to the diabetic group with moderate hyperglycemia. This subgroup of diabetic mice with blood glucose levels above 375 mg/dl appears to overcome the taste aversion behavior to NaCl solutions. There was especially evident at high NaCl concentrations (i.e. 600 mM) in which diabetic mice with severe hyperglycemia showed greater preference than the other two groups (Fig. 4.8A). Simple effects ANOVA revealed significant difference between diabetic mice, with moderate hyperglycemia, and control mice at NaCl concentrations of 150 mM (p=0.040), 270 mM (p=0.037), and 450 mM (p=0.018). Likewise, we found significant difference for NaCl preference at 300 mM (p=0.001), 450 mM (p=0.009) and 600 mM (p<0.001) within the both diabetic subgroups. In addition, NaCl preference at 600 mM was significantly different between diabetic mice with severe
hyperglycemia and non-diabetic mice (p=0.013). Clearly, diabetic mice have a
dramatic impairment for NaCl preference.

To test the functional role of ENaC in changes of salt taste perception in
diabetic mice we performed a series of short term behavioral assays using
amiloride (100 µM). Oral administration of amiloride reduces taste sensitivity to
NaCl by virtue of its antagonistic effect on ENaC (Eylam & Spector, 2003). We
added amiloride to all NaCl solutions. Amiloride was also included to ddH2O used
for water stimulus and rinses to eliminate any possible taste cues from amiloride
during the behavioral assays. Diabetes-induced differences in NaCl detection
were attenuated by amiloride. Both of the diabetic subgroups showed an
increase in NaCl preference that was not observed in the previous experiment
without oral amiloride (Fig. 4.9A). Thus, there was a difference in NaCl detection
between non-diabetic and diabetic mice with moderate hyperglycemia at
concentrations lower than 330 mM. In addition, diabetic mice with severe
hyperglycemia exhibited an elevation in NaCl preference at concentrations below
270 mM (Fig. 4.9A). Statistical analysis using simple effects ANOVA revealed
significant difference between diabetic mice with moderate hyperglycemia and
non-diabetic mice at 30 mM (p=0.042) and 270 mM (p=0.035) NaCl. Moreover,
the second subgroup, diabetic mice with severe hyperglycemia revealed a
significant difference at 270 mM NaCl (p=0.026). In addition, both diabetic groups
show significant differences at the following NaCl concentrations: 30 mM
Blood glucose levels were different among all three groups (Fig. 4.9B).

Discussion

Glucosuria, polydipsia and polyuria are defining characteristics of Type I diabetes. These changes in the salt and water balance result in osmotic diuresis, which force the kidney to adapt to the salt and water losses. Moreover, as glucosuria advances, a number of adaptive changes in gene expression and protein abundance of ENaC expressed along the renal tubule occurs (Bickel et al., 2002; Song et al., 2003). Because of the similarities between salt and water transport in the taste system and kidney, we initially began to investigate changes in ENaC expression and its function that may occur in the peripheral gustatory system during the onset of diabetes. In the present study, we have demonstrated adaptive changes in ENaC expression and function in mammalian taste receptor cells from Type I diabetic mice and, based upon our behavioral assays, hypothesize that ENaC expressed in the gustatory system plays a role in the compensatory mechanisms which are activated to stabilize the diabetic decompensation.

The importance of dietary patterns in diabetes is well established. For instance, diabetic rodents, which are allowed to free selection of diets, exhibited regression of many diabetic disturbances such as glucosuria (Tepper & Kanarek,
In addition, a variety of studies have shown similar results in which diabetic animals regulate their dietary intake to attenuate the effects of the disease (Bartness & Rowland, 1983; Kanarek & Ho, 1984; Vartiainen & Bastman-Heiskanen, 1950). These reports are consistent with the idea that the animal is capable of adjusting the pattern of nutrient choice, perhaps through changes in nutrient recognition (i.e. taste), to compensate for metabolic abnormalities. Using functional Na⁺ imaging, we showed that both control and diabetic taste receptor cells from fungiform and circumvallate taste buds evoked responses to a salty stimulus, 140 mM NaCl. Both cell types exhibited amiloride sensitivity suggesting the presence of functional ENaC (Tables 4.2 and 4.3).

Insulin plays a crucial physiological role in the regulation of Na⁺ movement through ENaC in both taste and kidney epithelia. Both ENaC activity and apical membrane insertion are stimulated by insulin. Therefore, the current proposed model is that insulin binds its cognate receptor in the cell membrane and produces a subsequent phosphorylation of insulin receptor substrate (IRS). This results in activation of PI3-kinase. PI3-K forms PtdIns(3,4,5)P₃ in the inner leaflet of plasma membrane and these changes in the lipid composition provide a direct mode for activation of ENaC. An alternative pathway is when PtdIns(3,4,5)P₃ activates the phosphatidylinositol-dependent protein kinase (PDK1). PDK1, activates serum and glucocorticoid–regulated kinase (SGK) which leads to both ENaC activation and insertion of the channel in the apical membrane (Blazer-Yost, Vahle, Byars, & Bacallao, 2004; Staruschenko,
Pochynyuk, Vandewalle, Bugaj, & Stockand, 2007; Tong, Gamper, Medina, Shapiro, & Stockand, 2000). Our preliminary findings have demonstrated insulin/ENaC-mediated responses are through the activation of PI3-kinase signaling cascade (unpublished data). In the present study, we have shown that the ability of insulin to enhance Na⁺ via ENaC is absent in TRCs from diabetic mice in both fungiform and circumvallate papillae (Figs. 4.3 and 4.6). Interestingly, the magnitude of Na⁺ influx was significantly increased in fungiform and circumvallate TRCs from diabetic mice compared to the control. Clearly, Type I diabetes abolishes ENaC regulation by insulin in the gustatory system, perhaps owing to the fact that diabetes leads to activation of the system to near maximum levels. However, further research needs to be done to clarify action of the lack of insulin in diabetic TRCs to determine whether insulin signaling transduction leading to ENaC activation would still be intact in mammalian taste cells from diabetic mice. In addition, we found that insulin stimulates Na⁺ influx in approximately 16% (n= 100) of fungiform diabetic cells. The significance of these results in fungiform cells as well as exact function however, remains unclear and more studies are needed to clarify these findings.

This study provides, to our knowledge, the first functional results of ENaC activity during the onset of diabetes in a mouse model of Type I diabetes. Since we hypothesized that ENaC expressed in TRCs will adapt to the metabolic abnormalities associated with the onset of diabetes, the gustatory system may play a role in the restoration of homeostasis within the body. To test this
hypothesis we explored the underlying differences among α-, β-, and γ-ENaC subunits in TRCs from diabetic and control animals using qRT-PCR analysis on α-, β-, and γ-ENaC subunits in all 3 types of taste buds. The molecular assays revealed that α-, β-, and γ-ENaC subunits were generally greater in fungiform taste cells from diabetic mice (Fig. 4.7). These results were supported by our functional Na\(^+\) imaging data showing a significantly greater magnitude of Na\(^+\) influx in diabetic mice. The differences for ENaC subunits in the posterior mouse tongue were most evident for γ-ENaC subunits among diabetic and control groups. Similar results have been observed in kidney in which γ-ENaC subunit protein abundance was significantly increased in animals with severe hyperglycemia (Song et al., 2003). In addition, the γ-ENaC subunit is involved in hormonal regulation of the channel since PtdIns(3,4)P\(_2\)/ PtdIns(3,4,5)P\(_3\) binding sites in ENaC are located in the COOH-terminal cytosolic domain of γ-ENaC subunit (Pochynyuk, Staruschenko, Tong, Medina, & Stockand, 2005). In the present study we have limited our investigation to the effects of insulin on ENaC function during the onset of diabetes. Though one possible explanation for a high expression of γ-ENaC subunit in diabetic TRCs may be that other hormones such as aldosterone and vasopressin stimulate ENaC gene expression in the taste system during diabetes (Gilbertson et al., 1993; Helms et al., 2005; Nicco et al., 2001; O'Neill et al., 2008) Moreover, plasma aldosterone levels have been reported to be enhanced in the early stages of diabetes (Song et al., 2003).
Clearly much more research is needed to identify compensatory mechanisms in the gustatory system during the onset of diabetes.

To relate the alterations of ENaC observed in our functional and molecular studies to the animal’s behavior, we performed a series of short-term behavioral assays to characterize NaCl preference in diabetic mice. Since ENaC plays a central role in the identification of NaCl in TRCs, one prediction from this data is that ENaC expressed in taste epithelia would adapt to the severe salt and water imbalance associated with diabetes and will vary gustatory preference to sodium salts. Using a computer-controlled lickometer we showed the ability of diabetic mice to recognize NaCl was altered. In this test, diabetic mice with moderate hyperglycemia (300-375 mg/dl) exhibited less preference for NaCl solutions at concentrations below 600 mM. This effect on NaCl may be attributed to the possibility that as the body’s osmolarity increases, a number of adaptive changes in gene expression and protein abundance of ENaC as well as other Na\(^+\) transporters expressed in taste and kidney epithelia occurs (Bickel et al., 2002; Oh et al., 2007; Song et al., 2004; Vidotti et al., 2008). Diabetic mice with severe hyperglycemia (375-566 mg/dl) overcome the aspects of high NaCl solutions. In particular, this subgroup with severe hyperglycemia displayed greater NaCl preferences than diabetic mice with moderate hyperglycemia. In contrast, the lick ratio for NaCl at 330 mM and 600 mM was dramatically enhanced in diabetic animals with severe hyperglycemia.

To elucidate ENaC involvement in salt taste during the onset of diabetes,
amiloride, a specific blocker of ENaC, was added into all solutions to reduce sensitivity to NaCl solutions. Our results showed that NaCl avoidance was diminished in both diabetic and control groups due to amiloride effects in blocking ENaC (Figs. 4.8 and 4.9). Taken together, our results from brief access behavioral assays are consistent with the prediction, based upon adjustments in ENaC expression and function detected in functional imaging and qRT-PCR experiments. It appears that with these changes the gustatory system may play a role in the restoration of salt balance during early stage diabetes by enhancing the avoidance of salt, a physiological response that dovetails well with the diabetic decompensation. Several previous reports in animals and humans have shown that diet selection, including drinking behavior, may help to improve diabetic symptoms (Bartness & Rowland, 1983; Hardy, Brennand, & Wyse, 1981; Isezuo et al., 2008; Kanarek & Ho, 1984; Le Floch et al., 1989; Miller, 1997; Smith & Gannon, 1991; Tepper & Kanarek, 1985; Vartiainen & Bastman-Heiskanen, 1950; Yirmiya et al., 1988). It should be taken into account that the changes in Na⁺ transport via ENaC in mammalian taste receptor cells from Type I diabetic mice and its role, salt and water homeostasis would thus be predicted on the assumption that other hormones (i.e. aldosterone and vasopressin) may contribute to control salt appetite.
REFERENCES


TABLE 4.1. Comparison of the area under the curve (AUC) in response to 140 mM NaCl ± amiloride (30 μM) in C57BL/6 mice, control (Na citrate-injected) mice, and diabetic (STZ-treated) mice using functional Na⁺ imaging with SBFI.

<table>
<thead>
<tr>
<th></th>
<th>Number of cells tested</th>
<th>140 mM NaCl</th>
<th>140 mM NaCl + amiloride</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>22</td>
<td>30.5 ± 2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.3 ± 2.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Control</td>
<td>59</td>
<td>36.8 ± 5.5</td>
<td>22.4 ± 3.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetic</td>
<td>59</td>
<td>42.4 ± 2.3</td>
<td>20.9 ± 1.3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data shown are (mean ± SEM) of the area under the curve determined by changes in SBFI ratio during application of either 140 mM NaCl ± amiloride (30 μM) as shown in Fig. 4.1, Fig. 4.2, and Fig. 4.3.

<sup>a</sup>Reported in Chapter 3 for 22 cells.

Significant values (P) for the comparison of amiloride inhibition between 140 mM NaCl and 140 NaCl+amiloride were determined using repeated measures ANOVA analysis.
TABLE 4.2. Relative proportion of taste cells containing functional amiloride-sensitive ENaC in both control (Na-citrate) and diabetic (STZ-treated) mice.

<table>
<thead>
<tr>
<th>Taste bud type</th>
<th>Control</th>
<th>Diabetic</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungiform</td>
<td>60% [98]</td>
<td>85% [86]</td>
<td>0.011</td>
</tr>
<tr>
<td>Circumvallate</td>
<td>63% [30]</td>
<td>92% [25]</td>
<td>0.090</td>
</tr>
</tbody>
</table>

Data shown reflect the percentage of cells containing functional amiloride-sensitive ENaC determined by the reversible decrease in SBFI ratio seen during the application of amiloride (30 μM) shown in Fig 4.2, Fig. 4.3, Fig. 4.4, and Fig. 4.5. Numbers in square brackets refers to the number of cells tested.

Significant values (P) for the comparison of the percentage of cells containing amiloride-sensitive ENaC between control and diabetic were determined using chi-square test. $X^2$ values for fungiform and circumvallate were 6.472 and 2.882 respectively.
Fig 4.1. Regulation of ENaC by insulin in mouse taste receptor cells. (A), our earlier functional Na\(^+\) imaging experiments using the Na\(^+\)-sensitive dye (SBFI) showed that insulin activates the amiloride-sensitive ENaC channel, which is expressed in mouse taste cells, and, therefore, increases Na\(^+\) influx. (B), proposed model of insulin signal transduction leading to ENaC activation in the peripheral gustatory system. According to this model, insulin binds its cognate receptor in the cell membrane, subsequently phosphorylating insulin receptor substrate (IRS), thereby activating PI3-kinase (PI3-K). PI3-K forms PtdIns(3,4,5)P\(_3\) (PIP\(_3\)) in the inner leaflet of plasma membrane; therefore these changes in the lipid composition provide a mode for direct activation of ENaC.
Fig. 4.2. Na⁺-citrate buffer treated mouse taste cells showed normal salt transduction mechanisms. (A), functional Na⁺ imaging using the Na⁺-sensitive dye, SBFI, in fungiform taste cells from a non-diabetic mouse. Taste cells like this one showed an enhancement of Na⁺ influx in response to insulin. (B), summary graph from Na⁺-citrate-treated taste cells showing changes in the area under the curve for 140 mM NaCl responses in the absence and presence of insulin (20 nM), as well as before and after amiloride (30 μM) treatment. Data are shown mean ± SEM. a & c denote significant reduction in SBFI ratio; b, denotes significant increase in SBFI ratio compared with 140 mM NaCl (p<0.01, ANOVA).
Fig. 4.3. Insulin’s effects on amiloride-sensitive fungiform cells were absent in the diabetic mice. (A), functional Na⁺ imaging from diabetic fungiform taste cells in the absence and presence of insulin (20 nM) and before and after amiloride (30 µM) treatment. Dotted line denotes the amplitude of the 140 mM Na⁺ response. (B), summary graph of the area under the curve of 140 mM NaCl responses in the presence and absence of insulin (20 nM) and subsequent application of amiloride (30 µM). Data are shown mean ± SEM. a, b, and c denotes significant reduction in SBFI ratio compared with 140 mM NaCl (p<0.01, ANOVA).
Fig 4.4. Insulin stimulates Na⁺ movement in a subset of STZ-treated cells. (A), functional Na⁺ imaging from diabetic fungiform taste cells in the absence and presence of insulin (20 nM) and before and after amiloride (30 µM) treatment. Dotted line denotes the amplitude of 140 mM Na⁺ response. (B), summary graph comparing insulin effects in fungiform taste cells from a diabetic mouse before and after amiloride (30 µM) treatment. Data are shown mean ± SEM. a & c denote significant reduction in SBFI ratio; b, denotes significant increase in SBFI ratio compared with 140 mM NaCl (p<0.01, ANOVA).
Fig. 4.5. Insulin effects on Na\(^+\) influx are similar in the posterior tongue of Na\(^+\)-citrate-treated mice. (A), circumvallate taste cells from non-diabetic mice evoked greater responses to 140 mM Na\(^+\) in the presence of insulin. These insulin-mediated Na\(^+\) responses were generally inhibited by amiloride (30 \(\mu\)M). Insulin effects on Na\(^+\) movement are similar in both circumvallate and fungiform taste cells from non-diabetic mice. (B), summary graph of the area under the curve from non-diabetic mice in the absence and presence of insulin (20 nM) and before and after amiloride (30 \(\mu\)M). Values shown are mean ± SEM. a & c denotes significant reduction in SBFI ratio; b, denotes significant increase in SBFI ratio compared with 140 mM NaCl (p<0.01, ANOVA).
Fig. 4.6. The magnitude of Na\(^+\) influx via ENaC is increased in the diabetic mice. (A), functional Na\(^+\) imaging from diabetic fungiform taste cells in the absence and presence of insulin (20 nM), before and after amiloride (30 µM) treatment. Circumvallate taste cells from diabetic mice evoke greater responses to 140 mM NaCl though insulin’s effects on amiloride-sensitive Na\(^+\) influx was abolished. Dotted line denotes the amplitude of 140 mM Na\(^+\) response (B), area under the curve graph from diabetic circumvallate cells showing the magnitude of amiloride-sensitive Na\(^+\) responses. Data are shown mean ± SEM. a & b denote significant reduction in SBFI ratio compared with 140 mM NaCl (p<0.01, ANOVA).
Fig. 4.7. Expression of α-, β-, γ-ENaC subunits from fungiform, circumvallate and foliate taste receptor cells in control and diabetic mice. The relative expression of (α-, β-, and γ-) ENaC subunits was calculated as described in methods. These results showed that one or more ENaC subunits are more highly expressed during the onset of diabetes, which dovetails nicely with differences in the magnitude of Na⁺ influx via ENaC observed in functional Na⁺ imaging studies.
Fig. 4.8. Diabetes impairs salt taste performance in mice. (A), lick ratio of NaCl/water (mean ± SEM) measured in short term taste assays using the Davis Rig in three groups of 21 mice. Diabetic animals have a significant avoidance to lower concentrations of NaCl solutions. Mice with severe hyperglycemia (i.e. blood glucose > 375 mg/dL; dark gray triangles) overcome the aversive aspects of high NaCl solutions. a, b and c denote significant difference of NaCl preference between both diabetic groups and control (p<0.01; simple effects ANOVA). (B), values shown are mean ± SEM for blood glucose levels (mg/dl) in each group. Asterisks denotes significant difference in blood glucose levels; * control vs. diabetic with moderate hyperglycemia (300-374mg/dl); ** control vs.
diabetic with severe hyperglycemia (375-566mg/dl). Blood glucose levels between diabetic with moderate hyperglycemia and diabetic with severe hyperglycemia was found to be statistically significant by one-way ANOVA (p<0.001).
Fig. 4.9. Diabetes-induced avoidance of NaCl is attenuated by amiloride. Amiloride (100 µM), a diuretic which inhibits ENaC, was added into all solutions in these short term behavioral assays. (A), preference ratio shown are (mean ± SEM) and each point represents 7 mice for each group. The presence of amiloride (100 µM) significantly reduced salt avoidance in both diabetic and non-diabetic groups due to the effects of amiloride in blocking the ENaC channel (compare to Figure 4.8). Interestingly, there were only differences in lick ratio between all three groups at lower NaCl concentrations (<270 mM). a, b and c denote significant difference of NaCl preference between both diabetic groups.
and control (p<0.01; simple effects ANOVA). (B), data are shown in mean ± SEM for blood glucose levels (mg/dl) in each group. Asterisks denote significant difference in blood glucose levels: * control vs. diabetic with moderate hyperglycemia (300-374 mg/dl); ** control vs. diabetic with severe hyperglycemia (375-566 mg/dl). Blood glucose levels between diabetic with moderate hyperglycemia and diabetic with severe hyperglycemia was found to be statistically significant by one-way ANOVA (p<0.001).
CHAPTER 5
SUMMARY AND FUTURE DIRECTIONS

The primary goal of this dissertation work was to explore three specific aims centered on the mechanisms regulating sodium salt transduction via epithelial sodium channels (ENaC) in mammalian taste receptor cells. The experiments outlined in Chapter 2 tested the hypothesis that ENaC expressed in taste epithelia is regulated by Cl⁻ ions. The results demonstrated that taste cells expressed a variety of Cl⁻ transporting proteins and that Cl⁻ movement throughout these proteins inhibited the amiloride-sensitive Na⁺ current carried by ENaC. The second aim dealt with the regulation of ENaC by insulin. The data in Chapter 3 provided evidence of a novel signaling pathway involved in ENaC regulation by insulin in the gustatory system. The third aim was designed to test the hypothesis that diabetes causes changes in salt transport by altering expression and/or function of ENaC. The findings in Chapter 4 established the first pathophysiological report detailing disease-related changes in the gustatory system. Using a multidisciplinary experimental approach, I was able to demonstrate alterations in expression and function of ENaC from the gene level, through cell-based assays and eventually to the animal's behavior. In the course of these experiments it became clear that some of the original outlined experimental designs and selected animal models were not the best way to test
these hypotheses in mouse taste receptor cells. Listed below are some of the major findings and conclusions from my dissertation project.

The physiological role of ENaC in identification of sodium salts is well understood. Previous studies in our laboratory have shown that ENaC expressed in taste epithelia is highly regulated by ions and hormones. In the regulation of ENaC by ions, I studied specifically the interaction between ENaC and chloride channels (Chapter 2). In this chapter, electrophysiological experiments using low intracellular chloride (10 mM) identified a relationship between intracellular chloride concentration and Na⁺ influx via ENaC. In addition, most of our previous knowledge about the magnitude of amiloride-sensitive currents came from studies in which ENaC currents were recorded with high intracellular Cl⁻ (140 mM) in the recording pipette (Doolin & Gilbertson, 1996; Gilbertson & Fontenot, 1998; Gilbertson, Roper, & Kinnamon, 1993). In agreement with these results, it would be interesting to revise previous studies in mechanism of NaCl adaptation (Gilbertson & Zhang, 1998). Self-inhibition is a common mechanism of NaCl adaptation in which ENaC activity is decreased by extracellular Na⁺. It appears that Cl⁻ regulation of ENaC may contribute to NaCl adaptation at the cellular level. Single channel recording of ENaC showed that low cytosolic Cl⁻ increases ENaC conductance and open probability. In contrast, Na⁺ inhibition of ENaC only changes ENaC open probability (Gu, 2008).

Using RT-PCR I identified 5 subtypes of ClC channels, CFTR and NKCC1 in TRCs. In addition, functional Cl⁻ imaging experiments with MQAE
exhibited that both fungiform and circumvallate taste cells are capable of transporting Cl⁻ ions across the cellular membrane. The functional and molecular characterization of chloride channels suggests that taste cells contained all the potential molecular candidates to increase intracellular chloride concentration and reduce ENaC activity. Based on these results, I wanted to determine whether chloride channels expressed in taste cells are able to raise intracellular chloride concentration and reduce ENaC activity. I performed a number of experiments using both a dual channel voltage clamp to record short-circuit (transepithelial) current across enzymatically isolated lingual epithelia from mouse tongue and whole cell patch clamping recording in isolate taste cells. The general results from this study are: (1) sodium transport in mouse lingual epithelia is regulated by increasing [Cl⁻]; (2) The magnitude of Na⁺-induced I_{SC} in lingual epithelia remains apparently equal after 60 mM Cl⁻; and (3) Specific pharmacological blockers of ClC channels and NKCC1 increase amiloride-sensitive currents in taste cells. These findings provided evidence that amiloride-sensitive ENaC currents in taste cells depend upon cytosolic Cl⁻ concentration. Taking all my results together from Chapter 2, I proposed a model for ENaC regulation in the peripheral gustatory system (Fig. 5.1).

Previous data has shown ClC₃ is a viable candidate of the CIC family for mediating I_{Cl,swell} (Gilbertson, 2002). I found, however, in the current study that ClC₃ is expressed in all three types of taste buds. Based upon these results, I attempted to identify ClC₃ protein expression in circumvallate taste.
Unfortunately, Western blot results for ClC₃ antibody were inconclusive, because the isolated immunoreactive protein has a molecular weight approximately of 154 KDa, which is different with the predict size of the ClC₃ protein of 84.5 KDa (Majid, Brown, Best, & Park, 2001). One possible justification is that there are several isoforms of ClC₃ protein in the taste system (Ogura et al., 2002). In addition, immunocytochemical techniques also failed to located ClC₃ protein in taste cells (Miyamoto, Fujiyama, Okada, & Sato, 1998). To identify the protein responsible for I_{Cl, swing} more research is needed.

Another interesting finding is the involvement of cytosolic Cl⁻ in osmoregulation of ENaC gene expression. Tissues under physiological conditions are routinely exposed to variable extracellular osmolarity that require cellular responses to maintain appropriate cellular volume and osmolarity. Hypotonicity, which is an example of osmolarity changes, causes initial cell swelling followed by regulatory volume decrease (RVD) to return cell volume to the original volume. RVD is a mechanism that decreases cytosolic Cl⁻ concentration, which in turn increases α-ENaC mRNA expression and ENaC activity (Niisato, Eaton, & Marunaka, 2004). Electrophysiological studies in the gustatory system showed that taste cells are capable of RVD by increasing the Cl⁻ conductance during cell swelling. Thus, these hypoosmotic induced currents were eliminated by Cl⁻ channel blockers (Gilbertson, 2002). Recently, our laboratory identified several aquaporin (AQP) channels in taste tissue which provided a molecular route for rapid water movement in the taste (Watson et al.,
The oral cavity is exposed to a variety of solution tonicities, ranging from extremely hypoosmotic (distilled water rinses) to high-salt solutions, that may be an order of magnitude greater than normal salivary ion concentrations (Feldman & Barnett, 1995). Due to this variety, it would be reasonable to evaluate the effects of hypoosmotic and hyperosmotic responses in salt transduction via ENaC.

ENaC function and activity are essential for local movement of electrolytes and water across epithelial barriers, as well as control of total body electrolyte and water homeostasis. In contrast, dysfunction and aberrant regulation of ENaC lead to a variety of diseases, including hypertension and hypotension (Pochynyuk, Tong, Staruschenko, & Stockand, 2007). Because the second and third aims of this dissertation deal with ENaC regulation by hormones, I have attempted in Chapter 3 to record insulin responses in TRCs. I found that insulin alters amiloride-sensitive currents. It has previously been shown that hormones (i.e. vasopressin) stimulate amiloride-sensitive ENaC currents in taste cells presumably by the action of V2 receptors in the cell membrane (Gilbertson et al., 1993). I have investigated a similar model for insulin, in which insulin binds its cognate receptor in the cell membrane, subsequently phosphorylating insulin receptor substrate (IRS). This results in activation of PI3-kinase. PI3-K forms PtdIns(3,4,5)P3 in the inner leaflet of plasma membrane changing the lipid composition and providing a mode for direct activation of ENaC (Blazer-Yost, Vahle, Byars, & Bacallao, 2004). Given our previous results in Chapter 2 about
ENaC inhibition by cytosolic Cl⁻ concentration, I decided to use functional Na⁺ imaging with SBFI (Na⁺ sensitive dye) to study ENaC function in TRCs. This technique is less invasive in nature allowing me to maintain the physiological resting intracellular chloride concentration in all experiments. In addition, whole cell patch clamp recording is not an optimal approach to studying second messengers.

The results in Chapter 3 showed insulin activates ENaC through PI3-kinase signaling cascade (Fig. 5.2). Thus, it is suggested that ENaC function is regulated by the direct effects of PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ (Tong, Gamper, Medina, Shapiro, & Stockand, 2004). Electrophysiological and functional studies, using a PI3-kinase blocker (LY294002), showed indirect evidence of the contribution of PtdIns(4,5)P₂/ PtdIns(3,4,5)P₃ in this signaling cascade. In addition, experiments with LY294002 and wortmannin revealed the involvement of other phosphoinositides such as PtdIns(4,5)P₂ in ENaC regulation. Similar results of ENaC regulation by PtdIns-(4,5)P₂ have been shown in other transporting epithelia (Ma & Eaton, 2005). The fact that PtdIns-(4,5)P₂ is necessary for ENaC function in TRCs implies the possibility of a salt receptor. It will be interesting to continue to investigate this hypothesis in greater depth because of its obvious importance in the taste responses to sodium salts.

I also looked for alternative pathways of ENaC regulation by insulin. I found both ENaC activation and insertion of the channel in the apical membrane depended upon serum and glucocorticoid–regulated kinase (SGK). Using a line
of transgenic mice that lack of SGK, I demonstrated the importance of SGK in the
gustatory system. The results suggested that insulin-mediated Na⁺ movement via
ENaC is impaired in these animals. Additionally, both insulin and aldosterone
signaling pathways converged at SGK to regulate ENaC activity. However,
insulin’s physiological role on SGK is additive to that of aldosterone because
insulin only increases phosphorylation of SGK while aldosterone increases SGK
expression (Faletti, Perrotti, Taylor, & Blazer-Yost, 2002; Tiwari, Nordquist,
Hlagappa, & Ecelbarger, 2007). Preliminary studies in lingual epithelia and
isolate taste cells support these results (Fig. 5.2; data not shown). I have further
identified Akt protein expression in taste cells with Western blotting. Akt may play
a physiological role in the activation of ENaC by insulin in TRCs. Little is currently
known about the properties of Akt in the gustatory system.

Following molecular and electrophysiological results from Chapter 3, I
began to explore insulin’s physiological role in salt taste. Using behavioral assays
with a mouse model of hyperinsulinemia, I showed that insulin regulates salt
intake. The main results from this study are: (1) insulin-treated mice significantly
avoid NaCl solution in the range of 150 mM-600 mM; and (2) These effects of
insulin in NaCl preference were attenuated by amiloride. These experiments
imply that the greater sensitivity to NaCl exhibited in insulin-treated mice is
attributable an increase in functional ENaC in the cell membrane of TRCs.
Together, Chapter 3 showed evidence that insulin effects on ENaC are extend
from the molecular level to the animal’s behavior.
One of the main goals of the proposed work was to evaluate the gustatory system’s physiological plasticity in restoration of salt and water balance in the body. In Chapter 4, I investigated alterations in salt taste transduction that occurred during the onset of diabetes. Because diabetes is an endocrine disease characterized by a severe water and electrolyte imbalance, I found that the gustatory system may play a role in the adaptation to these metabolic abnormalities. Previous experiments in Chapter 3 showed the importance of insulin in sodium salt transduction. I have evaluated the effects of Type I diabetes in salt taste transduction. The results revealed that there are at least two major changes in ENaC activity during the onset of diabetes; one is that insulin effects in Na⁺ influx via ENaC are abolished. The other is that the magnitude of Na⁺ movement was increased in taste cells from diabetic mice compared to control animals. Based on these findings I attempted to quantify the relative expression of α- β- and γ-ENaC subunits in fungiform, circumvallate and foliate taste buds. It appears that the expression of ENaC subunits was generally increased in diabetic taste receptor cells. Similar results have been observed in kidney epithelia in which protein abundance of ENaC increased in diabetic animals (Song, Knepper, Verbalis, & Ecelbarger, 2003).

Early behavioral studies showed a relationship between diabetes and the gustatory system since diabetic rodents exhibited an increase in fluid intake and hyperphagia (Smith & Gannon, 1991). Because of my interest in salt taste transduction, I have followed up on some early behavioral studies from Chapter 3
about the ability of insulin to contribute to the control of salt intake. Using short-term behavioral assays, I showed that diabetes impaired salt taste performance in mice. The data from these studies showed a correlation between blood glucose levels and NaCl preference. These differences in NaCl preference were attenuated by amiloride. The regulation of salt taste may be attributed to the possibility that the body’s osmolarity increases during the onset of diabetes. A number of adaptive changes in gene expression and protein abundance of ENaC, as well as other Na⁺ transporters expressed in taste and kidney epithelia occurred (Bickel, Knepper, Verbalis, & Ecelbarger, 2002; Oh et al., 2007; Song, Hu, Shi, Knepper, & Ecelbarger, 2004; Vidotti, Arnoni, Maquigussa, & Boim, 2008). During the course of these experiments it became clear to me that the Type I diabetic animal model (STZ treated) was not the best way to study diabetes due to the impairing nature of the drug. Since streptozotocin (STZ) directly destroys pancreatic β Cells, all treated-mice in the study showed severe physical signs and features of dehydration, weight loss, and lethargic behavior within 12 hours of injection in this animal model. It would be reasonable to look for alternative animal models of diabetes to continue this research.

Evidently, the gustatory system plays an essential role in the restoration of salt balance during early stage diabetes. Several previous studies have shown evidence of differences on the animal’s eating behavior during diabetes (Bartness & Rowland, 1983; Hardy, Brennand, & Wyse, 1981; Isezuo, Saidu, Anas, Tambuwal, & Bilbis, 2008; Kanarek & Ho, 1984; Le Floch et al., 1989;
Miller, 1997; Smith & Gannon, 1991; Tepper & Kanarek, 1985; Vartiainen & Bastman-Heiskanen, 1950; Yirmiya, Holder, & Derdiarian, 1988). Because diabetes is a generalized metabolic disorder, I attempted to determine any difference in body composition between diabetic and control group metabolic disorder. Using magnetic resonance imaging (MRI), I found that there was no significant difference in body water volume or fat content between diabetic and control mice (data not shown). Taken all results together, I hypothesized that the animals, through its gustatory system would change nutrient selection during the onset of diabetes to prevent osmotic diuresis. In addition, most diabetic animals and/or patients rarely present severe volume depletion, suggesting that the kidney is forced to adapt and compensate the osmotic diuresis to prevent hypovolemic shock (Vidotti et al., 2008).

In conclusion, the mechanisms of ENaC regulation in the gustatory system, like that in the kidney, are extremely complex. This dissertation offers an insight into the mechanisms of sodium salt transduction in mammalian taste buds. In addition, this is the first report, to our knowledge, to show functional changes of ENaC during diabetes. I anticipate that the findings of this work will be important for understanding the regulation of the gustatory system.

References


Fig. 5.1. Model of ENaC regulation by intracellular chloride. Ion transport pathways in mammalian kidney and taste cells. Cl⁻ influx through apical Cl⁻ channels (ClC and CFTR) and NKCC1 can directly inhibit Na⁺ influx through ENaC in sodium transporting epithelia (Nissato et al. AJP 287:F932, 2004). NHE3: sodium proton exchanger, type 3. NKCC: sodium-potassium-chloride cotransporter. ROMK: renal outer medullary K⁺ channel ENaC: Epithelial Na⁺ channel CFTR: cytofibrosys transmembrane receptor. NCC: sodium-chloride cotransporter.
Fig. 5.2. Model of insulin signal transduction leading to ENaC activation and apical insertion. Insulin binds its cognate receptor in the cell membrane, and produces a subsequent phosphorylation of insulin receptor substrate (IRS), which results in activation of PI3-kinase (PI3-K). PI3-K forms PtdIns(3,4,5)P3 in the inner leaflet of plasma membrane, therefore, these changes in the lipid composition provides a direct activation of ENaC. An alternative pathway in which PIP3 activates the phosphatidylinositol-dependent protein kinase (PDK1). PDK1, in turn, activates serum and glucocorticoid –regulated kinase (SGK) which leads to both ENaC activation and insertion of the channel in the apical membrane (Blazer-Yost et al. 2004) ENaC: Epithelial Sodium channel.PI3-K: phosphoinositide-3- kinase. PIP2: phosphatidylinositol 4,5- bisphosphate PIP3: phosphatidylinositol 3,4,5- trisphosphate
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Publications


Manuscripts in Preparation

Baquero AF, Gilbertson TA. Insulin activates ENaC via PI3-kinase in mammalian taste receptor cells.

Baquero AF, Watson KJ, Gilbertson TA. Regulation of ENaC by intracellular chloride and characterization of chloride channels in mammalian taste receptor cells.

Watson KJ, Baquero AF, Gilbertson TA. Differential expression of AQP5 in B6 and 129 mouse taste receptor cells.
Abstracts/Poster Presentations:


**Professional Societies:**

Association for Chemoreception Sciences

**Grants/Awards:**

2007 **Keystone Symposia Scholarship.** Travel award to attend The Keystone Symposium Chemical Senses: from Genes to Perception, Snowbird, UT received from Keystone Symposia.

2005 **E.L. and Inez Waldron Biotechnology Endowment Fund.** Travel award to attend the Association for Chemoreception Sciences Annual Meeting, Sarasota, FL received from the Center for Integrated BioSystems, Utah State University.

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