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Signaling Components Involved in the Hormone Induced Translocation of ENaC in Cultured Adult Human Fungiform (HBO) Taste Cells

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

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Table of Contents

List of Tables

Table

List of Figures

Figure

List of Abbreviations

Cultured Human Fungiform Taste Cells..... HBO
Epithelial sodium channel.....ENaC
Taste receptor cell..... TRC

Abstract

SIGNALING COMPONENTS INVOLVED IN THE HORMONAL-INDUCED TRANSLOCATION OF ENAC IN CULTURED ADULT HUMAN FUNGIFORM (HBO) TASTE CELLS

By Deanna Hojati, B.S.

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

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Director: Vijay Lyall, Ph.D., Assistant Professor, Department of Physiology

The amiloride-sensitive epithelial Na^+ channel, ENaC, is the Na^+-specific salt taste receptor in rodents. Compared to rodents, human salt taste perception is amiloride-insensitive. In rodents the ENaC is composed of αβγ-subunits. Whereas humans express an additional subunit, the δ-ENaC subunit. ENaC in human taste cells is composed of αβγ-subunits or δβγ-subunits, with the latter being amiloride-insensitive. Currently, it is not known if δβγ-ENaC expression and trafficking is regulated by hormones and their downstream intracellular signaling effectors. The aim of this study is to investigate if arginine vasopressin (AVP), aldosterone, and cAMP regulate δ-ENaC expression and trafficking in cultured fungiform human taste cells (HBO cells). Secondly, we want to demonstrate the expression of downstream signaling effectors involved in the trafficking

of δ-ENaC in HBO cells. Using molecular and immunocytochemical techniques, our results demonstrate that AVP, cAMP, and aldosterone increase expression of δ-ENaC mRNA and protein in HBO cells. Furthermore, AVP, cAMP and aldosterone increased trafficking of the δ-ENaC subunit from the cytosolic compartment to the apical pole of the HBO cells. Our results further demonstrate that HBO cells express several components of signaling cascade involved in ENaC translocation from cytosol to apical pole in HBO cells. The components of this signaling cascade include AVPR2, PKA, CREB, SGK-1, Nedd4-2, and GILZ-1. These hormones in mice and rats upregulate ENaC. Currently, we are not sure if these hormones affect ENaC this way in humans. By studying δ-ENaC with these hormones, we are able to see how human ENaC is regulated in the tongue.

Chapter 1: Introduction

1.1 An Introduction to Taste

Gustation, or taste, is a highly specialized system that guides organisms to obtain the appropriate energy and nutrients, while avoiding toxic substances (9, 22). Throughout time, evolution provided organisms the ability to distinguish between different chemicals, to approach some while avoiding others, thus developing our present day sophisticated gustatory system (9, 25, 33). There are five main taste qualities that humans can distinguish: sweet, salty, sour, bitter, and umami. These "taste qualities" that one perceives are derived from a variety of dissolved molecules and ions that stimulate the taste receptor cells found in taste buds, called tastants (33). Sweet taste signifies sugars and carbohydrates, denoting energy (4, 9, 14). Bitter taste serves as a protective mechanism against noxious substances (4, 9, 14). Umami taste in humans is mainly due to the presence of L- glutamate (monosodium glutamate; MSG) and to other L-amino acids, indicating protein content (4, 9, 14, 18). Salty taste is produced by Na^+ influx, and is essential for maintaining electrolyte homeostasis and blood volume (4, 9, 14). Lastly, sour taste is stimulated by mineral and organic acids. Sour taste is an aversive stimulus that prevents ingestion of acids *ad libitum* and helps in the maintenance of acid-base balance (4, 9, 17, 27). Other taste qualities, such as fat and metallic taste have also been suggested to be included as basic taste qualities (4). Threshold of tastant concentrations vary among individuals and is genetically determined (4, 22).

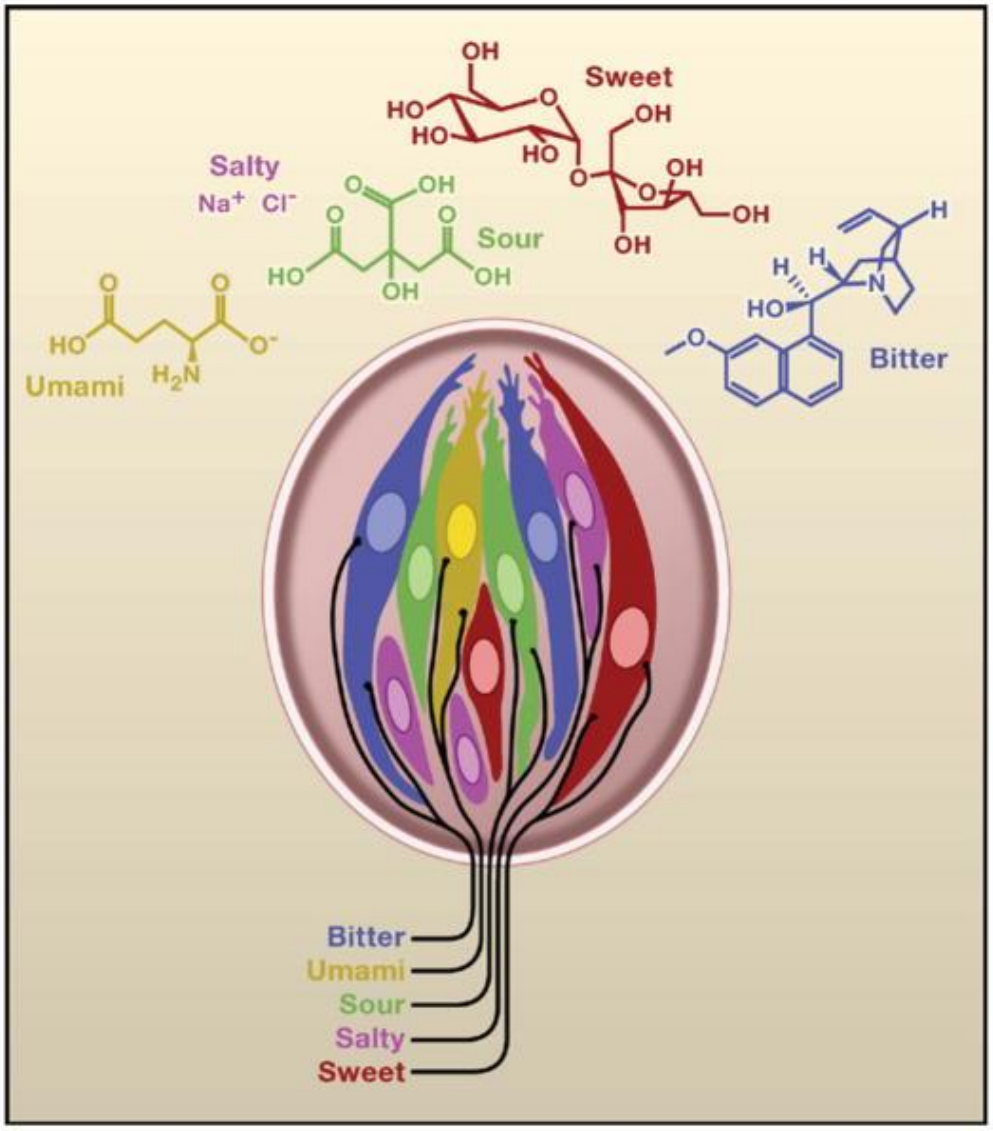
The tongue consists of an elaborate framework of vascular, neural, epithelial, and connective tissues, as well as specialized taste buds, to distinguish between various stimuli. Within the oral cavity, the tongue is the first organ to face the various complexities that organisms need to obtain to sustain life (25). The tongue acts similarly to several other organs in the human body, like the skin, the tongue consists of stratified squamous epithelium, sitting over a lamina propria and muscle (25). Also similar to the gastrointestinal system, the tongue is made up of specialized cells, and exhibits continuous cell turnover that is required for its function (25).

An important attribute of taste is that it not only detects nutrients and toxins, but it is the initial step to the physiological reflexes downstream, that prepare the body for absorption of the nutrients and other metabolic functions (4, 25). These reflexes are initiated by a combination of three sensory modalities; sight, smell, and, taste (4, 25, 33).

1.2 Taste Buds

Taste and flavor are two words commonly used synonymously, but the former has a stricter application, generated by the chemicals that activate the taste receptor cells in the taste buds, that send signals to specific parts of the brain (4, 33) (Figure 1). On the other hand, flavor, is the combined sensation of olfaction and gustation (4). Gustation solely relies on the sensory organs of the oral cavity, the taste buds, while olfaction is generated by neurons in the nasal epithelium (4, 25).

Figure 1. Diagrammatic representation of a taste bud. Diagram illustrates the shape of a taste bud and its constituents; the taste pore, microvilli, various taste receptor cells, and neural innervation (39).

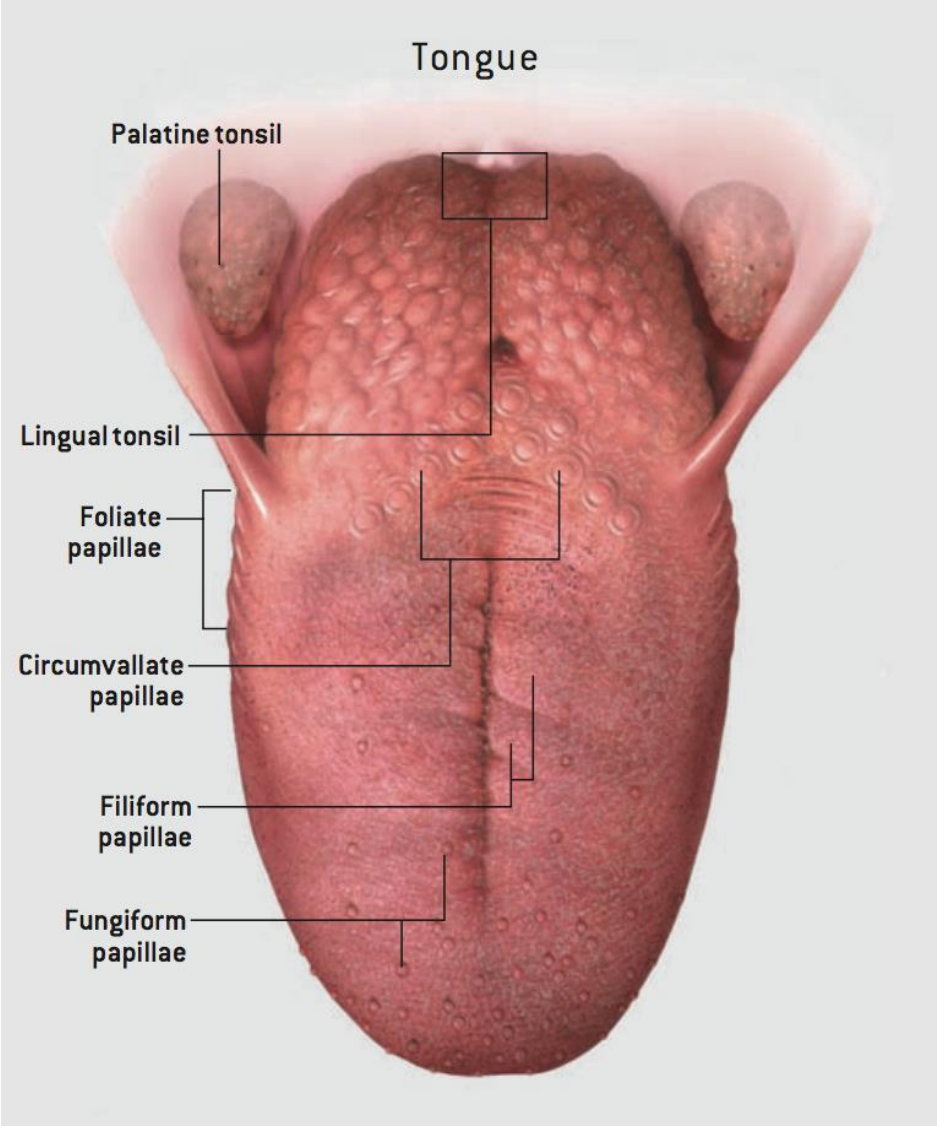

Sweet
Salty
Na+ Cl-
Sour
Umami
Bitter
Bitter
Umami
Sour
Salty
Sweet

Human taste buds are located in various types of papillae. These papillae are classified into four major types: filiform, fungiform, circumvallate, and foliate papillae (20, 28). Each of these papillae types are found at various locations on the tongue (Figure 2). Filiform papillae are the most abundant, distributed over a majority of the tongue surface, but are completely lacking in taste buds (9). Fungiform papillae are generally located along the anterior tip and lateral portions of the tongue (9). Circumvallate papillae are present at the posterior part of the tongue, just anterior to the sulcus, forming an upside down 'V' shape (9). The foliate papillae are located in two clusters on the posterior lateral portions of the tongue (9).

Taste buds are found mostly on the tongue, palate, and epiglottis. Humans can have anywhere from 500 to 10,000 taste buds, and the variety depends on ones genetics (4, 9, 22). Each taste bud contains between 50-150 taste receptor cells, which receive the initial signal for taste transduction (4, 9, 23).

The taste receptor cells detect taste stimuli (4, 33). Each taste receptor cell possesses microvilli, which project out through the opening of the taste bud called the taste pore onto the epithelial surface of the oral cavity (22, 33). Molecules from foods dissolve in one's mouth, and interact with the taste receptor cells via the microvilli through the taste pore. At the apical surface of the taste receptor cells are a variety of taste receptor

Figure 2. Diagrammatic representation of a human tongue and papillae. Diagram illustrates location of various types of papillae on the human tongue; filiform, fungiform, circumvallate, and foliate. This research utilized isolated human fungiform papillae taste buds to study salt taste receptors in humans.


Tongue
Palatine tonsil
Lingual tonsil
Foliate papillae
Circumvallate papillae
Filiform papillae
Fungiform papillae

proteins, that interact with the various tastant stimuli to trigger action potentials that send signals to specific parts of the brain that deciphers the taste quality of the taste stimuli (22, 33).

1.3 Taste Receptor Cells

Taste receptor cells are innervated by one of the cranial nerves, depending on their location. The anterior 2/3rd of the tongue is innervated by the chorda tympani (CN VII), the posterior 1/3rd is innervated by the glossopharyngeal nerve (CN IX), and the root of the tongue/esophagus is innervated by the vagus nerve (CN X) (9). These innervations between taste receptor cells to the nervous system allow the information from multiple taste buds to be sent to the brain to code a distinct taste quality (9).

Vertebrate taste receptor cells regenerate after about 10-11 days, with some lasting even longer (9, 22). The polarized taste receptor cells project their microvilli onto the epithelium of the oral cavity, so that they can sense the chemicals in the environment (22, 33). At the apical surface of the taste receptor cells is a variety of proteins; ion channels, ligand-gated channels, (LGICs) and/or G-protein coupled receptors (GPCRs) that are the receptors for the various tastant molecules (22). Each taste quality is registered by a different receptor type, and thus each tastant signals a different downstream transduction cascade that generates an action potential that reaches the brain

to signal the tastant identified (22). Taste receptor cells are also described as neuroepithelial cells, due to their ability to transduce an external stimuli (45). Furthermore, TRCs can fire action potentials either spontaneously or in response to chemical/electrical stimulation, and they express voltage gated Na^+, K^+ and Ca^{2+} channels (46).

1.4 Physiology of Taste

Taste receptor cells have various protein complexes at their apical membrane that specifically bind to taste stimuli belonging to a specific taste quality.

Umami, sweet, and bitter tastants interact with G protein-coupled receptors (GPCRs), that are seven transmembrane domain receptors. GPCRs interact with molecules on the apical side of the taste receptor cell to activate downstream intracellular signal transduction pathways (8, 14, 21, 37). There are two types of GPCRs that are involved with these tastants (T1Rs and T2Rs). T1Rs are sub-classified into T1R1, T1R2 and T1R3. The T1R1/T1R3 heterodimer, T1R2/T1R3 heterodimer, detect umami and sweet taste stimuli, respectively (8, 14, 21, 23, 26, 37). For bitter taste, human TRCs have approximately 25 genes that express different T2Rs involved in bitter taste detection. While sweet, umami, and bitter taste is detected by different GPCRs, the downstream signaling transduction is the same and involves α-gustducin, PLCβ2 and TRPM5 ion channel (37).

Whereas, sour stimuli are detected by a separate set of taste receptor cells that express PKD2L1 and PKD1L3 channels, which are members of the transient receptor potential (TRP) family (6, 17, 27). However, weak organic acids (acetic acid, CO_2) can permeate the apical cell membrane and decrease intracellular pH of taste receptor cells. In the case of strong acids (HCl) H^+ permeates the cell membrane via Zn^{2+}-sensitive proton channels. This suggests that a decrease in intracellular pH of sour sensing taste receptor cells is the proximate signal for sour taste transduction (47). Salty (Na^+) taste is detected by the amiloride-sensitive epithelial Na^+ channel (ENaC), while the amiloride-insensitive component of salt taste is detected by a non-selective cation channel. This pathway has been shown to involve Na^+ influx via vanilloid receptor-1 (VR-1) (51). It is important to note that unlike in rodents, salt taste perception is humans is amiloride-insensitive. In rodents the ENaC is composed of αβγ-subunits. Human taste cells express an additional subunit, the δ-ENaC subunit, allowing human taste cell ENaC to be composed of either αβγ-subunits or δβγ-subunits. The δβγ-ENaC is amiloride-insensitive (15, 16, 19).

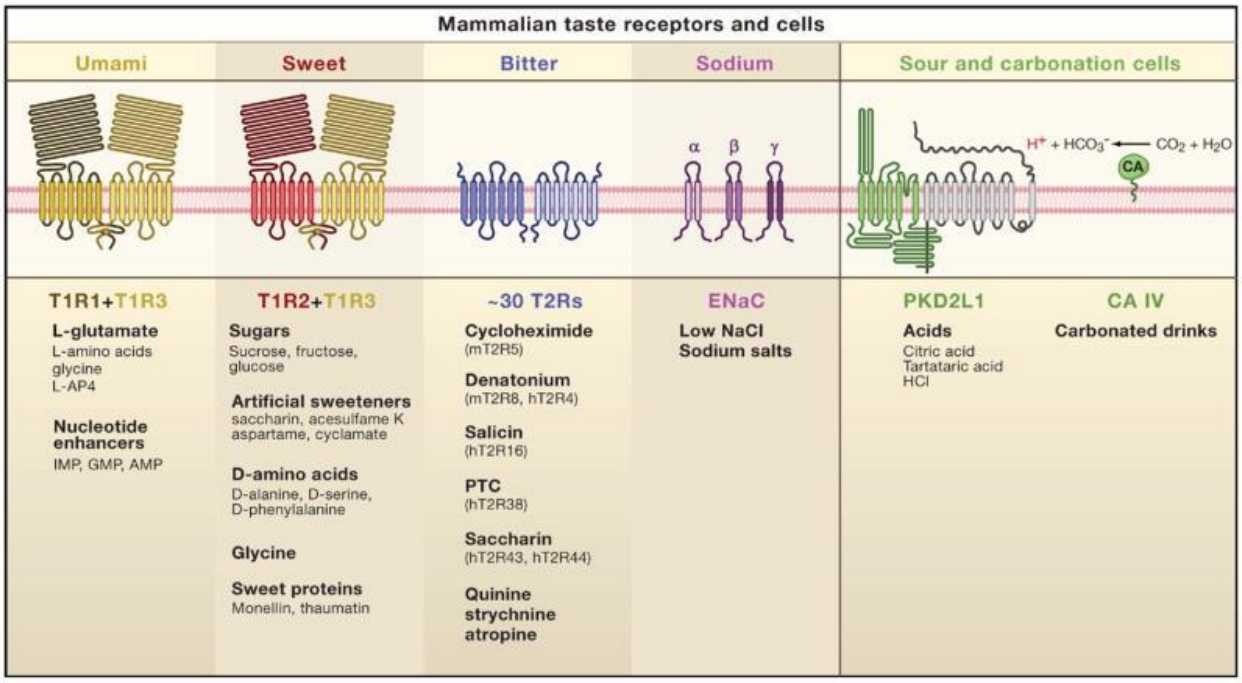
1.5 Transduction of the Five Tastes

The penultimate step in the taste transduction involves an increase in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$ that causes neurotransmitter release, which acts on the afferent neuron attached to the basolateral aspect of the taste receptor cell, to send a signal to the brain for the identification of the specific tastant (2, 4, 17, 18, 21, 22, 27,

33). The taste specific neurotransmitter is ATP. This is supported by the observation that double P2X2 and P2X3 purinergic knockout mice are completely agusic (48).

Several taste receptors types that have been identified in the oral cavity, have also been identified elsewhere in the body, like the gastrointestinal tract, lungs, and kidneys (Figure 3) (6, 13, 22).

Figure 3. Mammalian taste receptors and cells. Diagram depicts the various types of tastes and their receptors, as well as the ligands they specifically detect (39).


Mammalian taste receptors and cells
Umami
Sweet
Bitter
Sodium
Sour and carbonation cells
α β γ
H+ + HCO3- ← CO2 + H2O
CA
T1R1+T1R3
L-glutamate
L-amino acids
glycine
L-AP4
Nucleotide enhancers
IMP, GMP, AMP
T1R2+T1R3
Sugars
Sucrose, fructose, glucose
Artificial sweeteners
saccharin, acesulfame K
aspartame, cyclamate
D-amino acids
D-alanine, D-serine,
D-phenylalanine
Glycine
Sweet proteins
Monellin, thaumatin
~30 T2Rs
Cycloheximide
(mT2R5)
Denatonium
(mT2R8, hT2R4)
Salicin
(hT2R16)
PTC
(hT2R38)
Saccharin
(hT2R43, hT2R44)
Quinine
strychnine
atropine
ENaC
Low NaCl
Sodium salts
PKD2L1
Acids
Citric acid
Tartataric acid
HCl
CA IV
Carbonated drinks

1.5.1 Sweet taste

The sensation of sweet taste is initiated by sweet taste stimuli interacting with a specific heteromer of T1R2+T1R3 G protein-coupled receptors (GPCRs) at the apical membranes of taste receptor cells. The receptors for sweet taste responses were mapped in murine and human DNA sequencing databases (23). The sweet sensing receptor, T1R3, was identified by scanning human genes for GPCRs in the region orthologous to the mouse Sac locus (23, 37). The Sac locus is what controls mouse sensitivity to various sweet tastants, for example, sucrose or fructose (23). Murine studies showed that T1R3 was expressed in approximately 15-30% of taste receptor cells in all of the taste-cell containing papillae (21, 23). The T1R3 receptor was found to be closely related to T1R1 and T1R2 GPCRs, and was further found that T1R3 expressing cells usually express at least one of the other GPCRs, denoting that these T1Rs may function as heterodimers (21, 23, 26). Also, unlike T2Rs (bitter sensing receptors), T1R expressing cells do not express gustducin (23, 37). This signifies that sweet and bitter tastants are detected by different subset of taste receptor cells.

Several models for sweet transduction have been suggested; one is a GPCR-G_s-cAMP pathway that is stimulated by sucrose and other sugars (23, 37). The second is a GPCR-G_q/$G\beta\gamma$-IP3 pathway, which is stimulated by artificial sweeteners (23, 37). The former stimulates $G\alpha$, which activates adenylyl cyclase, elevating cAMP levels that cause the basolateral K^+ channel to close, depolarizing the taste cell, proceeding to a voltage-

dependent Ca^{2+} influx, and finally neurotransmitter release. For the latter, one or more GPCRs are activated, coupled to PLCβ2, activating $G\alpha_q$ or releasing Gβγ, which activates PLCβ2 to generate IP_3 and DAG, causing Ca^{2+} release from internal stores, depolarizing the taste receptor cell, and finally leading to neurotransmitter release (22, 23, 37).

1.5.2 Bitter taste

Mapping of DNA sequence databases identified the T2R receptor family as the bitter tasting receptors by isolating DNA sequences to produce its final functional protein form (33). The T2R receptor family is a seven-transmembrane domain GPCR family (26). In murine models, T2R receptors are only expressed in about 15-20% of the taste buds, mostly in circumvallate and foliate, with very few present in fungiform papillae (26). Several studies have shown that a single taste receptor cell can express multiple *T2r* genes, thus it is possible that we are unable to distinguish between various bitter compounds (26). Confirming this, behavioral studies have shown that rats cannot distinguish between two different bitter compounds, quinine and denatonium (26).
It was found that a majority of taste cells that express T2R receptors also express gustducin (26). α-Gustducin is an α-transducin-like G protein subunit selectively expressed in 25-30% of TRCs (35). In vitro and in vivo analysis of murine models has shown that α-gustducin played a role in bitter taste transduction, as well as others (23). The signaling cascade begins with the T2R receptors at the apical membrane of the taste receptor cell. When a bitter tastant is dissolved in the mouth, the gustducin heterotrimer

splits into its respective α and βγ subunits, to activate two different paths (35, 37). α-Gustducin activates PDE (phosphodiesterase), which decreases cAMP levels to stimulate intracellular Ca^{2+} release, followed by neurotransmitter release (23, 37). The second path, βγ-Gustducin subunits activate PLCβ2, to make IP3 and DAG, which stimulates intracellular Ca^{2+} release, and finally neurotransmitter release (23, 37). This second path was noted when α-gustducin KO mice still showed residual response to bitter compounds, denoting that there must be other G-proteins working in the bitter taste transduction pathway (23).

1.5.3 Umami taste

The term "umami" originated from a Japanese term *umai*, meaning "delicious" (22). This word denotes a tastant that is pleasant, but cannot be described as sweet, salty, sour, or bitter. Umami is represented mostly by a common amino acid, L-glutamate, which is signified by meat-like tastes (18, 21, 22). Also, umami taste is potentiated by several ribonucleotides like inosine-5'-monophosphate (IMP) or guanosine-5'-monophophate (GMP), which by themselves also display the same umami taste (22).

Several receptor types have been found to bind L-glutamate and/or nucleotides in taste receptor cells. Among these are: T1R1/T1R3 heterodimer, the taste specific isoforms of metabotropic glutamate receptors (mGluR1,2,3,4), and several ionotropic glutamate receptors (NMDA and kainate receptors) (18, 21). Note that glutamate is also a neurotransmitter, and for these receptors to be considered as taste receptors, they must be

expressed at the apical membrane of taste receptor cells (18). Thus, receptors localized on the basolateral membrane, like NMDA and kainite receptors, are noted to be responding to glutamate as a neurotransmitter rather than as a tastant (18, 21). Thus far, KO studies of T1R1 and T1R3 receptors completely eliminated any oral responses to L-glutamate (18).

The T1R1/T1R3 heterodimer is coupled to a heterotrimeric G protein consisting of α, β, and γ subunits (18, 22). Ligand binding to the receptor activates the $\beta\gamma$ subunits, which activate phospholipase Cβ2, which produces IP_3 and DAG (18, 21, 37). IP_3 binds to the type III IP_3 receptor, causing the release of Ca^{2+} from internal stores and Ca^{2+} dependent activation of TRPM5, a monovalent selective cation channel (18). TRPM5 depolarizes taste receptor cells, resulting in action potential, and final release of the neurotransmitter ATP (18).

This pathway was confirmed by KO mouse studies of phospholipase Cβ2, type III IP_3 receptor, and TRPM5, which all showed reduced umami taste responses in a similar manner to a KO of the T1R3 receptor (18). Also, pharmacological inhibitors of phospholipase Cβ2 and Ca^{2+} ATPase, which maintains intracellular Ca^{2+} stores, eliminated all responses to L-glutamate and nucleotides applied to the oral cavity (18, 22).

1.5.4 Sour taste

The receptors for salty and sour taste are ion channels. Sour taste, is used to identify complex foods, and allows the organism to be able to recognize rotten or spoiled foods (9, 22). A sub-set of taste receptor cells that express PKD2L1 ion channel (also called Type III cells) are involved in sour taste sensing (49). Upon stimulating with sour taste stimuli, a decrease in intracellular pH (pH_i) is the proximate stimulus for sour taste transduction (47). Several different mechanisms are involved in sour taste transduction. In the case of strong acids (HCl), H^+ ions enter taste receptor cells via Zn^{2+}-sensitive proton channels (47, 50). Chorda tympani responses to HCl are enhanced by increasing cAMP in taste receptor cells, suggesting that proton channels in the apical membranes of taste receptor cells are activated by cAMP (47). The un-dissociated weak organic acids permeate apical cell membranes as neutral molecules and decrease taste receptor cell pH_i. While in the case of CO_2, both apical carbonic anhydrase 4 (CA4) and intracellular carbonic anhydrases are involved in CO_2 sensing (47,49).

Patch clamp studies showed that protons depolarize taste cells by interaction with K^+ ion channels located in the apical membrane (27). Microelectrode studies suggested that the K^+ channel blocker tetraethylammonium (TEA) completely inhibited any response to sour stimuli, which confirmed the role of K^+ channels in sour taste transduction (27). Also, by utilizing whole-cell voltage-clamp recordings, it was shown that citric acid reduced the voltage-dependent K^+ current, Na^+ current, and Ca^{2+} current (27). Thus concluding that voltage-sensitive K^+ channel block is a plausible mechanism

for sour taste transduction when K^+ channels are located in the apical membrane (27). The transduction pathway includes H^+ ions in the oral cavity, which block the K^+ channels in the apical membrane (17, 27). A decrease in pH_i results in depolarizing the taste receptor cells, by preventing a resting efflux of K^+ ions via proton block of K^+ channels which eventually opens voltage-gated Ca^{2+} channels resulting in increase in $[Ca^{2+}]_i$ and the release of the neurotransmitter ATP (9, 17, 27).

1.5.5 Salt taste

Salt taste is used to denote nutrient and mineral concentration, which provides for maintaining ion and water homeostasis in the body (22). Salty tastants are mainly comprised of the Na^+ ion, which elicits the salt sensation via either Na^+-specific, or Na^+-nonspecific mechanism (6, 22). The Na^+-specific mechanism utilizes amiloride-sensitive epithelial sodium channels (ENaC) (6, 22). In mice in which the α-ENaC subunit is knocked out only in taste receptor cells, they do not display an amiloride-sensitive component of the NaCl chorda tympani response (30). In behavioral experiments, α-ENaC subunit knockout mice demonstrate significantly reduced licking for appetitive NaCl concentrations (30). In contrast to mice, the human salt taste perception is amiloride-insensitive. In healthy humans, amiloride only suppresses sodium intensity by 21%, thus denoting that approximately 80% of the sodium response in these humans is amiloride-insensitive (6). In rodents the ENaC is composed of αβγ-subunits. Human taste

cells express an additional subunit, the δ-ENaC subunit. In human taste cells the ENaC is composed of αβγ-subunits or δβγ-subunits, with the latter being amiloride-insensitive.

TRPV1 is a nonspecific cation channel that is permeable to Na^+, K^+, NH_4^+, and Ca^{2+} (51). In rats and mice, TRPV1 agonists (capsaicin, resiniferatoxin, and heat) produced biphasic changes in chorda tympani responses to NaCl + amiloride (51). At low concentrations these agonists enhanced and at high concentrations inhibited the NaCl chorda tympani response (51). Additionally, the NaCl + amiloride chorda tympani responses in the absence and presence of the TRPV1 agonists were blocked by TRPV1 antagonists, SB-366791 and capsazepine (51). These led to the hypothesis that the amiloride- insensitive component of the NaCl chorda tympani response is derived from apical Na^+ flux via the TRPV1 channel (51). However, current studies suggest that TRPV1 is not expressed in rat and mice fungiform taste receptor cells (51). However, TRPV1 is expressed in the trigeminal nerve endings (51). Currently, the signaling steps between TRPV1 activation and increase in NaCl flux across taste receptor cell apical membrane are not known.

1.6 Hormonal Regulation of ENaC

The epithelial sodium channel, ENaC, is the mammalian Na^+ specific taste receptor. Several hormones and intracellular signaling effectors have been shown to regulate ENaC in taste receptor cells. In adult rats AVP, cAMP, aldosterone and insulin

increase and angiotensin II attenuate TRC ENaC activity (10, 64, 65, 66, 58, 67). These hormones effect receptors on the basolateral aspect to create their effect (58).

1.6.1 ENaC regulation by AVP and cAMP

AVP binds to the arginine vasopressin receptor 2 (V2R) coupled to $G\alpha_s$ (Figure 4). $G\alpha_s$ stimulates adenylyl cyclase and increases intracellular cAMP. Cyclic AMP activates downstream effectors resulting in the trafficking of ENaC to the apical cell membrane (30, 68, 69, 70, 71, 72).

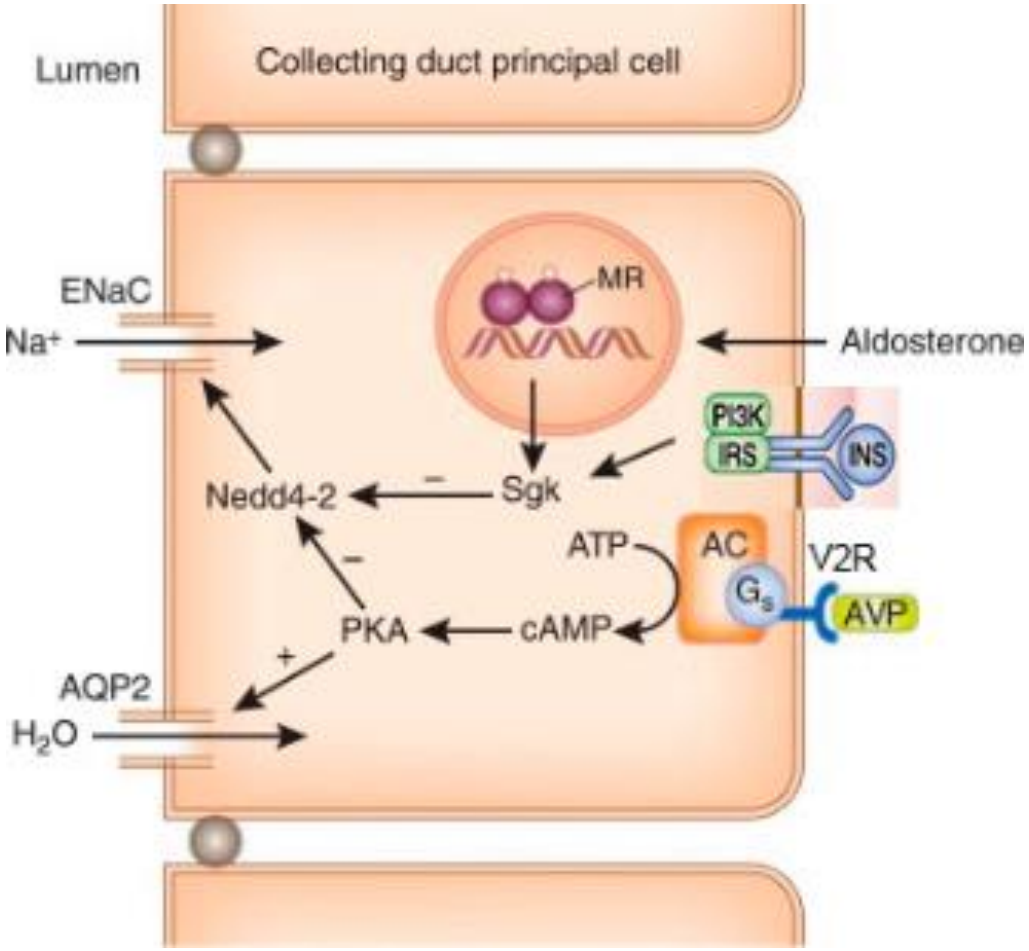
1.6.2 ENaC regulation by aldosterone

Aldosterone acts through the mineralocorticoid receptor (MR) to alter the transcription of specific genes (Figure 4), including SGK-1 (24, 66). The effect of cAMP and aldosterone on ENaC trafficking is due to the inhibition of ubiquitin ligase Nedd4-2-induced ENaC ubiquitination *via* phosphorylation of Nedd4-2 on Ser^{327}, Ser^{221}, and Thr^{246} by PKA and SGK-1 (38). Nedd4-2 controls ENaC surface expression by catalyzing its ubiquitination, which targets ENaC for degradation (38). Nedd4-2 binds to the PY motif located in the cytoplasmic C terminus of each ENaC subunit decreasing ENaC surface expression. SGK-1 binds to Nedd4-2 and phosphorylates it, which reduces the binding of Nedd4-2 to ENaC (38).

1.6.3 ENaC regulation by insulin

Insulin acts via the insulin receptor (IR) and stimulates ENaC activity by activating SGK-1 via PI3-kinase (Figure 4) and phosphoinositide-dependent kinase (PDK1) (73).

Figure 4. ENaC, hormones, and downstream signaling effectors. Arginine vasopressin (AVP), insulin (INS) and aldosterone signal transduction. AVPR (V2R); Adenylyl cyclase (AC); protein kinase A (PKA); aquaporin-2 (AQP2); insulin receptor substrate (IRS), PI3-kinase (PI3K), mineralocorticoid receptor (MR); serum and glucocorticoid-inducible kinase (Sgk); neural precursor cell expressed developmentally down-regulated protein 4-2 (Nedd4-2) [39].


Lumen
Collecting duct principal cell
ENaC
Na+
MR
Aldosterone
PI3K
IRS
INS
Nedd4-2
Sgk
ATP
AC
V2R
Gs
AVP
PKA
cAMP
AQP2
H2O

IR and SGK-1 are expressed in FF and circumvallate (CV) TRCs (58). In isolated TRCs from wildtype (WT) adult mice, insulin (5-20 nM) enhanced Na^+ influx (58). Treating TRCs with LY294002, an inhibitor of PI3-kinase, abolished insulin-induced changes in ENaC (58). Adult mice given a single I.P. injection of insulin showed significant avoidance of NaCl at lower concentrations than the control group (58). In contrast, SGK KO mice demonstrated a reduction in functional ENaCs in TRCs (58). Insulin was unable to increase Na^+ influx in TRCs isolated from SGK KO mice (58). This suggests that SGK is essential to maintain normal ENaC function and for the action of insulin on TRC ENaC.

In additional to the above hormones, intracellular and extracellular signaling effectors also regulate ENaC in rat taste receptor cells, including extracellular and intracellular pH, $[Ca^{2+}]_i$, osmolarity, cell volume, proteases and small molecular weight activators of ENaC (7).

1.7 Sodium & its Concerns in Health

The amount of salt ingested to be pleasurable varies from person to person. Even in murine models, it was found that sodium-depleted animals presented with a lower salt taste threshold than others, and found higher concentrations of salt aversive (6, 13). While sodium-replete animals had a higher salt taste threshold and found increased concentrations of salt to be appetitive (6).

It has been noted that increased levels of sodium intake can cause a multitude issues to human health. Issues like hypertension, increased risk of cardiovascular and/or renal disease, are all outcomes of high sodium levels (5). Studies show that excess sodium intake is a significant risk to public health and as a possible solution the sodium content of foods should be decreased and regulated by the United States Food and Drug Administration (5).

Sodium chloride, otherwise known as "salt", is essential for life and is thus consumed regularly (5). The human body does not efficiently store salt, and as a consequence there is a constant need to consume more of it (1, 5, 12). The epithelial sodium channel, ENaC has long been studied as the main sodium recognizer in taste cells. There is also a non-ENaC mechanism that obtains salt in the human diet, but is currently unknown. This was confirmed by the fact that ENaC does not respond to potassium chloride, and thus there must be another receptor that is involved in salt taste perception (5). While salt intake is necessary to sustain life, with increase in age humans have a greater demand for higher concentrations of salt to please their taste preferences (5, 12, 22).

The issues with salt taste sensation is that humans consume a much more substantial amount of salt than they need. The taste of salt is more pleasurable than aversive and as such, people consume much more salt than needed to maintain a normal sodium balance. This increased salt-intake is not a huge issue for healthy individuals, but

poses a health issue for those with hypertension (5, 6). In addition, patients with diseases like Liddle Syndrome (a rare genetic disorder associated with severe hypertension and hyperaldosteronism), pseudohypoaldosteronism, cystic fibrosis, and other diseases that implicate uncontrollable levels of sodium influx, can benefit from these studies as they can better understand how to manipulate ENaC channels and their function (3, 24). For example, patients with Liddle Syndrome display gain of function mutations that enhance their ENaC activity (52). In these patients, the renal collecting ducts reabsorb all of the Na^+ resulting in the retention of Na^+ and water (52). This causes hypertension and expansion of blood volume. Thus, Liddle Syndrome provides a direct link between NaCl and hypertension.

1.8 Objective of this Study

ENaC is regulated by channel expression/synthesis, intracellular channel trafficking, and single channel properties via PKA-dependent or PKA-independent mechanisms (72,75-78). In rodents the ENaC is composed of αβγ-subunits. Human taste cells express an additional subunit, the δ-ENaC subunit. Humans have two kinds of functional ENaCs, the amiloride-sensitive αβγ and the amiloride-insensitive δβγ, where the latter ENaC predominates in humans. Currently, it is not known if δβγ-ENaC expression and trafficking is regulated by hormones and their downstream intracellular signaling effectors. This research has two objectives: (1) We will investigate if δ-ENaC expression is regulated by hormones via in-vitro treatment with confluent isolated HBO

cells, thus allowing the hormones to penetrate the basolateral aspect of the cells. (2) To identify key signaling molecules involved in the hormone-induced trafficking of δ-ENaC from the cytosolic compartment to the apical pole of human fungiform taste receptor cells. This information can potentially be able to provide us with the ability to increase or decrease salty taste sensation. For example, it may be possible to change how individuals perceive salt tastes, or to create artificial salt tastants for people with high blood pressure or cholesterol. In addition, a deeper understanding of the brain and its connections to taste receptor cells can provide information on how our conscious and subconscious decisions of food are ascertained. This study of ENaC in human taste can thus provide a broader understanding of gustation, which can eventually provide greater neural and clinical implications for diseases related to salt overload (22, 26).

In summary, today's society has difficulty maintaining a proper amount of sodium in their diets. The excess amount of processed and fast foods readily available contain far more sodium than necessary and thus pose an issue for everyone, especially those with poor health. By studying the salt taste receptor mechanisms, we can understand how salt taste is identified and possibly find ways to maintain the salty taste at even lower sodium concentrations (6).

Chapter 2: Materials & Methods

2.1 Human Fungiform Taste Papillae (HBO) Cells

HBO cells were kindly provided by Dr. Hakan Ozdener of Monell Chemical Senses Center, Philadelphia PA. HBO cells were cultured as described earlier (28).

2.2 Western Blot to detect δ-ENaC subunit protein expression

HBO cells were treated with 10 μM 8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate (8-CPT-cAMP, a membrane permeable form of cAMP), 1 μM aldosterone, 10nM dDAVP (1-desamino-8-D-arginine vasopressin), or 10 μM 8-(4-chlorophenyl-thio) guanosine 3′,5′-cyclic monophosphate (8-CPT-cGMP) for 24 hrs in incubator at 36 °C in 5% CO_2. 8-CPT-cGMP was used as a negative control for dDAVP and cAMP.

The HBO cells (2×10^6) treated with the above modulators were lysed in 200 μl modified M-PER buffer (Thermo Fisher Scientific, MA, USA). Samples containing 30 μg total protein were used for the Western blots. The Western blots were performed with SDS-PAGE electro-blotting system (Bio-Rad). 30 μg total Protein samples (30 μg total protein) were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes (Cat: 162-0094, Bio-Rad). Membranes were immune-blotted with rabbit antiserum containing δ-ENaC antibody (aa411-460, antibody from Lifespan Biosciences, Inc (LS-Cl 19717)) and a primary β-actin antibody (Santa Cruz Biotechnology). Followed by HRP-conjugated

secondary antibodies; anti-Rabbit for δ-ENaC and anti-Mouse for β-actin. Reactions were visualized by ECL Western Blotting Substrate. β-actin was used as a protein loading control.

2.3 RT-PCR to detect ENaC downstream signaling elements

Total RNA from HBO cells was purified using TRIZOL reagent (cat# 15596018, Thermo Fisher Scientific, MA, USA) and reverse transcription was performed using High-Capacity cDNA Reverse Transcription Kit (cat# 4368814, Thermo Fisher Scientific, MA, USA). RT-PCR for the detection ENaC downstream signaling effectors listed in Table 1, was carried out by using MyTaq red mix (Bioline, Luckenwalde, Germany). Briefly, 2 µg total RNA was mixed with 2X Reverse Transcription Master Mix to total 20 µL per reaction. Reverse transcription were performed at 25ºC x 10 min, then 37ºC x 120 min, followed by 85ºC x 5 sec and cooled to 4ºC. Reverse transcription was done in three separate experiments due to the various primers needing different temperatures. Afterwards, 200 ng total cDNA was used as template, 35 cycles of PCR amplification were performed (initial denaturation at 95ºC for 1 min, denaturation at 95ºC for 15 sec, annealing for 15 sec at various temperatures according to specific primers listed in Table 1, and extension for 10 sec at 72ºC). RT-PCR products were subjected to electrophoresis on a 1% agarose gel to determine the expression of the downstream signaling effectors. The primers used to detect the ENaC downstream signaling elements were made using Primer-BLAST program (NCBI) and are listed in Table 1.

Table 1. Primers used for RT-PCR. Primers of the downstream signaling elements of ENaC made on Primer-Blast program from NCBI.

Gene	NCBI Accession Number	Primer	Length (bp)	Annealing Temperature (°C)
GILZ-1	NM_001015881.1		489	58
Forward		CCTAGCTAGCTTCAGAGCCG		
Reverse		GCTCTTGTCAGGGGTCTGTC		
CREB	NM_134442.4		349	57
Forward		TGAACGAAAGCAGTGACGGA		
Reverse		ATCTGTGTTCCGGAGAAAAGTC		
SGK-1	NM_005627.3		430	57
Forward		GCTAAGGGCACCCTCACTTA		
Reverse		ACAGAACATTCCGCTCCGAC		
PKA	AF037439.1		392	60
Forward		AACGGACATATGCCGGGAAG		
Reverse		CCCCATTCTCTCCCTGGAAAC		
Nedd-4	NM_006154.3		493	60
Forward		GGAGGACGAGGAAAATTCACG		
Reverse		GTCCAAAACAACCCAGCCAG		

HEK 293-cells were used as positive control. HEK cells were obtained from (American Type Culture Collection, Manassas, VA) and grown in a complete 293 SFMII growth medium (Thermo Fisher Scientific) supplemented with 4 mM L-glutamine.

2.4 Single cell real-time RT-PCR

Single cell RT-PCR was used to perform expression analysis of signaling effectors in single cells using the Single Cell-to-CT^{TM} Kit (ThermoFisher Scientific) as per manufacturer's instructions. The final real-time PCR products were separated by electrophoresis on a 2% agarose gel containing 1 μl/ml ethidium bromide.

2.5 cAMP Assay

Cyclic AMP levels were measured in the presence of 3-Isobutyl-1-methylxanthine (IBMX) a phosphodiesterase blocker (55, 56). HBO cells (2 x 10^6 cells/mL) were incubated for 10 min in the presence of 100 μM IBMX in the absence and presence of 0.01 μM dDAVP, 0.1 μM dDAVP, or 10 μM forskolin (an activator of AC). The reaction was terminated with 1 mL of 6% ice-cold trichloroacetic acid, and cAMP was extracted via freeze thawing. The acid was removed by ether extraction, and cAMP was measured in triplicates by radioimmunoassay using 10 μL aliquots of reconstituted samples. The results were expressed in picomoles per mg protein.

2.6 Immunohistochemistry & Confocal Imaging

HBO cells were plated into 8-well chamber slides and treated with 10 uM 8-CPT-cAMP or 10 uM 8-CPT-cGMP or 10 nM dDAVP for 10 min. HBO cells were fixed with

methanol for 10 min at -20°C. Slides were then washed with 1X PBS 3 times for 5 min each, then blocking buffer with 3% donkey serum for 1 h at room temperature. Slides were stained with were rabbit antiserum containing γ-ENaC antibody (1:400 dilution in 3% donkey serum) or rabbit polyclonal δ-ENaC antibody (1:200 dilution in 3% donkey serum). After washing, cells were incubated in the dark with fluorescent-conjugated secondary antibodies for 1 h at room temperature. Nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI; 1 μg/mL). Images were acquired with a 63X (1.4 numerical aperture) oil immersion objective on a Zeiss LSM 700 confocal laser scanning microscope and processed using Photoshop software (Adobe System).

Chapter 3: Results

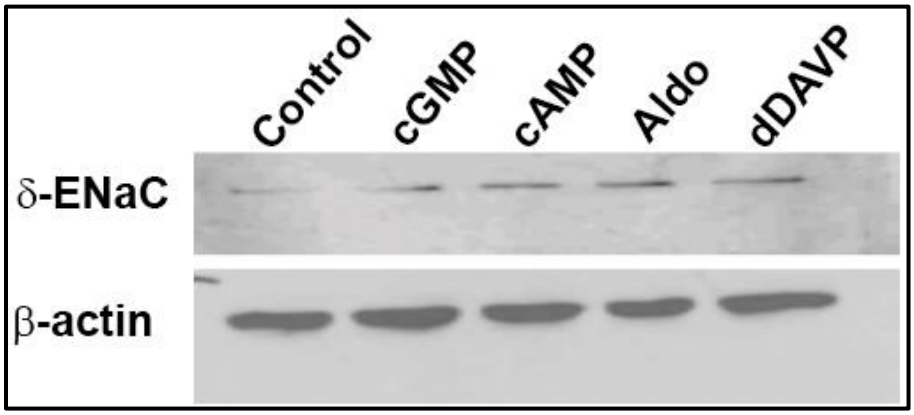
Experiments were designed to investigate δ-ENaC expression and trafficking by hormones and intracellular signaling effectors.

3.1 Expression of δ-ENaC subunit

HBO cells treated with 8-CPT-cAMP, aldosterone, and dDAVP for 24 h showed increase in δ-ENaC subunit protein expression (Figure 5A). Western blot of β-actin was done as the endogenous control to calculate the fold increase in ENaC expression relative to control (Figure 5B). This denotes channel expression is increased in the presence of the above specific signaling effectors and hormones. Cells treated with aldosterone showed the greatest fold increase in δ-ENaC protein expression. In HBO cells treated with 8-CPT-cGMP, no increase in δ-ENaC protein expression was observed relative to the control group. Both aldosterone and AVP have been shown to increase ENaC expression in TRCs and in other epithelial tissues (57, 66, 79, 80). In our earlier studies, exposure of rats to membrane permeable 8-CPT-cAMP, aldosterone, and dDAVP via IV/IP injections increased NaCl chorda tympani responses in young and adult rats (30). These studies suggest that increase in ENaC subunit expression by the above hormones and intracellular signaling effectors results in increased ENaC activity and, hence, in an increase in the neural responses to NaCl.

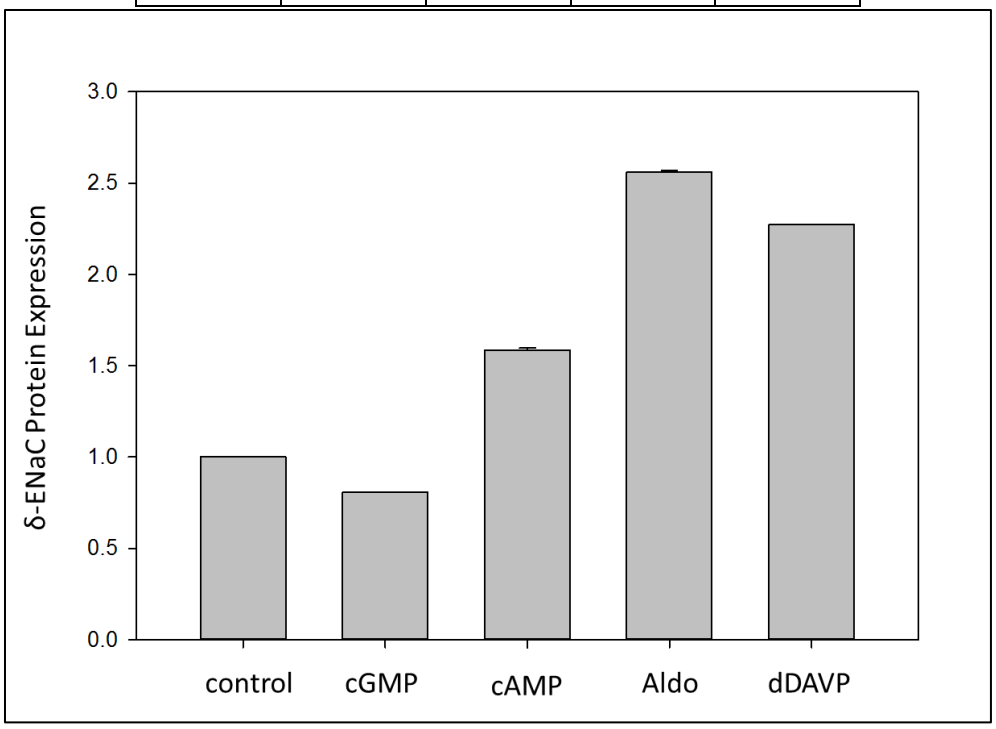
Figure 5A & 5B. The effect of cGMP, cAMP, aldosterone, or dDAVP on δ-ENaC subunit expression in HBO cells. Cells treated for 24 h with 10 μM 8-CPT- cAMP, 1 μM aldosterone, or 10 nM dDAVP showed increased levels of δ-ENaC protein expression by 1.59, 2.56, 2.27 fold, respectively, compared to untreated cells or cells treated with 8-CPT-cGMP.

5A



5B

Control	cGMP	cAMP	Aldo	dDAVP
1	0.81	1.59	2.56	2.27



3.2 Trafficking of δ-ENaC Subunit

In isolated mouse TRCs, the amiloride-sensitive Na^+ current is increased by AVP and membrane-permeable cAMP analogues. This effect was predominantly due to an increase in the number of functional apical ENaCs (62,10). Similarly, insulin-induced increase in amiloride-sensitive Na^+ current was also due to an increase in the number of functional apical ENaCs (58). Accordingly, we tested if the above hormones and intracellular effectors increase the number of functional ENaC channels via δ-ENaC trafficking in HBO cells.

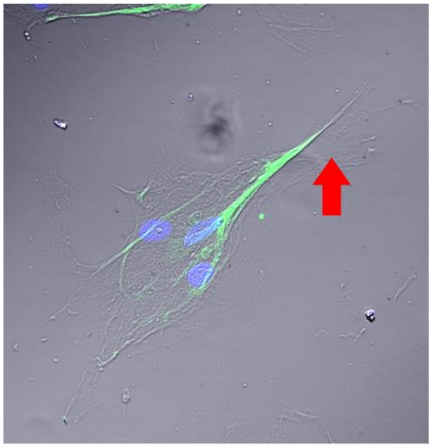
Treatment of the HBO cells for 10 minutes with 8-CPT-cAMP and dDAVP (Figure 6) showed remarkable visual changes compared to the untreated cells or cells treated with 8-CPT-cGMP (Figure 6). The blue stain (DAPI, 4',6-diamidino-2-phenylindole) binds to the taste cell nucleus, while the green-FITC-labeled secondary antibody binds to the primary δ-ENaC subunit antibody. The cells treated with 8-CPT-cGMP or the untreated group displayed staining that was evenly distributed throughout the cell. Whereas in cells treated with 8-CPT-cAMP or dDAVP, the δ-ENaC staining is more concentrated at the apical tips, with minimal dispersed staining in the cytosolic compartment around the nucleus. This data clearly, indicate that the above modulators induce trafficking of δ-ENaC subunit from the cytosolic compartment to the apical pole of the cell. However, a possible change in cell shape may also occur in the presence of the above modulators. Similar results were observed in HBO cells when stained for the

primary antibodies to the γ-ENaC subunit (Figure 7), under the same experimental conditions.

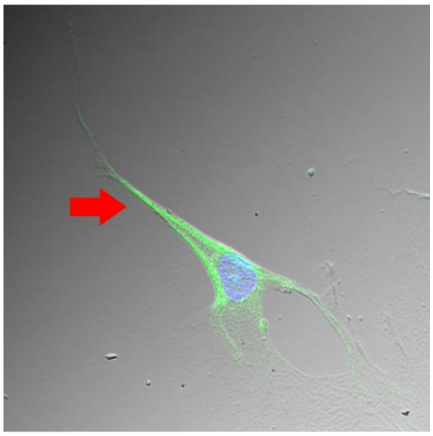
Figure 6. Effect of dDAVP or 8-CPT-cAMP compared to untreated or 8-CPT-cGMP on δ-ENaC trafficking in HBO cells.

HBO cells treated for 10 min with 10 μM dDAVP or 5 μM 8-CPT-cAMP. Shown here is a decrease in staining at the cytosolic compartment, denoting subunit translocation to the apical compartment (marked with the arrows). In the untreated or HBO cells treated for 10 min with 5 μM 8-CPT-cGMP, the δ-ENaC stain dispersed evenly throughout the taste cell, denoting no movement of the δ-ENaC subunit.

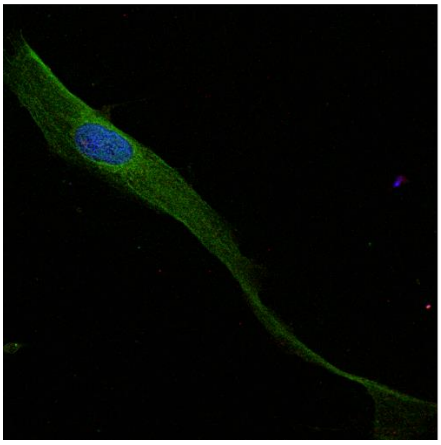
HBO δENaC treated with ddAVP 10 uM



HBO δENaC treated with cAMP 5uM



HBO δENaC untreated



HBO δENaC treated with cGMP 5uM

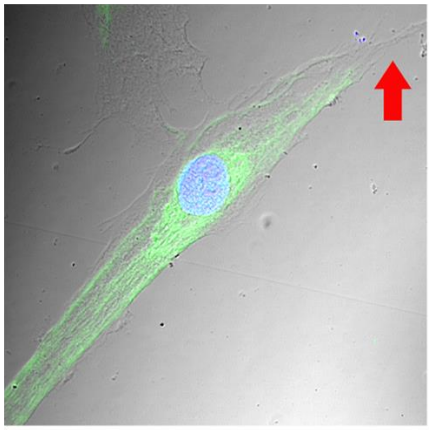
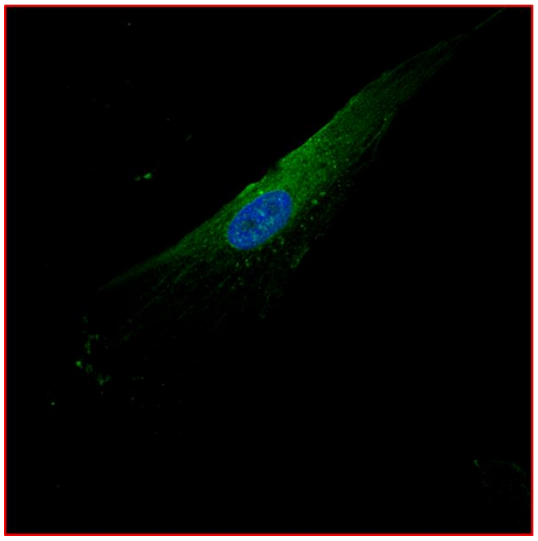


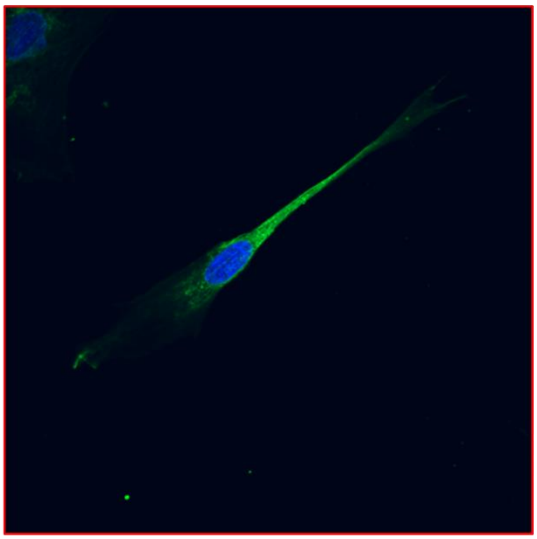
Figure 7. Effect of untreated cells or 8-CPT-cAMP on γ-ENaC trafficking in HBO cells.

Untreated HBO cells or cells treated for 10 min with 10 μM 8-CPT-cAMP. Shown here you can see the similarity between the γ-ENaC subunit to the δ-ENaC subunit, both displaying similar staining patterns in their respective untreated or cAMP-treated examples.

HBO γENaC no treatment



HBO γENaC treated with 10 uM cAMP

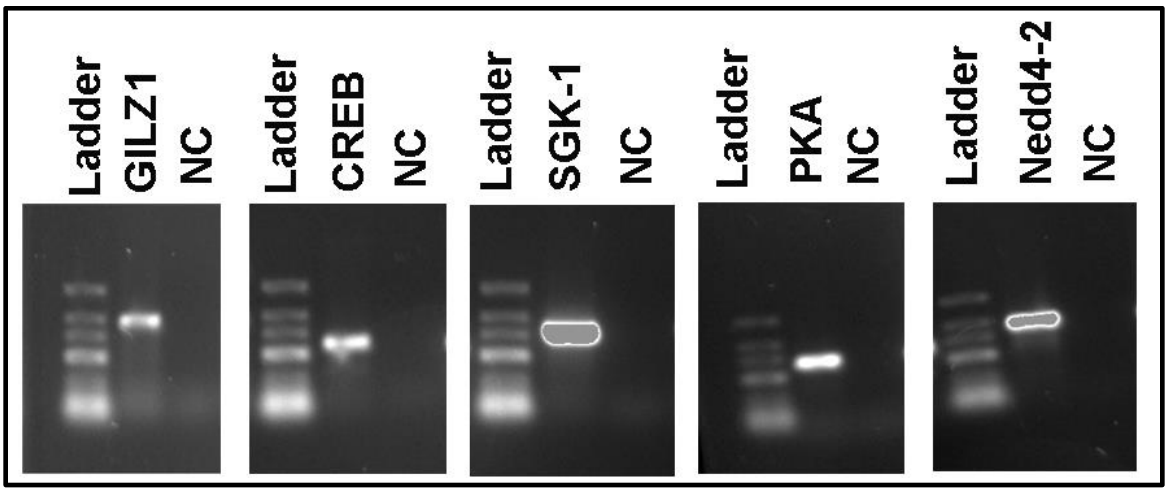


3.3 Detection of Downstream Signaling Effectors in HBO cells visualized by RT-PCR

Figure 4, summarizes the various transduction effectors that are involved in the ENaC trafficking by of AVP, aldosterone and insulin. Accordingly, we first investigated if some of the above signaling effectors are expressed in HBO cells.

In a cDNA library from HBO cells, using specific primers and RT-PCR demonstrated the presence of mRNAs of PKA (protein kinase A), CREB (cAMP response element-binding protein), SGK-1 (serine/threonine-protein kinase), Nedd4-2 (E3 ubiquitin-protein ligase), and GILZ-1 (glucocorticoid-induced leucine-zipper protein) (Figure 8). Inhibiting PKA or PI3 kinase inhibits the increase in Na^+ currents in isolated mouse taste cells or the increase in the NaCl chorda tympani responses induced by cAMP (10, 58, 63). The cDNA library was made from total RNA.

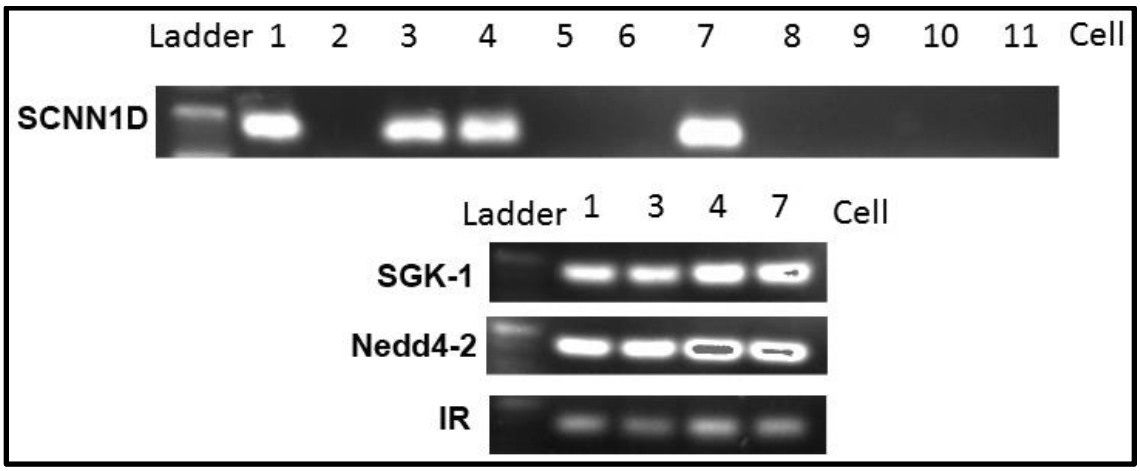
Figure 8. RT-PCR of ENaC downstream signaling elements. Using specific primers and RT-PCR, a cDNA library from HBO cells displays the presences of mRNAs for GILZ1, CREB, SGK-1, PKA, and Nedd4-2. These signaling effectors are all involved in the regulation of ENaC surface expression.


Ladder
GILZ1
NC
Ladder
CREB
NC
Ladder
SGK-1
NC
Ladder
PKA
NC
Ladder
Nedd4-2
NC

The effect of the hormone aldosterone, induces SGK-1 to phosphorylate Nedd4-2, thus reducing its ability to ubiquinate, retrieve, or degrade ENaC subunits (12). We investigated the AVP binding to V2R by using a strong specific V2R agonist, dDAVP. The binding of dDAVP to V2R stimulates the Gs pathway, activating adenylyl cyclase (AC) to convert ATP into cAMP. The cAMP produced in the cytosol activates PKA, which phosphorylates Nedd4-2, also inhibiting its function, maintaining the δ-ENaC subunit in the apical membrane. The hormone insulin, which binds to the insulin receptor (Figure 9) also plays a role in ENaC trafficking and transduction. Insulin binding to its receptor activates phosphoinositide 3-kinase OH (PI3-kinase) which leads to phosphyorylation then activation of 2 different pathways. One pathway leads to phosphorylation of ENaC, and the other activated phosphoinositide-dependent kinase 1 (PDK1). PDK1 goes on to activate SGK-1, which goes on to phosphorylate Nedd4-2 and inhibit its function. These pathways mentioned previously both converge at Nedd4-2, which ubiquinates the ENaC subunits by binding to the PPxY motifs, beginning their retrieval from the apical membrane into endosomes, to be degraded in lysosomes or proteasomes, or to be recycled back to the apical membrane (12). Therefore, providing us a common factor in the ENaC regulation pathway where Nedd4-2 poses to be the first step in the ENaC retrieval and degradation process. Several of these downstream signaling effectors were found using single cell RT-PCR technique, where 4 out of the 11

HBO cells expressed δ-ENaC (SCNN1D) mRNA positive cells. Expression of mRNAs for SGK-1, Nedd4-2, and IR were found (Figure 9).

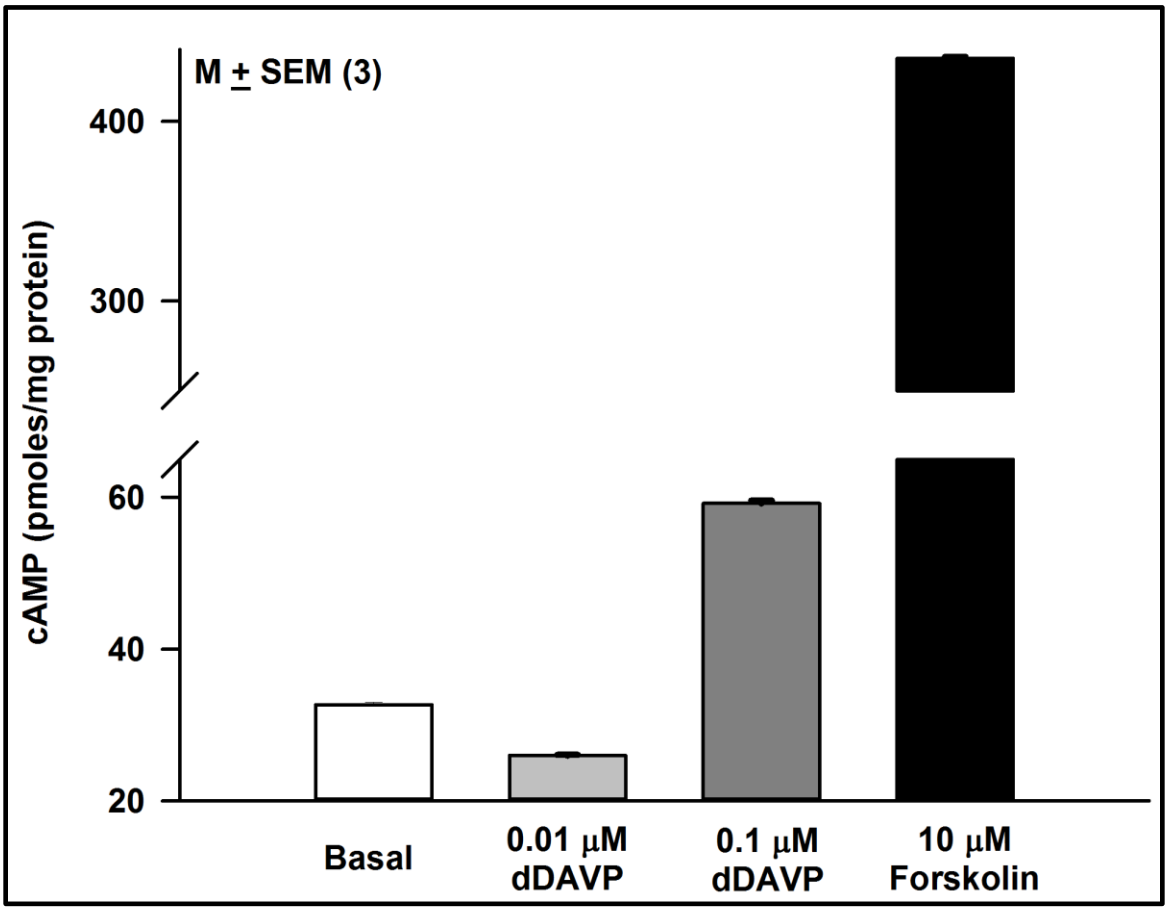
Figure 9. Single Cell real-time RT-PCR. The mRNA for δ-ENaC (SCNN1D) was detected in 4 out of 11 HBO cells examined. Detection of mRNAs for SGK-1, Nedd4-2, and IR mRNA found in single HBO cells.


Ladder 1 2 3 4 5 6 7 8 9 10 11 Cell
SCNN1D
Ladder 1 3 4 7 Cell
SGK-1
Nedd4-2
IR

3.4 Relationship of cAMP generation and δ-ENaC trafficking

Cyclic-AMP levels were measured in HBO cells in the presence of a phosphodiestrase inhibitor (IBMX), and treated with dDAVP or forskolin (an activator of AC) for 10 min (Figure 10). It was found that cAMP levels increased with a dose-dependent increase of dDAVP treatment. In addition, cAMP levels were the highest when cells were treated with forskolin, which bypasses the need for AVP to bind V2R to activate AC. Within this time frame, application of cAMP also increases behavioral and neural responses of developing rats (30). These results indicate that cAMP induces translocation of ENaC subunits from cytosolic to apical pole within minutes.

Figure 10. cAMP generation in presence of IBMX. HBO cells in the presence of phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), and treated with 100 nM dDAVP or 10 μM forskolin (activator of AC) for 10 min increase cAMP levels.


M ± SEM (3)
400
300
60
40
20
cAMP (pmoles/mg protein)
Basal
0.01 μM dDAVP
0.1 μM dDAVP
10 μM Forskolin

Chapter 4: Discussion

4.1 Arginine Vasopressin (AVP)

Arginine vasopressin, also known as antidiuretic hormone (ADH), is a neurohypophysial hormone that is essential for the body's regulation of water volume (53). There are several receptors AVP can bind to, namely V1a, V1b, and V2 which all belong to the family of GPCRs (53). The V1a receptor is expressed in vascular walls and is responsible for vasoconstriction (53). The V1b receptor is found in the anterior pituitary and is responsible for the secretion of ACTH (Adrenocorticotropic hormone) (53). Finally, the V2 receptor is found in the renal distal tubules and collecting ducts to stimulate water absorption, as well as the basolateral aspect of taste cells to stimulate sodium influx (10, 53). Previous studies on hamster fungiform taste cells displayed an increase in sodium current following a 10-20 minute treatment with AVP (10). This effect was also seen in other reports showing that AVP increase the density of apical Na^+ channels in transporting epithelia (10, 54). The difference between these receptors is that V1a and V1b stimulate the G_q pathway, activating PLCβ, which activates IP3, which stimulates intracellular Ca^{2+} release, and its downstream effects of vasoconstriction and ACTH secretion, among other things (53). On the contrary, the V2 receptor, stimulates the G_s pathway, to activate adenylyl cyclase (AC), which increases cAMP levels, to

activate PKA, and finally phosphorylate Nedd4-2 to inhibit its function to retain the ENaC channel in the apical membrane.

4.2 Effect of dDAVP on intracellular cAMP levels

In this study, we utilized a strong synthetic V2R agonist named 1-desamino-8-D-arginine vasopressin, otherwise known as dDAVP. While previous studies were performed on hamsters, rats, mice, and frogs, our study shows dDAVP's impact on human FF taste cells. Treatment of HBO cells with 100 nM dDAVP or 10 μM forskolin (an activator of AC) for 10 min increased cAMP levels beyond the basal levels (Figure 10). The results displayed a dose-dependent increase in cAMP levels with increasing doses of dDAVP (30). Furthermore, treatment of HBO cells with a membrane permeable form of cAMP (8-CPT-cAMP) induced trafficking of δ- and γ-ENaC subunits (Figure 6 & 7). Thus denoting that cAMP is a key effector in the transduction pathway of ENaC.

4.3 Relationship between cAMP generation and δ-ENaC trafficking

It has been previously noted that the amiloride sensitive Na^+ current is increased by AVP and membrane permeable cAMP analogs (30). This effect is mainly due to the increase of functional ENaC channels in the apical membrane (30). A maximal increase in Na^+ current was seen between 15-20 min post-AVP treatment (30). In conjunction with these results, it can be concluded that AVP induces an increase in intracellular cAMP levels (Figure 10) as well as ENaC trafficking within minutes (Figure 6 & 7). In addition, cAMP treatment to MDCK (Madin-Darby canine kidney epithelial cells) cells increased

the density of ENaC subunits in the apical membrane in direct proportion to amiloride-sensitive Na^+ transport (57). In our previous studies, IV injections of dDAVP increased the expression and trafficking of γ-ENaC from the cytosolic compartment to the apical membrane of rat FF TRCs (30). Importantly, 15 day old rats express low levels of the TRC V2 receptor, and repeated injections of dDAVP has been shown to include ENaC expression and trafficking (30). This data suggests that TRC ENaC intracellular signaling and trafficking mechanisms are regulated similarly in rodents and human models. Furthermore, our previous results demonstrated a dose-dependent increase in the magnitude of the ENaC-dependent NaCl CT response in FF TRC cAMP treated rats (30). Signifying an increase in cAMP treatment results in an increased amount of functional ENaC in the apical membrane of TRCs. 8-CPT-cGMP was used as a control and it did not have any effect on the ENaC-dependent NaCl CT response in rats (30). Incubating cells with 8-CPT-cAMP or dDAVP for 10 min displayed δ-ENaC trafficking from the cytosolic pole to apical pole. Whereas HBO cells treated with 8-CPT-cGMP for 10 min, the δ-ENaC antibody was seen mainly in the cytosolic compartment of the TRC.

4.4 Aldosterone

Aldosterone stimulates sodium transport in the renal collecting duct by activating ENaC channels, which is seen when elevated levels of circulating aldosterone is present (24). In addition, aldosterone acts through the mineral corticoid receptor (MR) to alter the

transcription of SGK-1. Aldosterone has been shown to increase ENaC expression and translocation of ENaC subunits from cytosolic to apical pole (30).

Studies on SGK-1 KO mouse models displayed a mild phenotype where aldosterone is still able to activate ENaC, indicating that aldosterone has other effects not reliant upon SGK-1 function (12). Another aldosterone induced protein, GILZ-1, a scaffolding protein, disrupts the Raf-1/ERK pathway, to stimulate ENaC (12, 41).

While the other signaling effectors and hormones induced various events within minutes, aldosterone effects on CT response are observed between 48-72h post-IP injections in rats. This increase in time is due to aldosterone inducing a transcriptional event. In addition, our studies present increase in protein expression of δ-ENaC subunit (Figure 5), as well as translocation of the δ-ENaC from cytosolic to apical pole.

4.5 Insulin

Insulin has been studied to contribute to Na^+ movement in kidney epithelial ENaC. It has been found that both ENaC open probability and membrane expression is increased by insulin stimulation (58). Insulin works by binding to the insulin receptor (IR), to activate phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3-kinase), leading to a increased ENaC activity and open channel characteristics, as well as activation of SGK-1 (58). Recall, this is the same SGK-1 that is induced transcriptionally by aldosterone stimulation. Whole cell patch-clamp recordings displayed results that insulin does stimulate Na^+ movement through amiloride-sensitive ENaC channels (58). Insulin

increased inward Na^+ currents in fungiform and circumvallate taste cells, and these enhancing effects were reduced by amiloride treatment (58). In addition, using several PI3-kinase blockers (wortmannin and LY294002) it was found that PI3-kinase and PI4-kinases are able to enhance or maintain ENaC channel activity (58). SGK-/- mice were also studied in conjunction with SGK+/+ mice. Results indicated that SGK is essential for ENaC function and that absence of SGK protein drastically inhibits insulin's effects on ENaC taste transduction (58).

4.6 Nedd4-2

With the variety of signaling effectors and hormones implicated on ENaC's function and regulation, this channel can be manipulated from multiple points along its transduction cascade. By understanding each of the proponents of the signaling cascade it is possible to overcome mutations and issues effecting ENaC regulation. A commonality between the two transduction cascades studied in this research was a convergence upon a ubiquitin ligase, Nedd4-2. This enzyme poses to be the first step in the degradation process of the ENaC subunits. Lack of Nedd4-2 results in sodium sensitive hypertension, and increase in the presence of ENaC subunits (12). Conversely, excess Nedd4-2 activity should result in the decrease of ENaC subunits at the apical membrane, and thus less sodium intake. Some studies indicate that ubiquitination and deubiquitination of ENaC by Nedd4-2 occurs constitutively, to be able to maintain and recycle ENaC subunits to the apical membrane (12).

4.7 δ-ENaC

δ-ENaC, the fourth ENaC subunit, still presents as a gustatory "mystery". The well-characterized αβγ-subunits have been found and studied in many other models, while the δ-subunit is thought to be absent in rat and mouse tissues (59). So far, δ-ENaC subunit has been found in many human tissues, including the heart, placenta, lung, liber, kidney, thymus, prostate, colon, and taste buds (59). In our studies, it has been suggested that δ-ENaC displays the same signaling effectors and trafficking as α- and γ-ENaC.

4.8 Trafficking versus Cell shape

While our studies implicated that the δ-ENaC subunit is being trafficked from cytosolic to apical pole, there is also a cell shape change occurring simultaneously. In previous studies, hormonally induced increase of intracellular cAMP or direct application of membrane permeable forms of cAMP have been shown to induce cell shape changes in epithelial as well as non-epithelial cells (30). Incubation of cells with 1 or 2 mM dibutyryl-cAMP, displayed cell shape change within 40 minutes (60). Dose-dependent increase in cAMP studies were also performed that changed cell morphology (61). That is why in our studies, it can be deduced that the HBO cells treated with 8-CPT-cAMP, or cells treated with a hormone that increases intracellular cAMP (like dDAVP), will observe a cell shape change as well. But the significant finding is the translocation of δ- and γ-ENaC subunits from cytosolic to apical pole in several, but not all treated HBO

cells. Thus it is plausible that this cell morphology change and trafficking occur simultaneously.

4.9 Implications of this study

So far in this research, it has been found that δ-ENaC trafficking is the same as α- and γ-subunits (β-subunit has a poor antibody). At this time, it is not known whether the δ-ENaC subunit is regulated differently than the other subunits. By studying these various effectors and hormones, it is possible to find several methods of controlling regulation of the ENaC channel. Inhibition of one effector can present different outcomes than another, and their mere presence indicated in this study has helped allude to the similarities of the ENaC subunits and channels throughout the body.

In conclusion, ENaC activity can be regulated in one of three ways; by expression, trafficking, or open channel characteristics. This study interpreted the expression of δ-ENaC via various treatments with 8-CPT-cAMP, aldosterone, dDAVP, and 8-CPT-cGMP/control groups. As well as visualization of δ-ENaC movement and/or cell shape change, without the synthesis of new subunits, seen by treatment of downstream signaling effectors.

4.10 LaCunae

With the convergence upon SGK-1 with aldosterone or insulin, an SGK-1 blocker can be used to identify if PKA alone will phosphorylate Nedd4-2 and inhibit its function, thus retaining the same level of expression of δ-ENaC.

It is difficult to deduce from the confocal imaging which side of the cell is apical versus basolateral, but further studies will be done to mark either side of the cell to make it distinguishable. Previous studies using anti-NHE1 or anti-NHE3 antibodies to bind to the basolateral or apical membranes of fungiform and circumvallate TRCs has proven to be conclusive in murine models, respectively (42). By using the anti-NHE3 antibody in conjunction with δ-ENaC antibody, the NHE3 stain should bind at the more concentrated tips of the 8-CPT-cAMP or dDAVP treated cells.

Treatment of cells with MG132, a potent cell-permeable inhibitor of proteasomes, was performed but results were inconclusive. Hypothetically, treatment with MG132 was supposed to inhibit the δ-ENaC subunit from being degraded in the proteasome, and thus we would be able to see more δ-ENaC in the apical membrane. Confocal imaging studies showed no change to the δ-ENaC subunits, but further studies need to be done. MG132 was only used in treatment for 10 min, whereas previous studies done with HEK-293 cells were incubated for up to 8 h (43). Possible explanation for this is that Nedd4-2 function still remains active in the presence of MG132, and thus can still ubiquitinize the δ-ENaC subunit and pull it back into the cytosolic compartment. Future studies should allow for longer treatment time of the inhibitor, as well as western blot analysis to see if δ-ENaC protein expression is still increased in the presence of 8-CPT-cAMP, aldosterone, or dDAVP, alongside the inhibitor. In addition, a Nedd4-2 inhibitor, to

"mimic" the effects of PKA or SGK-1, should be studied to understand the full capabilities of Nedd4-2 on ENaC function.

Further investigation of ENaC regulation should aim to study open channel characteristics using patch clamp studies, to understand quantitative values of sodium influx.

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