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

by

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List of Abbreviations

Capsazepine……...CZP
Chorda Tympani Nerve……...……CT
Cranial Nerve……...CN
Cultured Human Fungiform Taste Cells……...……HBO
Endoplasmic reticulum……ER
Amiloride Sensitive Epithelial Sodium Channel……..…..……ENaC
G Protein Coupled Receptor……GPCR
Human Embryonic Kidney Cells……...……HEK
N-(3-methoxyphenyl)-4-chloro-cinnamide……..…SB-366791
Resiniferatoxin……..RTX
Taste Receptor Cell……TRC
Transient Receptor Potential Variant 1……..……TRPV1
Scnn1d……...……δENaC

Abstract

ACTIVATION OF TRPV1 BY CAPSAICIN REGULATES ENAC

By Alexander Saffran, 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., Associate Professor, Department of Physiology and Biophysics

ENaC is a constitutively open heterotrimeric channel which regulates Na^+ transport in tight epithelia of the kidney, lungs, colon and anterior tongue containing fungiform taste buds. The amiloride-sensitive ENaC is comprised of αβγ subunits. Humans express an additional subunit, the δ subunit. Therefore, humans contain both αβγ-ENaC and δβγ-ENaC functional channels. Relative to αβγ-ENaC, the δβγ-ENaC is 10-fold less sensitive to amiloride. In the mammalian anterior tongue, ENaC is expressed in fungiform salt sensing taste receptor cells and is the Na^+-specific salt taste receptor. In mammals, salt elicits an inverted U shaped behavioral response. Lower concentrations of salt are appetitive while high salt concentrations are aversive. The appetitive salt concentrations are sensed via ENaC. Thus, modulating ENaC activity in fungiform taste receptor cells will, in turn, regulate salt intake. The aim of this project is to investigate the effect of a common food ingredient, capsaicin, on ENaC expression and function in two cell lines, HEK293 cells and cultured adult human fungiform taste bud cells (HBO cells). Capsaicin, a TRPV1 agonist was chosen because in previous studies, it modulated chorda tympani taste nerve responses to NaCl in a dose-dependent manner. Most importantly, capsaicin and other agonists of TRPV1 were effective in modulating human salt taste perception. It is likely that the effect of capsaicin is due to its interactions with TRPV1, because TRPV1 and

ENaC subunits are co-expressed in cortical collecting duct cells (CCD) and in a subset of human taste bud cells. In support of this hypothesis, TRPV1 has been shown to regulate ENaC expression and function in CCD cells of rats and mice.

Using immunohistochemical techniques, our results demonstrate that TRPV1 is co-localized with the δ-ENaC subunit in HBO cells. Additionally, the results in HEK-293 cells suggest that the activation of TRPV1 via capsaicin has a modulatory effect on δ-ENaC mRNA and protein expression as well ENaC channel function measured as Na^+ flux.

Chapter 1: Introduction

1.1 An Introduction to Taste

Taste, also known as gustation, is a feature of the sensory system which is a critical aspect to the survival of many organisms. This modality has been selected during evolution to allow organisms to differentiate between essential nutrients and those which may be noxious to the organism. Many mammals, particularly humans, have the ability to differentiate between five 'fundamental' tastes; bitter, salty, sour, sweet, and umami. It is understood that these basic tastes comprise the physiological requirements for the preservation of life (8).

Bitter taste detection evolved as a central warning system to prevent the consumption of toxic substances (48). Similar to bitter taste, sour taste is aversive. It also serves as a protective mechanism in humans; sour taste is an indicator of acidic foods (26). Because it is aversive, the taste system, in addition to lungs and kidneys, limits its *ad libitum* intake of acid, which helps to maintain acid-base balance (26). Salt taste is produced predominantly by the Na^+ ion, which is vital to the preservation of water and blood homeostasis in the body (23). Sweet taste is a highly pleasurable sense designed to detect food with elevated levels of saccharides. These foods function as an energy source for organisms (8). Umami, the Japanese word for savory taste, denotes the presence of amino acids in a particular food. Monosodium L-glutamate, MSG, which occurs naturally in many foods is often used as a food additive and elicits the savory taste of umami (17). Although these five primary tastes are shared among humans, individuals can differ considerably in taste thresholds (4). These differences are likely due to genetic variation in receptors and play a role in food preference (8).

The tongue occupies a majority of the oral cavity as well as the oropharynx and is a multifunctional organ in the mouth. It acts as the primary organ of taste in the gustatory system

and supports the mastication and deglutition of food. The tongue originates during the first month of gestation from the branchial arch and is innervated by five cranial nerves (1). The dorsal surface of the tongue contains lingual papillae, which are projections of lamina propria and have sensory endings for taste (1). There are four types of papillae which include filiform, foliate, fungiform, and circumvallate. Foliate, fungiform, and circumvallate papillae contain taste buds which depolarize in the presence of a taste molecule, causing an increase in intracellular calcium and the release of a specific neurotransmitter (1).

The sense of taste provides organisms with a vast amount of information regarding the characteristics of the foods they will consume. Information including the quality, toxicity, and nutritional value are processed and distinguished by various taste receptors and sent to the brain to prepare the organism complex biochemical and physiological processes.

1.2 Taste Buds

Taste buds are specialized structures that reside primarily on the dorsal surface of the tongue and soft palate, but can be found throughout the oral cavity (38). These structures are responsible for determining the chemical composition of the food organisms ingest (38). Taste buds can be distinguished by a characteristic oval shaped arrangement of elongated cells. These cells are comprised of three different cell types; functioning as supporting cells as well as taste receptors for salty, bitter, sour, sweet and umami taste stimuli (43). They are located within projections of loose areolar connective tissue called papillae (1).

The body of the tongue is comprised of four types of lingual papillae, while three of which contain taste buds (39). Fungiform papillae are mushroom-shaped structures which are located at the apex and lateral surfaces of the tongue. Foliate papillae consist of mucosal folds on the lateral aspect of the tongue (1). On the posterior aspect of the tongue, circumvallate papillae

are distributed in an inverted V shape. The most abundant papillae are filiform papillae, located on the dorsum of the tongue. Although filiform papillae are not involved in taste, they supply the tongue with valuable information about tactile sensation (1).

Electron micrographs of taste buds have indicated that there are cells within taste buds which have been classified as Type I, II, and III cells. Type I cells are the most numerous and express genes with multifunctional purposes (8). These cells are thought to be involved in glutamate uptake as well as K^+ homeostasis within the taste bud (8). Their general function serves to terminate synaptic transmission and restrict the spread of neurotransmitters. ENaC subunits are most likely expressed in Type I cells. Type II cells are predominately responsible for signal transduction and are located in the plasma membrane of the taste bud. These cells bind to bitter, sweet, and umami compounds but are not associated with salty or sour stimuli. Type III cells are characterized as presynaptic cells with neuronal properties and transduce sour taste (8).

The mammalian taste bud cell (TBCs) are polarized neuro-epithelial cells with apical and basal regions separated by tight junctions (44). TBCs elicit spontaneous action potentials and demonstrate the presence of voltage-gated Na^+, K^+ and Ca^{2+} channels. Microvilli project from the taste pore on the apical surface and have direct contact with the saliva, while the basal region is devoted to cell communication (44). Taste buds release the taste specific neurotransmitter, ATP, to relay taste information to the brain (44).

1.3 Taste Receptor Cells

In the gustatory system, taste receptor cells (TRCs) are located within the taste bud (1). These cells have microvilli, which are projections that allow for the detection of different stimuli within the oral cavity (44). Taste receptor cells express GPCRs, ion channels, and specific proteins in the apical membrane which function as taste receptors.

Figure 1. Diagrammatic representation of a taste bud: This diagram illustrates the structure of a taste bud and its structural components; taste receptor cells, tight junctions, supporting cells, gustatory neural innervation, and taste specific neurotransmitters (15). This image was obtained from Dr. Janet Fitzakerley's – 2014 Sensory Physiology.

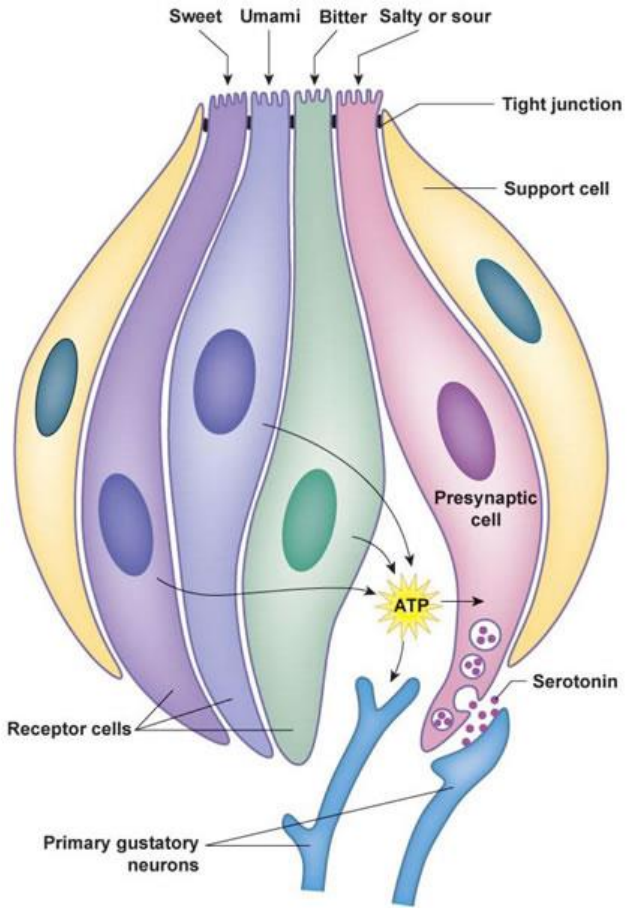
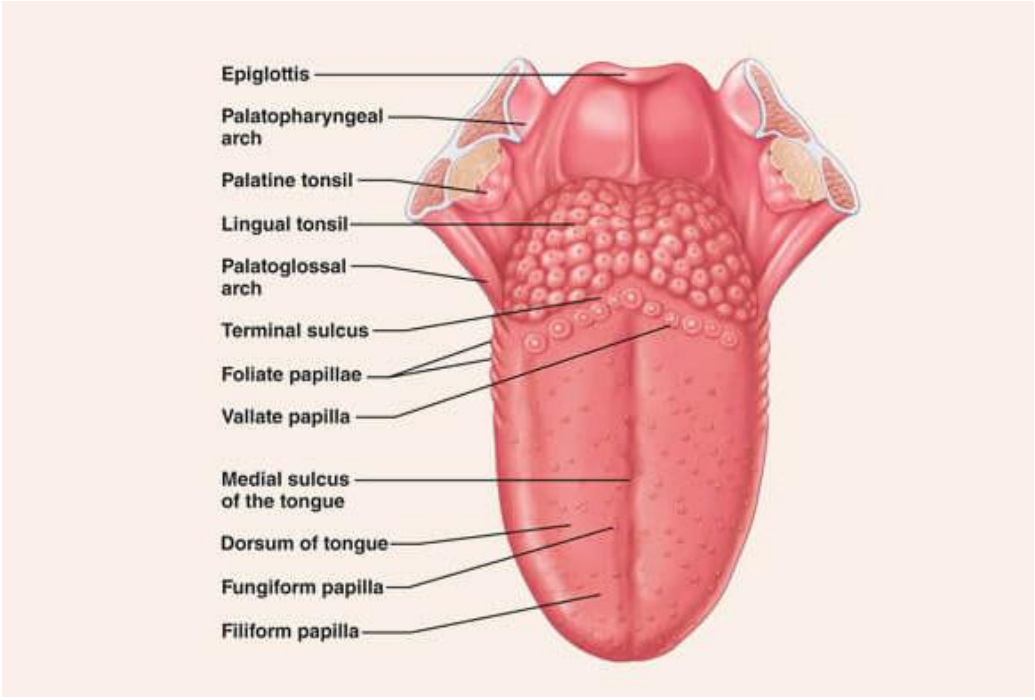

Sweet
Umami
Bitter
Salty or sour
Tight junction
Support cell
Presynaptic cell
ATP
Serotonin
Receptor cells
Primary gustatory neurons

Figure 2. Diagrammatic representation of the human tongue and papillae: This diagram illustrates the various anatomical structures on the human tongue as well as the location of the filiform, fungiform, foliate, and vallate papillae (47). This image was obtained from Organs of the Body – Human Tongue Parts, Functions with Details and Diagram.


Epiglottis
Palatopharyngeal arch
Palatine tonsil
Lingual tonsil
Palatoglossal arch
Terminal sulcus
Foliate papillae
Vallate papilla
Medial sulcus of the tongue
Dorsum of tongue
Fungiform papilla
Filiform papilla

Following the binding of a tastant to its receptor, an essential downstream intracellular taste transduction step is the depolarization of TBCs and the release of the neurotransmitter, ATP.

In the case of sour and salty sensing TBCs, the entry of H^+ and Na^+ via proton channels and ENaC respectively, leads to the depolarization of the cell. Depolarization activates voltage-gated Ca^{2+} channels (VGCCs) and Ca^{2+} flows into the cells and triggers the release of ATP (1). In the case of sweet, bitter, and umami cells, intracellular Ca^{2+} is released from IP_3-recptors in the endoplasmic reticulum (ER) and activates a basolateral membrane Transient receptor potential non-specific cation channel subfamily M member 5 (TRPM5) that allows Na^+ and K^+ entry, resulting in cell depolarization (16). Each taste stimuli, representing a different taste quality, is detected by a different subset of TBCs expressing a quality specific receptor, which activates a unique transduction cascade and leads to the specific taste quality to be identified (1). In the case of sweet, bitter, and umami taste, the receptors are GPCRs. In the case of sour and salty taste, the receptors are ion channels, proton channels and ENaC, respectively.

Different cranial nerves innervate taste receptor cells based on their localization within the oral cavity. The chorda tympani is a branch of the facial nerve (CN VII) which innervates the fungiform taste bud cells and is activated by stimuli on the anterior aspect of the tongue (1). Information presented on the posterior aspect of the tongue is directed by the lingual-tonsillar branch of the glossopharyngeal nerve (CN IX) (1). The vagus nerve (CN X) and trigeminal nerve (CN V) also play a role in the transduction of taste and sensation to the organism (1). The site at which the taste receptor cell is stimulated determines the specified afferent neural pathway, which transmits information from the sensory system to the brain.

1.4 Physiology of Taste

Sweet, bitter, and umami taste sensations are initiated by the interaction of tastants with seven transmembrane domain G protein-coupled receptors (GPCRs). This occurs in the apical membranes of the taste receptor cells which leads to a signal cascade and ultimately the release of the taste specific neurotransmitter, ATP (16).

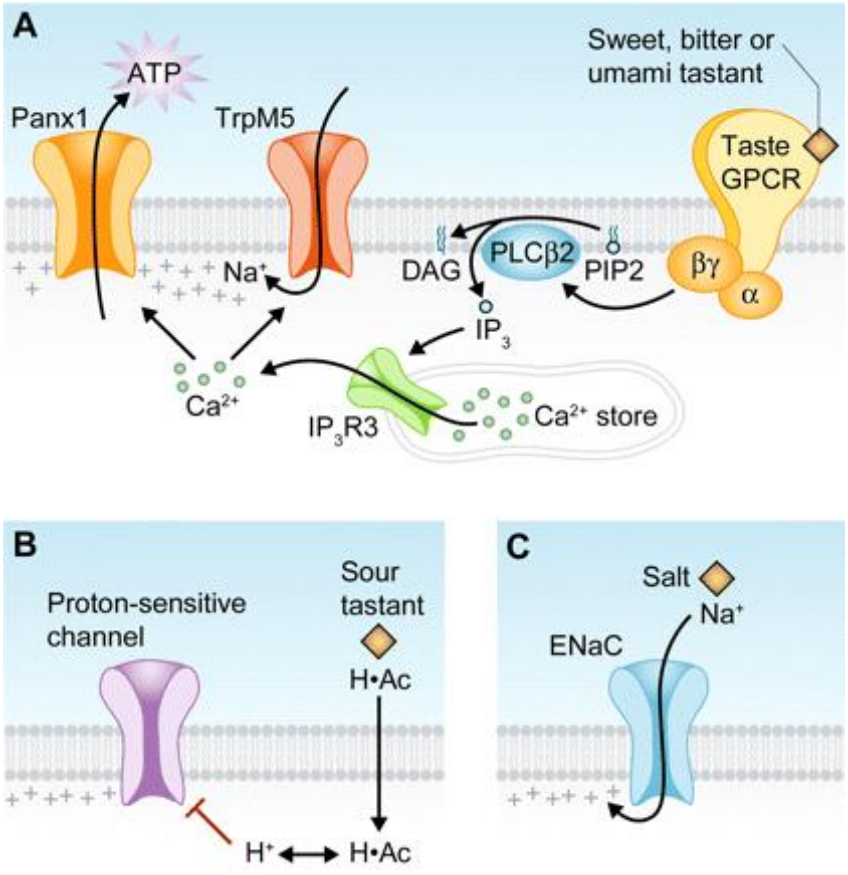
When sweet, bitter, or umami tastants bind to their receptors in Type II cells, taste specific GPCRs activate GTP-binding proteins. This frees α and $\beta\gamma$ subunits to interact with a phospholipase, PLCβ2. PLCβ2 then stimulates the synthesis of IP_3 and DAG from PIP_2. IP_3 becomes the second messenger which causes the IP_3R_3 ion channels on the endoplasmic reticulum (ER) to open, releasing Ca^{2+} (16). The influx on Ca^{2+} into the cytosol of taste receptor cells targets TRPM5, a taste-specific cation channel, and Panx1, a gap junction hemi-channel (16). When TRMP5 opens, it causes the cell to depolarize and evokes an action potential. The action potential results in the taste specific neurotransmitter, ATP, to be secreted through the hemi-channel into the extracellular space (16). This data is supported by the observation that KO mice lacking ionotropic purinergic P2X receptor units P2X2 and P2X3 demonstrated no neural responses in chorda tympani and glossopharyngeal taste nerves to tastants (16).

DNA sequencing discovered a multigene family of GPCRs involved in the transduction different compounds. The fundamental taste receptors are broken into T1R and T2R GPCR families. Downstream from the GPCRs, the transduction mechanism for sweet, bitter, and umami tastes are the same (8). Bitter compounds are detected by the T2R family of GPCRs and display a diverse receptive range (7). The T1R family of GPCRs are comprised of T1R1, T1R2, and T1R3 (7). The T1R1/T1R3 heterodimer detects umami stimuli which are often found in foods with high levels of L-glutamate and guanosine monophosphate (GMP) (8). It is believed that additional taste receptors may be involved in the transduction of umami taste because T1R3

knockout (KO) mice because they demonstrate residual neural and behavioral responses to umami taste stimuli. Lastly, the T1R2/T1R3 heterodimer detects sweet stimuli which are often found in foods with high levels of sugars and carbohydrates (8).

The mechanism of sour taste transduction differs from the other tastants because this stimulus excites presynaptic Type III cells. PKD2L1 and PKD1L3 ion channels have been proposed as a membrane receptor for sour taste (8). The transduction of salt taste stimuli is regulated by the amiloride-sensitive and amiloride-insensitive pathways. The Na^+-specific salt taste is detected by the amiloride-sensitive ENaC (10). The amiloride-insensitive component of salt taste is most likely detected by a non-selective cation channel that is permeable to Na^+, K^+, NH_4^+ and Ca^{2+}, one possibility being a TRPV1 or TRPV1 like non-specific cation channel (8).

Figure 3. Diagrammatic representation of taste transduction: This diagram illustrates sweet, bitter, and umami ligands binding to their specific GPCR and activating the phosphoinositide pathway which leads to Ca^{2+} stores to be released and ultimately leading the taste specific neurotransmitter, ATP, to be released (8). This image was obtained from Dr. Nurupa Chaudhari's – The cell biology of taste.


A
ATP
Panx1
TrpM5
Sweet, bitter or
umami tastant
Taste
GPCR
Na+
DAG
PLCβ2
PIP2
βγ
α
IP3
Ca2+
IP3R3
Ca2+ store
B
Proton-sensitive
channel
Sour
tastant
H•Ac
H+
H•Ac
C
Salt
Na+
ENaC

1.4.1 Bitter Taste

Mammals have the ability to discriminate between a vast range of bitter compounds. The multigene family of taste receptor cell GPCRs, known as T2Rs, have been shown to function as bitter taste receptors and are predominately located in fungiform, circumvallate, and foliate papillae (8). The T2R family is known to be encoded by 20-35 separate genes which are expressed by overlapping subsets of mRNAs (8).

When bitter tastants bind to GPCRs, the bitter T2Rs activate α-gustducin, which serves as the taste-selective G-α subunit. This pathway follows that of sweet and umami taste, where βγ-gustducin activates the PLCβ2/TRPM5-dependent pathway, which leads to an increase in intracellular Ca^{2+} and neurotransmitter release (8, 39). Upon release of the neurotransmitter, the organism is able to detect bitter stimuli and help it to avoid it as a potential toxic compound.

1.4.2 Salt Taste

Salt taste plays an essential role in maintaining physiologic processes in the nervous system as well as hormonal and behavioral regulation. The sodium ion (Na^+), is the predominant ion which is known to impart a 'salt' taste (23). Evidence from animal models indicate amiloride, a diuretic compound known to block ENaC channels, reduces the specificity and quality of salt taste perception when used to block the channel (12). Previous observations showed that a conditional α-ENaC KO mice in which the channel is silenced only in taste receptor cells, did not display the amiloride-sensitive component of the NaCl chorda tympani response and showed significantly reduced licking for appetitive NaCl concentrations (11). However, in humans, amiloride is less effective in blocking salt taste perception than in animal models (20). Humans differ from rats, mice, and hamsters in that their salt taste perception is primarily less sensitive to the application of amiloride on the anterior tongue (34). Humans

express a δ-ENaC subunit in addition to the αβγ subunits. Relative to αβγ-ENaC, the δβγ-ENaC subunit is 10-fold less sensitive to inhibition by amiloride. In human behavioral experiments, even when amiloride was added at 10 times the concentration that inhibits αβγ-ENaC and δβγ-ENaC channels, it only reduced salt taste perception by 21% (11). These results suggest that unlike rodents, a significantly smaller component of salt taste is ENaC-dependent (11). Since humans can perceive Na^+, K^+, NH_4^+ and Ca^{2+} salts as salty, it is quite likely that one or more non-specific cation channels are the major contributors to human salt taste perception.

Mammals have developed an exquisite sensory system and can differentiate between mineral salts through the utilization of two different taste receptor systems. Fungiform taste receptor cells express functional amiloride-sensitive epithelial Na^+ channel (ENaC) (11). The glossopharyngeal taste nerve responses to NaCl are not affected by amiloride (11). Although, ENaC subunits are expressed in circumvallate taste receptor cell located in the posterior aspect of the tongue, no functional ENaC channels are present in the apical membrane of circumvallate taste receptor cells (11). In different species of mammals, contribution of amiloride-sensitive and amiloride-insensitive component of salt taste is variable.

1.4.2.1 TRPV1 and Salt Taste

TRPV1 is a member of the transient receptor potential channel (TRP) of the vanilloid class (34). Dorsal root and trigeminal ganglion neurons express TRPV1, which are primarily responsible for the detection of pain and thermal nociception (34). This polymodal non-selective cation channel is permeable to Na^+, K^+, NH_4^+ and Ca^{2+} (34). Unlike ENaC, TRPV1 is constitutively closed but can be opened by capsaicin, heat, and low pH (30). Due to the high permeability of Ca^{2+} ions through TRPV1 channels, TRPV1 agonists increase influx of Ca^{2+} ions through the channel (30). The prolonged increase in intracellular Ca^{2+}causes an overload of the

capsaicin-sensitive nerve terminals and subsequent desensitization of nociceptive sensory endings. These structural and functional changes serve as the basis of the therapeutic use of capsaicin as a topical analgesic (48).

Stimulating rat or mouse anterior tongues with mixtures containing NaCl and TRPV1 activators capsaicin (CAP), resiniferatoxin (RTX), and elevated temperature (>38°C) induced biphasic effect on the chorda tympani (CT) taste nerve responses (34). At low concentrations of agonists, NaCl CT response increased and at high concentrations inhibited the NaCl CT response (34). These responses were also observed in the presence of amiloride or benzamil, specific ENaC blockers, indicating that TRPV1 agonists modulated the amiloride-insensitive component of NaCl CT response (34). NaCl CT taste nerve responses were blocked by TRPV1 antagonists, N-(3-methoxyphenyl)-4-chloro-cinnamide (SB-366791) and capsazepine (CZP) (34). In TRPV1 KO mice, no changes in the amiloride-insensitive CT responses were observed in mixtures containing NaCl + amiloride + TRPV1 agonists, but did not show behavioral differences to NaCl relative to wildtype mice (34). Although data suggests that TRPV1 plays a role in Na^+ detection, there may be additional receptors involved in the transduction of Na^+ through the amiloride-insensitive non-specific cation channels (46). Presently, it is not known how TRPV1 agonists and antagonists modulate NaCl CT responses in rodent taste receptor cells. Thus, it is most likely that TRPV1 activation indirectly modulates NaCl CT responses in rodents.

1.4.3 Sweet Taste

Genetic mapping of rodent models has provided underlying data to locate the loci for sweet taste. The murine loci, sac, governs the preference and electrophysiological response to sweeteners including sucrose and saccharin (39). This loci is known to be the main genetic factor involved in sweet taste perception (39).

Previous observations have shown that the taste receptor cell contains sac (T1R3) but its heterologous expression isn't enough to yield a functional sweet receptor (39). This suggests that a heterodimeric form of T1R2/T1R3 is required to produce a functioning receptor (39). Researchers examined T1R2+T1R3 using KO mice which lacked functioning receptors. A group of sugars were provided to test sweet response and the subsequent response was impaired when the T1R2+T1R3 receptor was knocked out or specific subset of cells were obliterated from the taste buds using diphtheria toxin (7). These findings support that the heterodimeric T1R2+T1R3 is the mammalian sweet taste sensor (53).

1.4.4 Sour Taste

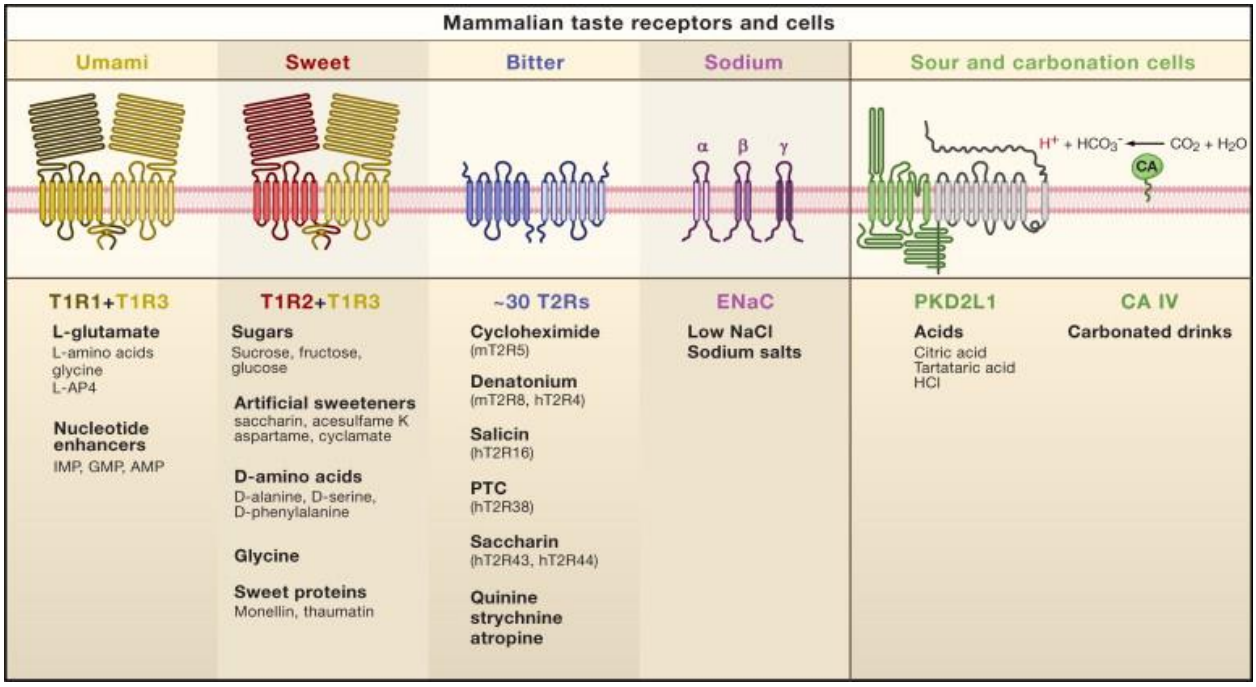
Sour taste is a sensation which has developed as a mechanism to prevent organisms from ingesting spoiled and acidic food (26). Sour taste is detected by a different subset of cells (Type III cells) that express polycystic kidney disease-like ion channel, PKD2L1 (26). Using a cell ablation method dependent upon diphtheria toxin, ablating type III cells from the taste buds abolished CT responses to sour taste stimuli without affecting CT responses to other taste stimuli (26). However, in PKD2L1 KO mice, sour taste responses were diminished but not eliminated (26). PKD2L1 cells also express carbonic anhydrase 4, which is involved in the detection of CO_2, a sour stimulus (26). Acid-induced decrease in intracellular pH of sour sensing cell is the proximate intracellular signal for sour taste transduction (26).

1.4.5 Umami Taste

Umami receptors respond to the combination of multiple compounds, including GMP/IMP and L-glutamate (53). Observations suggested that mGluR4t, a variant of the glutamate receptor-4 functions as the umami taste receptor (53). These findings are contradicted because mGluR4t is postulated to function by a cAMP dependent pathway, which differs from

the umami taste PLCβ2/TRPM5 transduction cascade (53). Evidence suggests that umami taste is transduced through taste receptor cells which express the heterodimeric GPCR T1R1+T1R3 (53). This heterodimeric receptor has characteristics that share similarities in selectivity and sensitivity that attributed to amino acid taste. T1R1+T1R3 can be amplified by inosine monophosphate (IMP) and GMP which are known enhancers of umami taste and is activated by L-Asp which is an umami agonist (53). T1R1+T1R3 knockout (KO) mice were examined and results showed that there was a substantial decrease in physiological response when they were exposed to umami tastants and their enhancers (53). These results were further supported by the demonstration that T1R1 KO mice lack all umami taste response. This demonstrated that the combination of T1R1+T1R3 is the mammalian umami receptor (53).

Figure 4. Mammalian taste receptors and cells: This diagram illustrates the five mammalian taste receptors and the respective cells that they bind (52). This image was obtained from Dr. Yarmolinsky's – Common sense about taste: from mammals to insects.


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 ENaC

The amiloride- and benzamil-sensitive epithelial Na^+ channel (ENaC) functions as a heterotrimeric channel which transports Na^+ across the apical plasma membrane in tight epithelial tissues (3). ENaC plays a critical role in the regulation of salt and water homeostasis in the kidneys, lungs, distal colon, and the anterior fungiform taste papillae. ENaC expression and function is regulated by hormones (arginine vasopressin, aldosterone, and insulin) and intracellular signaling components, pH_i, $[Ca^{2+}]_i$, cell volume, osmolarity and by sodium self-inhibition (9).

ENaC works in conjunction with the basolateral Na^+/K^+-ATPase which regulates salt reabsorption and plays a chief role in the maintenance of blood pressure and water homeostasis in the body (3). In rodents, ENaC is comprised of αβγ subunits which are structurally related but can undergo posttranslational modifications to alter their function (3). Humans differ from rodents in that they express an additional δ-subunit, which is expressed in human epithelial tissues (19). The δ-gene is a pseudo gene in rodents. The δ-subunit is able to combine with βγ subunits to form δβγ-ENaC. Relative to αβγ-ENaC, δβγ-ENaC is about 10-fold less sensitive to inhibition by amiloride and benzamil (19).

1.7 Capsaicin

Capsaicin, (8-methyl-N-vanillyl-trans-6-nonenaminde), is the predominant pungent ingredient which is derived from plants of the genus Capsicum (46). This hydrophobic compound is responsible for thermal and nociceptive sensations experienced when ingesting ‘hot’ chili peppers, which is initiated by the activation of sensory neurons of the trigeminal nerve (CN V) on the anterior aspect of the tongue (46). Capsaicin is a TRPV1 agonist and activates primary afferent neurons (46). This activation allows for TRPV1 receptor cells in the trigeminal

nerve to communicate with the central nervous system to warn the organism against consuming noxious stimuli (5, 46).

Capsaicin also serves a beneficial role in mammals. It has been shown to stimulate metabolic activity, aid in weight loss, and promote the oxidation of fatty acids (46). Additionally, capsaicin may have tumor suppressing effects and have a cardio-protective role in organisms (46). These findings have led researchers to continue searching for novel functions capsaicin may play in the metabolism of mammals.

1.8 Health Concerns Relating to Excess Sodium Intake

Sodium is a staple in the diet and is the most widely consumed mineral around the world. Sodium is essential to humans and the main challenge is that the human body cannot efficiently store it for extended periods of time. Without the ability to store sodium, humans are required to consume it on a regular basis. While there is a spectrum of sodium preference among individuals, high salt consumption is a common practice in most cultures and has compromised the health of many (10).

It has been reported that genetic and environmental factors play a role in the pathogenesis of hypertension and cardiovascular disease. One of the leading environmental causes of hypertension is due to increased dietary sodium intake (30). The kidney's ability to regulate urinary sodium excretion establishes the organism's intravascular fluid volume and blood pressure. In humans, the ENaC channel is a primary mechanism for sodium reabsorption and regulation in the kidney collecting ducts and aberrations of this function may lead to hypertension (30). These observations have led to an emergent public health concern which has been addressed by the International Life Sciences Institute and has been reported as a primary concern of the US food and Drug Administration (FDA) (10).

Diseases implicated with irregular sodium intake can benefit from gaining a greater understanding of ENaC channels and the ability to manipulate its function. For example, patients with Liddle Syndrome display a deletion of the PY motif in ENaC, which leads to an increase in the channel number expression as well as the open probability of channels (40). The unregulated, continual Na^+ reabsorption across the collecting tubules of the kidney results in volume expansion, suppression of plasma renin activity, aldosterone secretion, and consequent hypertension (40). Alternatively, pseudohypoaldosteronism displays reduced ENaC activity, resulting in decreased Na^+ reabsorption in the kidney, salt wasting, and volume reduction which subsequently leads to hypotension (40). These syndromes exemplify the importance of ENaC in maintaining health and show the consequences of a diseased state.

The FDA and healthcare community have been working to develop strategies to prevent the growth and development of hypertension in the population. A solution to this concern entails reducing sodium intake while increasing the consumption of vegetables. An alternative to healthy eating is the use of thiazide diuretics. These diuretics target the distal convoluted tubule by inhibiting renal sodium absorption, leading to an increased urinary sodium excretion and decreased extracellular fluid volume (30).

1.9 Objective of this Study

In humans, sodium specific salt taste perception is predominantly regulated by the amiloride- sensitive epithelial Na^+ channel, ENaC. ENaC is a heterotrimeric channel which has profound effects on the functioning of physiologic systems. While the amiloride-sensitive ENaC is comprised of αβγ-subunits in rodent models, human taste cells contain αβγ-ENaC and δβγ-ENaC subunits. Currently, it is unknown if ENaC subunits expression and function can be modulated to alter Na^+ specific salt taste in humans. Activation of TRPV1 can act either directly

or indirectly modulate ENaC activity. Activation of TRPV1 can release calcitonin gene related peptide (CGRP) or substance P that can indirectly affect ENaC activity. Alternately, if TRPV1 is co-expressed in ENaC containing cells, it can directly affect ENaC expression and function (30). In rodents, TRPV1 is not expressed in taste buds cells but is located in the surrounding cells. However, in humans, TRPV1 is expressed in taste buds (24). Capsaicin is an agonist of TRPV1 and enhances amiloride-insensitive non-ENaC component of salt taste in wildtype mice but not in TRPV1 KO mice. In humans, TRPV1 modulators, especially those that are non-pungent, have been shown to modulate human salt taste perception in psychophysical evaluation. My hypothesis is that long term activation of TRPV1 can modulate ENaC subunit expression and function in human cells that co-express TRPV1 and ENaC. I specifically tested the effect of capsaicin on human-specific δ-ENaC subunit expression and function in HEK293 cells and HBO cells. The pursuit to identify mechanisms that alter the perception of salt taste in humans could have profound effects on dietary and clinical applications.

Chapter 2: Materials & Methods

2.1 Human Embryonic Kidney Cell (HEK293)

HEK293 cells were obtained from (American Type Culture Collection, Manassas, VA). HEK293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (VWR Life Science) supplemented with 10% Fetal Bovine Serum (FBS) (Hyclone, Logan, UT), 25 mM 4-(2-hydroxyethly)-1-piperazineethanesulfonic acid (HEPES), and 100 mL Penicillin-Streptomycin (Invitrogen, Carlsbad, CA) at 37 °C in 5% CO_2.

2.2 Cultured Adult Human Fungiform Taste Papillae (HBO) Cells

HBO cells were obtained from Monell Chemical Senses Center, located in Philadelphia PA. HBO cells were cultured as described earlier (41).

2.3 Western Blot to detect TRPV1 and δ-ENaC protein expression in HEK293 Cells

HEK cells were treated with 0.1 μM, 0.25 μM, 0.5 μM, 0.75 μM, and 1.0 μM capsaicin dissolved in DMEM for 7 hours in the incubator at 37°C in 5% CO_2. Untreated HEK cells were used as a negative control.

HEK cells treated with capsaicin were lysed in 200 μL modified RIPA buffer (Thermo Fisher Scientific, MA, USA). Western blots used 30 μg of total protein sample. Protein samples were resolved by 10% SDS-PAGE (Bio-Rad) and transferred to nitrocellulose membranes (Cat: 162-0094, Bio-Rad). Three separate membranes were immune-blotted with either δ-ENaC antibody (Lifespan Biosciences, Inc (LS-C119717), TRPV1 antibody (Lifespan Biosciences, Inc LS-Cl72124) or a β-actin antibody (Santa Cruz Biotechnology). Subsequently, HRP-conjugated secondary antibodies were added to the membrane of interest; anti-Rabbit IgG HRP for TRPV1, anti-Rabbit IgG HRP for δ-ENaC, and anti-Mouse IgG HRP for β-actin, were added. β-actin served as the protein loading control. ECL Western Blotting Substrate was used to for detection

of HRP activity from the antibodies. CL-XPosure film (ThermoFisher Scientific #34090) was developed using an auto-processor in a darkroom.

2.3 Detection of TRPV1 and δ-ENaC using qRT-PCR

Total RNA from HEK cells was purified using TRIZOL reagent (cat# 15596029, Thermo Fisher Scientific, MA, USA) and reverse transcription was performed using High-Capacity cDNA Reverse Transcription Kit (cat# 1308188, AB applied biosystems, CA, USA). 4 μg total RNA was mixed with 2 μL 10X RT Buffer, 0.8 μL 25x dNTP, 2.0 μL 10xRT Random Primer, 1.0 μL MultiScribe Reverse Transcriptase 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. Afterwards, 200ng of total cDNA was used as a template and mixed with 8 μL of H_2O, 10 μL of 2x MyTaq Red Mix, and 1 μL of specific primer (GAPDH, TRPV1, or δENaC) and PCR amplification was performed using BioRad C1000 Thermal Cycler. The products were added to 1% agarose gel electrophoresis to determine the expression of TRPV1 and δ-ENaC. Dilute sample with 20 μL of RNase-free water to give 40 μL total volume of solution. qRT-PCR for the detection δ-ENaC and TRPV1 was carried out by using AB applied Biosystems reaction mix. 1 μL of 20x TaqMAn Gene Expression Assay, 10 μL of 2x TaqMan Gene Expression Master Mix, 2 μL of cDNA template and 5uL of RNase-free water was added to a test tube and briefly centrifuged. 20 μL of PCR reaction mix solution was transferred into each well of a 96-well reaction plate and loaded into Applied Biosystems StepOne/StepOnePlus Real-Time PCR system and run at standard thermal cycling conditions.

2.4 Immunoflourescence and Confocal Imaging to co-localize TRPV1 and δ-ENaC

HBO cells were plated into a collagen coated 8-well chamber and were fixed with methanol for 10 min at -20°C. Slides were washed with 200 μL PBS x 3 for 5 min each, then

blocking buffer with 3% donkey serum was added for 1h at room temperature. Subsequently, slides were stained with δ-ENaC antibody (1:100 dilution in 3% donkey serum) or TRPV1 antibody (1:100 dilution in 3% donkey serum).

Slides were washed again with 200 uL PBS x 3 for 5 min each and incubated in the dark with 1 μL donkey anti-rabbit 488 (1:1000) for 1 h at room temperature. Slides were washed with 200 μL PBS x 3 for 5 min each. Opposite primary antibody (δ-ENaC and TRPV1) and 3% donkey serum to negative control was added and stored overnight at 4°C. Slides were washed again with 200uL PBS x 3 for 5 min each, then incubated in the dark with 1 μL donkey anti-goat TR (1:200) for 1 h at room temperature. Slides were washed with 200 μL PBS x 3 for 5 min each. Cells were incubated on (DAPI; 1 μg/mL) for 1 min and rinsed with PBS. Coverslip was mounted with a drop of mounting medium. Images were then viewed using a 63x (1.4 numerical aperture) oil immersion objective on a Zeiss LSM 700 confocal microscope and processed using ImageJ software by Dr. Jie Qian and Ms. Shobha Mummalaneni.

2.5 Calcium Imaging

Temporal changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) were measured in HEK293 cells as a response to capsaicin to demonstrate the presence of functional TRPV1 channels in cells. HEK cells were plated onto glass coverslips (Warner Instruments, Hamden, CT, USA) and grown in the incubator at 37°C in 5% CO_2 until 75% confluency. 50 μL of DMSO was mixed into a Fura-2- AM (ab120873) solution followed by 50 μL of 10% pluronic. Fura-2 solution was added to 3 mL of normal Ringer's solution and placed on vortex for 10 seconds. Slides were added to the chamber and washed with 500 μL Ringer's solution x3 containing (in mM) 140 NaCl, 5 KCl, 1 $CaCl_2$, 1$MgCl_2$, 10 glucose, 10 HEPES, 7.4 pH and 650 μL of Fura-2-AM solution was loaded onto each slide and incubated in the dark at room temperature for 2 hours. Slides were washed 3x

with Ringer's solution and coverslips were mounted in an experimental chamber (RC-26GLP, Warner Instruments; 0.7 ml volume) and fitted onto a Series 20 Chamber Platform (Warner Instruments). The cells were visualized through a water immersion 40X objective (Zeiss; 0.9 NA) with a Zeiss Axioskop 2 plus upright fluorescence microscope. The cells were excited with 340 (nm) and 380 (nm) which was visualized at 15 s intervals. Relative changes in Ca^{2+} were expressed as changes in FIR (fluorescence intensity ratio; F_{340}/F_{380}) in individual cells relative to control. The FIR obtained in cells in control Ringer's solution were normalized to 1. Changes in FIR were analyzed using TILL Vision V3.3 software (TILL Photonics, Martinsried, Germany).

2.6 Sodium Imaging

Temporal changes in intracellular Na^+ ($[Na^+]_i$) were measured in HEK-293 cells as a response to a change in Na^+ from zero to 140 mM in Ringer's solution to evaluate the ENaC channel activity at room temperature. The composition of zero Na^+ Ringer's solution was (in mM) 140 N-methyl-D-glucamine (NMDG), 5 KCl, 1 $CaCl_2$, 1$MgCl_2$, 10 glucose, 10 HEPES, 7.4 pH. Relative changes in intracellular Na^+ activity ($[Na^+]_i$) were monitored in HEK cells by loading the tissue with sodium benzofuran isophthalate (SBFI)-AM as described earlier (31). Changes in HEK ($[Na^+]_i$) were monitored in control cells and cells treated for 7 h with 2.5 μM capsaicin dissolved in DMEM. Similar to the case with Ca^{2+} imaging, for Na^+ measurement, the cells were alternately excited at 340 (nm) and 380 (nm) and the images were captured at 350 ± 10 nm at 15 s intervals. The relative changes in HEK ($[Na^+]_i$) were expressed as changes in FIR (F_{340}/F_{380}) relative to control Ringer's solution in individual cells. The FIR obtained in cells in control Ringer's solution were normalized to 1. The data were presented as means ± SEM of N, where N represents the number of regions of interest (ROIs) in HEK293-cells in the viewing area.

Table 1. Primers used for qRT-PCR. Primers used to detect the mRNA of δ-ENaC and TRPV1 are listed below.

Gene	NCBI Accession Number	Primer	Length (bp)
δ-ENaC	NM_00130413		186
Forward		CCATCAGCATCCGAGAGGAC	
Reverse		GAGGGTGGAGGTAGTAGCCA	
TRPV1	NM_018727.5		322
Forward		TCCAGCAGATGGGCATCTATG	
Reverse		AGGACAAGTGGGACAGATTCG	

Chapter 3: Results

Experiments were designed to investigate if activation of TRPV1 can modulate the expression and function of ENaC. Our original plan was to investigate modulation of ENaC activity by TRPV1 in HBO cells and its implications regarding human salt taste perception. We had initiated a Material Transfer Agreement (MTA) with Monell Chemical Senses Center, Philadelphia, PA for this project. We received the permission to use HBO cells to investigate the role of TRPV1 in regulating ENaC in the April of 2018. Due to this delay in executing the MTA, we did initial studies in HEK293 cells and later part of the study in HBO cells. Since, both HEK and HBO cells are of human origin, our studies were focused on δ-ENaC subunit, a taste specific human subunit of ENaC that forms a function channel comprising δ, β, and γ subunits. The δβγ-ENaC channel is 10-fold less sensitive to blockage by amiloride and benzamil than the αβγ-ENaC.

3.1. mRNA expression of δ-ENaC subunit in HEK-293 cells

The δ-ENaC subunit is expressed in human epithelial tissues, including a subset of TBCs. As expected, using specific primers and RT-PCR, we detected a single band of 186 bp (base pair) corresponding to δ-ENaC mRNA (Fig. 5A). These results indicate that δ-ENaC subunit is expressed in HEK cells.

3.2 Protein expression of δ-ENaC subunit in HEK293 cells

The expression of δ-ENaC protein was detected by immunofluorescence in HEK cells. As shown in Fig. 6, blue-fluorescent DAPI nucleic acid stain was used to identify individual cell nuclei. All the cells in the visual field were stained with the δ-ENaC antibody (green fluorescence label). The antibody binding was observed in both the cytosol and the cell membrane. In control cells, in which the primary antibody step was omitted, no green

immunofluorescence was observed. Similar ENaC antibody binding is observed in many tight epithelial cells. It is suggested that translocation of ENaC subunits from the cytosolic compartment to the apical membrane increases the number of functional channels and is a major mechanism of ENaC regulation. The specific ENaC location in the apical cell membrane could not be determined. However, it is expected that when the cell will reach 100% confluency and form a function single epithelial monolayer, the ENaC will be exclusively localized in the apical cell membrane.

3.3. HEK293 cells express functional ENaC channels

To test if HEK express functional ENaC channels, we recorded temporal changes in FIR in cells loaded with SBFI in response to the changes in bath Na^+ concentration from zero to 140 mM or vice versa. As shown in Fig. 7, decreasing bath Na^+ from 140 mM to zero, induced a rapid efflux of Na^+ and increasing Na^+ from zero to 140 mM induced a Na^+ influx into the cells. This rapid influx and efflux as a response to changes in external Na^+ is mostly due to Na^+ flux across the ENaC channels expressed in HEK293 cell membrane. In summary, the above data indicate that HEK293 cells express functional ENaC channels.

3.4. TRPV1 mRNA expression in HEK-293 cells

TRPV1 is expressed in nociceptive neurons and in many other cells types. TRPV1 has been shown to be expressed in rodent kidney cells and in cortical collecting duct cells in culture (30). Consistent with its expression in CCD cells, using specific primers and RT-PCR, we detected a single band of 322 bp corresponding to TRPV1 mRNA (Fig. 5B). These results indicate that TRPV1 RNA is expressed in HEK cells.

3.5. HEK293 cells express functional TRPV1 cation channels

TRPV1 is a non-specific cation channel that is permeable to Na^+, K^+, Ca^{2+} and NH_4^+. Under normal conditions TRPV1 channel is constitutively closed. The channel is opened in response to capsaicin, heat and low pH. To test if HEK cells express functional TRPV1 channels, we recorded temporal changes in FIR in cells loaded with Fura-2 in response to 1 μM capsaicin (Fig. 9). Immediately, following capsaicin treatment, the cells responded with a transient increase in $[Ca^{2+}]_i$ (indicated by a rapid increase in FIR). The changes in $[Ca^{2+}]_i$ were transient, and, in the continuous presence of capsaicin, returned near their normal value in all cells in the next 30-45 s. This is a typical $[Ca^{2+}]_i$ response observed in nociceptive neurons (30).

In summary, the above results indicate that HEK293 cells express TRPV1. The transient changes in $[Ca^{2+}]_i$ reflect that HEK293 cells are healthy and regulate $[Ca^{2+}]_i$ to maintain cell homeostasis even in the presence of capsaicin.

3.6. Co-localization of TRPV1 and δ-ENaC in individual HBO cells.

TRPV1 can regulate ENaC activity indirectly or directly. The direct effect of TRPV1 on ENaC is likely, if both ENaC and TRPV1 are co-expressed in the same cells. As shown in Fig. 6, all HEK293 cells investigated were stained with δ-ENaC antibody. Although I did not stain HEK293 cells with TRPV1 antibody, I expect that most of the HEK293 cells will also stain for TRPV1.

However, in order to confirm this hypothesis, I used dual labeling experiment to investigate if indeed TRPV1 and δ-ENaC are co-expressed in a subset of HBO cells. HBO cells contain subset of cells that responded to taste stimuli for all five taste qualities in a cell and taste receptor specific manner. Previous work from our laboratory using single cell PCR and immunofluorescence has demonstrated that a subset of HBO cells express all four ENaC

subunits (42). This subset of cells most likely represents the salt sensing TBCs in human fungiform taste buds.

As shown in Fig. 10, the δ-ENaC antibody binds to a subset of HBO cells. This cell also showed binding to the TRPV1 antibody. The two labels co-localize in individual cells. These results strongly support my hypothesis that TRPV1 and δ-ENaC are co-expressed in ENaC containing HEK293 and HBO cells. Further experiments were carried out to investigate the effect of capsaicin on ENaC expression and function in HEK293 cells.

3.7 Effect of capsaicin on TRPV1 mRNA Expression in HEK293 cells

To determine the effects of capsaicin on TRPV1 mRNA expression, cultured HEK cells were incubated with culture containing Cap (0.1-2.5 μM) for 7h. The results of qRT-PCR experiment showed a significant decrease in expression at 0.1 μM and 0.25 μM [p = 0.002 and 0.004, respectively] (Fig. 11A). However, there was a significant increase in expression at 1.0 μM and 2.5 μM [p = 0.002 and 0.013, respectively]. These results indicate that TRPV1 mRNA expression can be altered by prolonged exposure to capsaicin in a dose dependent manner. At present, the exact significance of a decrease in TRPV1 mRNA expression at lower capsaicin concentrations is not clear to me. In other cell types, prolonged exposure to capsaicin have shown changes in TRPV1 mRNA expression (2).

3.8. Effect of TRPV1 protein expression in HEK2913 cells

The expression of TRPV1 protein was detected by immunoblotting in control and capsaicin treated HEK293 cells. Cells were incubated with incrementally (0.1-1.0 μM) with Cap for 7h. HEK cells treated with 0.1 μM capsaicin showed a significant decrease [p = 0.016] in TRPV1 subunit protein expression while 0.75 μM and 1.0 μM capsaicin showed a significant increase [p = 0.011 and 0.032, respectively] (Fig. 12B). Western blot of β-actin was performed

as the control to calculate the fold increase in TRPV1 expression. The changes in protein expression are similar to the changes in mRNA shown in Fig. 11A. However, at present, the significance of a decrease in TRPV1 protein expression at low capsaicin concentrations is not clear.

3.9. Effect of capsaicin on δ-ENaC mRNA expression in HEK293 cells.

To determine the effects of capsaicin on δENaC mRNA expression, cultured HEK cells were incubated with Cap (0.10-2.5 μM) for 7h. While δENaC mRNA significantly increased at 0.75 μM Cap [p = 0.006], it significantly decreased after 1.0 μM and 2.5 μM exposure to Cap for 7h [p = 0.001 and 0.008, respectively] (Fig. 11B).

3.10. Effect of capsaicin on δ-ENaC protein expression in HEK293 cells.

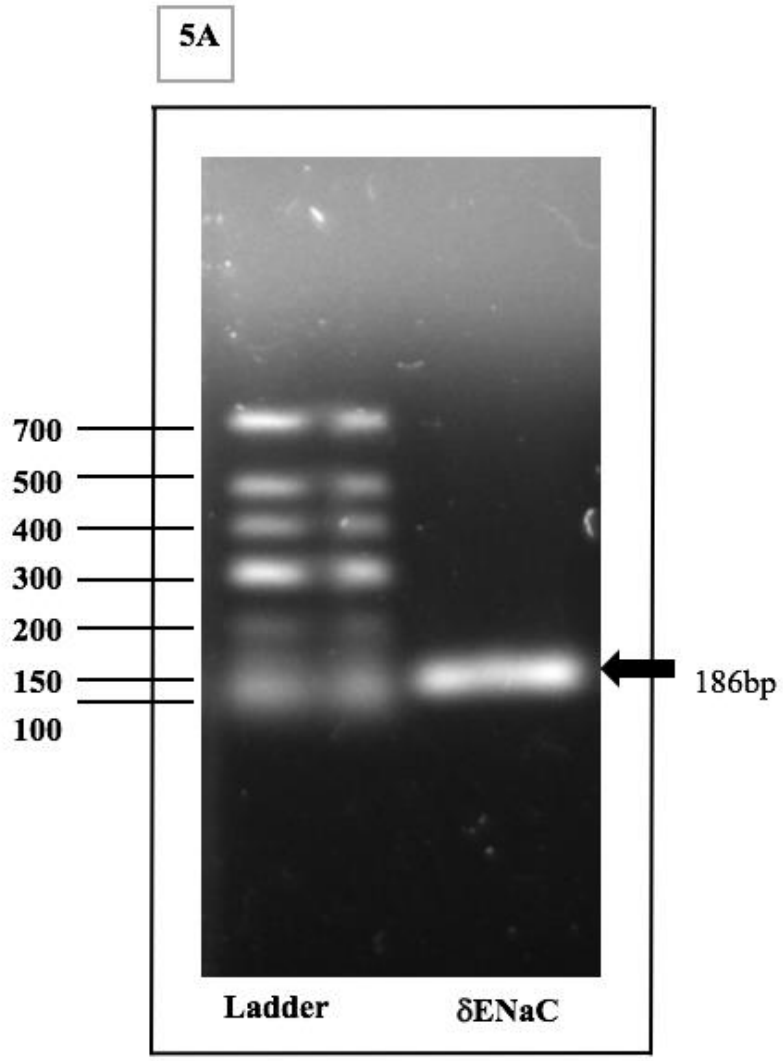
The effect of a TRPV1 agonist, capsaicin, on δ-ENaC protein expression was investigated in HEK cells, treated incrementally (0.1 to 1.0 μM) with Cap for 7h. The δ-ENaC protein expression was evaluated using Western blots. Cells treated with 0.1 μM and 1.0 μM capsaicin showed significant decrease [p = 0.037 and 0.007, respectively] in δ-ENaC subunit protein expression. Western blot of β-actin was done to calculate the fold increase in δ-ENaC expression. Consistent with mRNA expression data (Fig. 11B), the δ-ENaC protein expression was decreased at 1 μM Cap. Although I did not test δ-ENaC protein expression at 2.5 μM Cap, I predict that protein expression will further decrease relative to 1 μM Cap. This denotes that the δ-ENaC protein expression was decreased in the presence of elevated levels of capsaicin.

3.11. Capsaicin induced decrease in δ-ENaC expression alters ENaC channel function in HEK293 cells.

Na^+ can enter TRCs located in the apical membrane via two routes. The first pathway characterizes Na^+ flux through ENaC and can be blocked by the presentation of amiloride on the

apical membrane (24). The second pathway is characterized by Na^+ flux through the TRPV1 cation channel, which is inhibited by SB-366791, and is amiloride insensitive (24). To test ENaC function in control HEK cells and cells treated for 7h with Cap, we measured Na^+ flux across HEK cell membranes, induced by a change on extracellular Na^+ from zero to 140 mM. The Na^+ flux was estimated at the change in FIR per unit time. Upon a change in bath Na^+, the mean normalized changes in FIR in the first 120 s were analyzed using linear regression analysis to calculate the slope in each cell. The slope of the relationship between FIR and time reflects indirectly the flux of Na^+. In addition, V_{max}, the difference in FIR values at zero Na^+ and 140 mM Na^+ was calculated to estimate maximum Na^+ influx. As shown in the tables above Figure. 14A and 14B, HEK cells treated with 2.5 μM Cap Na^+ efflux, Na^+ influx and V_{max} are significantly lower ($p = 0.039$, 0.028, and 0.0124 respectively) relative to control. Taken together, my mRNA and protein expression data along with Na^+ imaging data indicate that long term exposure to HEK293 cells to Cap modulates ENaC expression and function.

Figure 5A & 5B. **Gel Electrophoresis after DNA amplification via PCR:** Shown below is the detection of δ-ENaC (5A) and TRPV1 (5B) DNA fragments based off of size and charge after the amplification of the respective DNA segment via PCR. A single band of expected size was yielded for both δ-ENaC and TRPV1 (25, 42).


5A
700
500
400
300
200
150
100
186bp
Ladder
δENaC

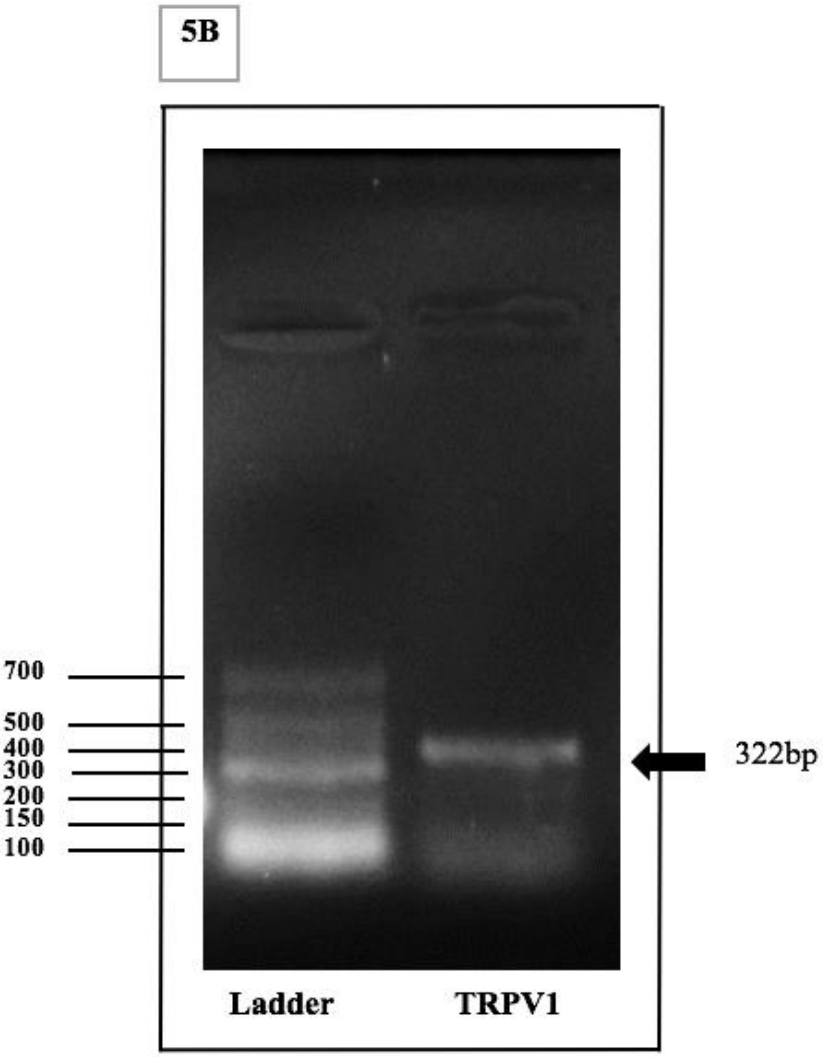

5B
700
500
400
300
200
150
100
322bp
Ladder
TRPV1

Figure 6. Immunofluorescence of δ-ENaC in HEK Cells: Using immunofluorescence, δ-ENaC is visualized in HEK cells. DAPI indicates the cell nucleus.

6

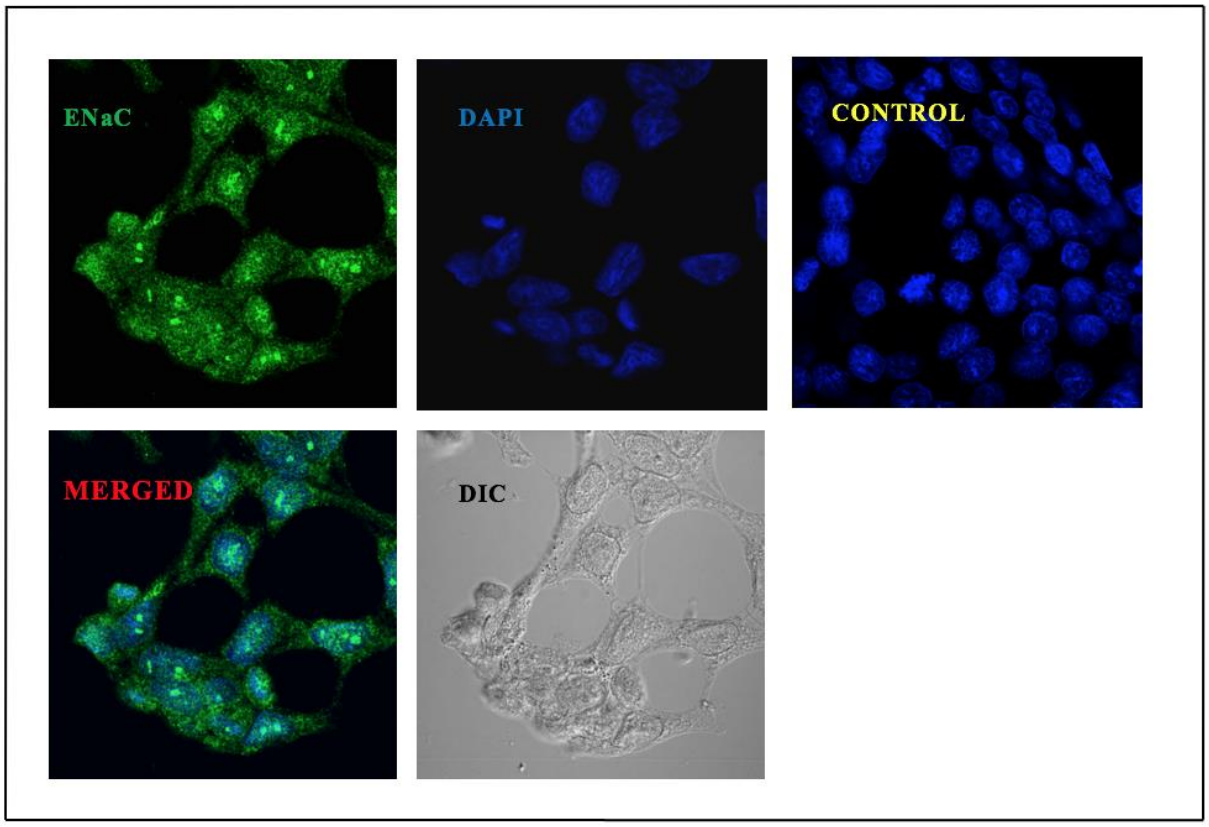


Figure 7. HEK293 cells express functional ENaC Channels: Temporal changes in FIR in cells loaded with SBFI in response to the changes in bath Na^+ concentration from zero to 140 mM or vice versa. Decreasing bath Na^+ from 140 mM to zero, induced a rapid efflux of Na^+ and increasing Na^+ from zero to 140 mM induced a Na^+ influx into the cells.

7

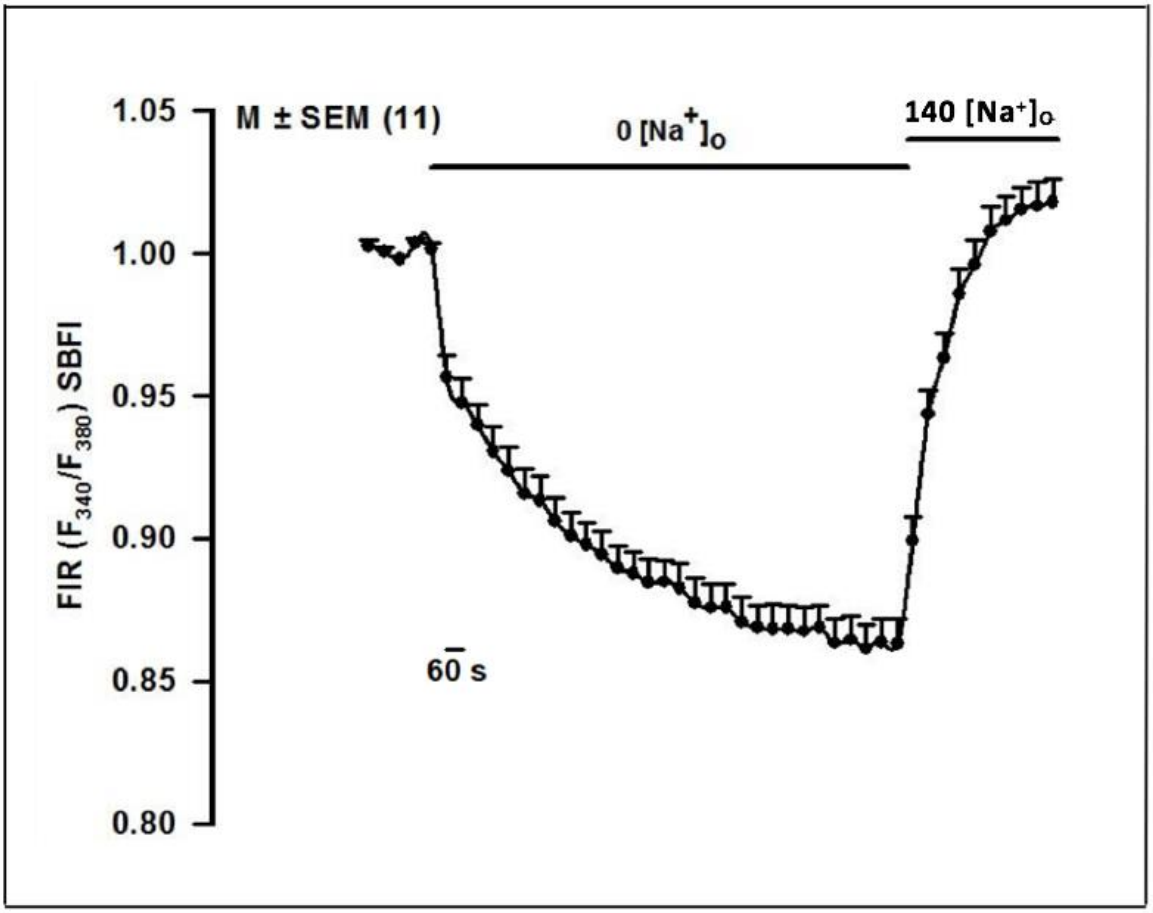

1.05
1.00
0.95
0.90
0.85
0.80
FIR (F_{340}/F_{380}) SBFI
M ± SEM (11)
0 $[Na^+]_o$
140 $[Na^+]_o$
60 s

Figure 8. HEK293 cells loaded with Fura-2 at 340 (nm) and 380 (nm): The images represent HEK293 cells loaded with Fura-2 uniformly distributed in the cytosol and retained inside the cell.

8

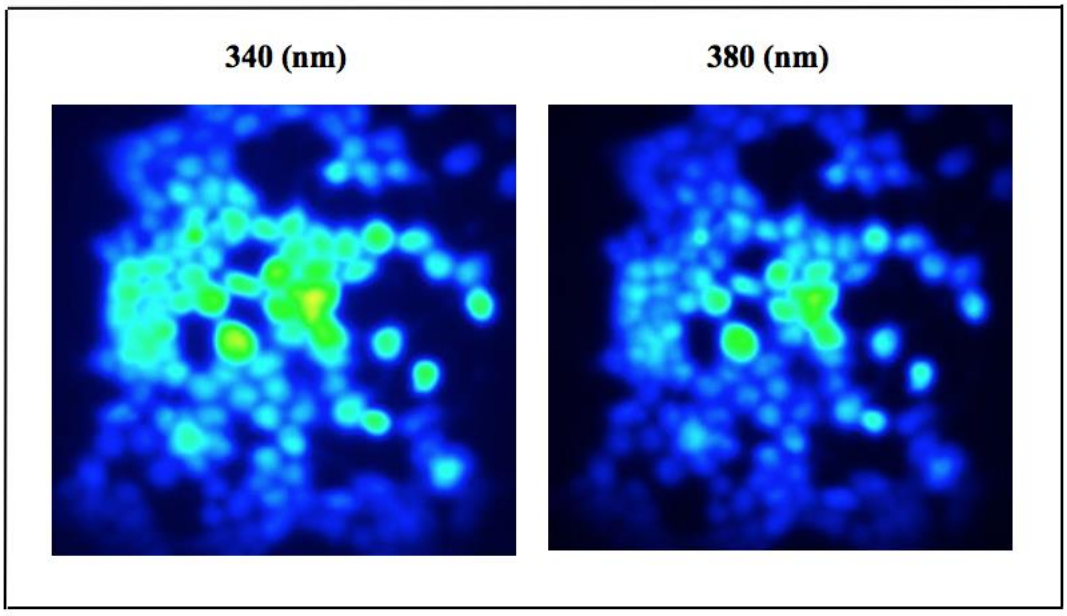


Figure 9. Ca^{2+} measurement in HEK Cells: This graph shows changes of $[Ca^{2+}]_i$ upon presentation of 1.0 μM capsaicin in Ringer's solution. The FIR values in control Ringer's solution were normalized to 1. Data is presented as the mean ± SEM; n=28.

9

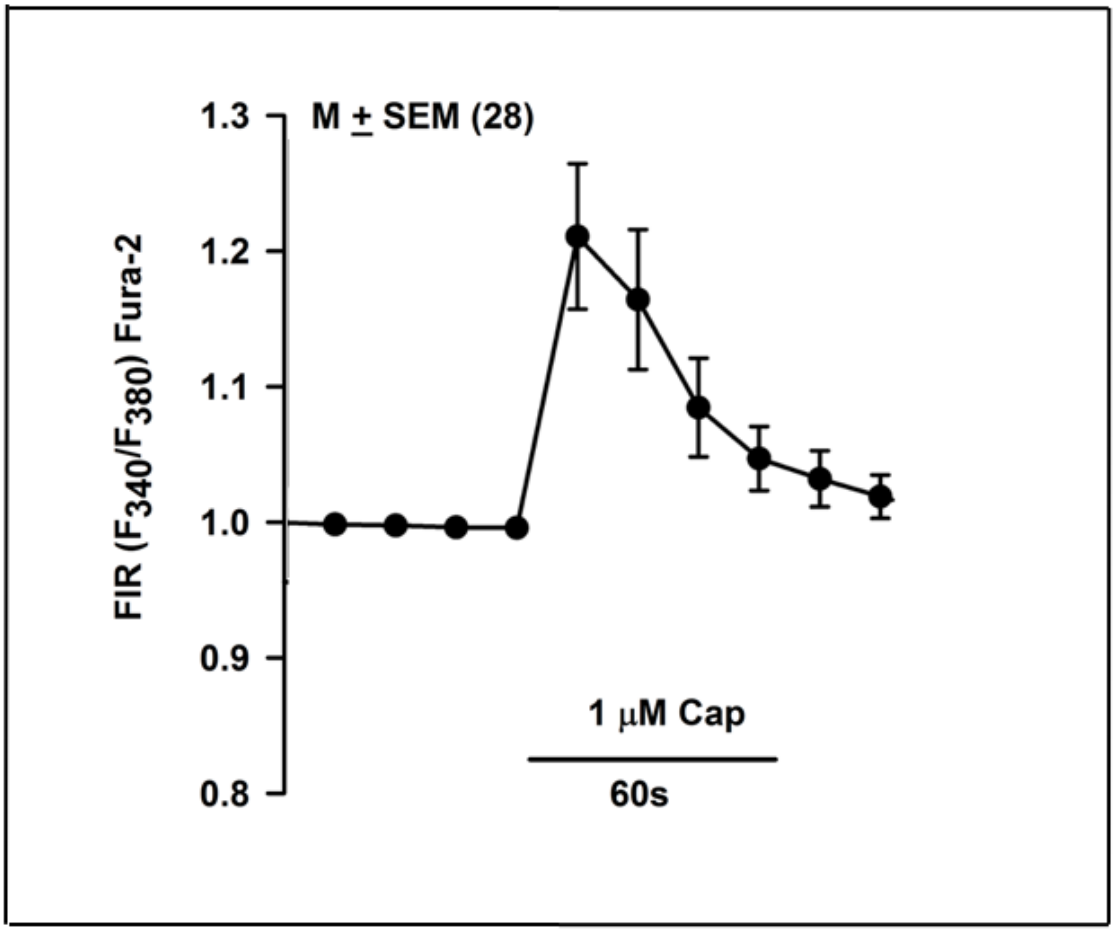


Figure 10. Double-Immunofluorescence TRPV1 and δ-ENaC in HBO Cells: Using double-immunofluorescence, the co-localization of TRPV1 and δ-ENaC is visualized in an HBO cell. DAPI indicates the cell nucleus.

10

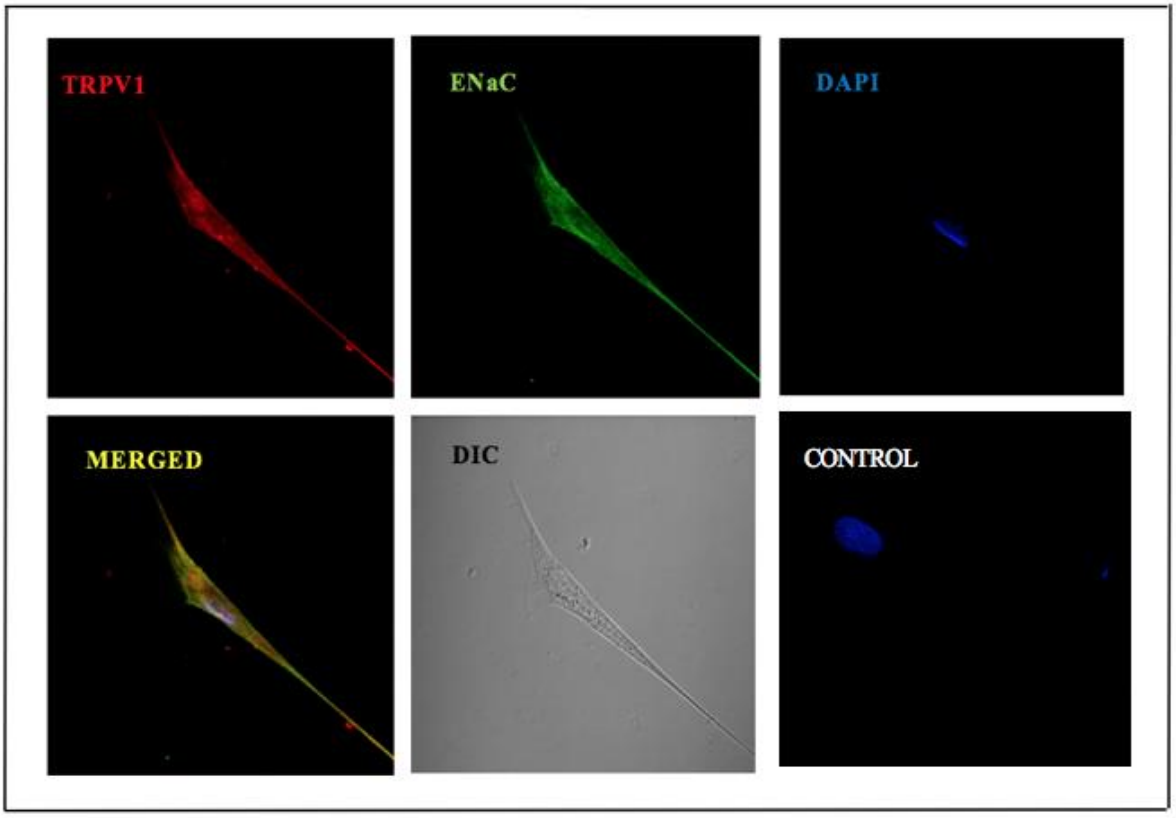


Figure 11A. qRT-PCR for TRPV1 mRNA expression: The values are presented as the mean ± SEM mRNA TRPV1 expression between negative control and treatment variables. The asterisk represents a significant difference ($p < 0.01$) between control and treatment conditions. TPRV1 mRNA expression was shown to significantly decrease ($p = 0.002$ and 0.004 respectively) at 0.1 μM and 0.25 μM Cap and significantly increase at 1.0 μM and 2.5 μM capsaicin [$p = 0.002$ and 0.013, respectively]. Figure data was normalized to GAPDH.

11A

	Control	0.1uM Cap	0.25uM Cap	0.50uM Cap	0.75uM Cap	1.0uM Cap	2.5uM Cap
Fold Change	1	0.4281	0.639	0.941	0.952	1.336	1.259

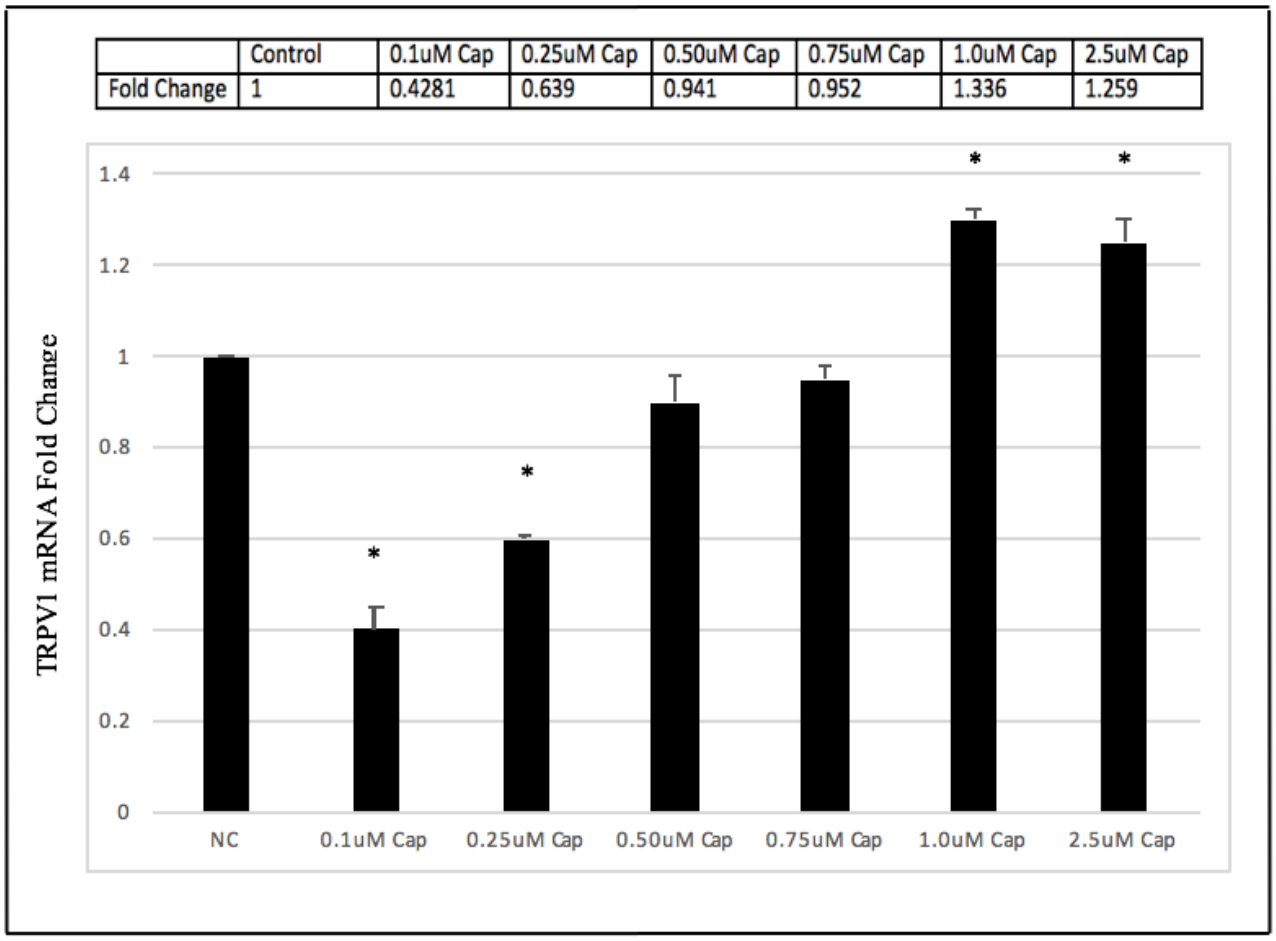


Figure 11B. qRT-PCR for δ-ENaC mRNA expression: The values are presented as the mean ± SEM mRNA δ-ENaC expression between negative control and treatment variables. The asterisk represents a significant difference ($p < 0.01$) between control and treatment conditions. δ-ENaC mRNA expression was shown to significantly increase ($p = 0.006$) at 0.75 μM and decrease at 1.0μM and 2.5μM capsaicin ($p = 0.001$ and 0.008 respectively) in δ-ENaC after exposure to capsaicin for 7h. Figure data was normalized to GAPDH.

11B

	Control	0.1uM Cap	0.25uM Cap	0.50uM Cap	0.75uM Cap	1.0uM Cap	2.5uM Cap
Fold Change	1	0.834	0.851	0.986	1.237	0.482	0.579

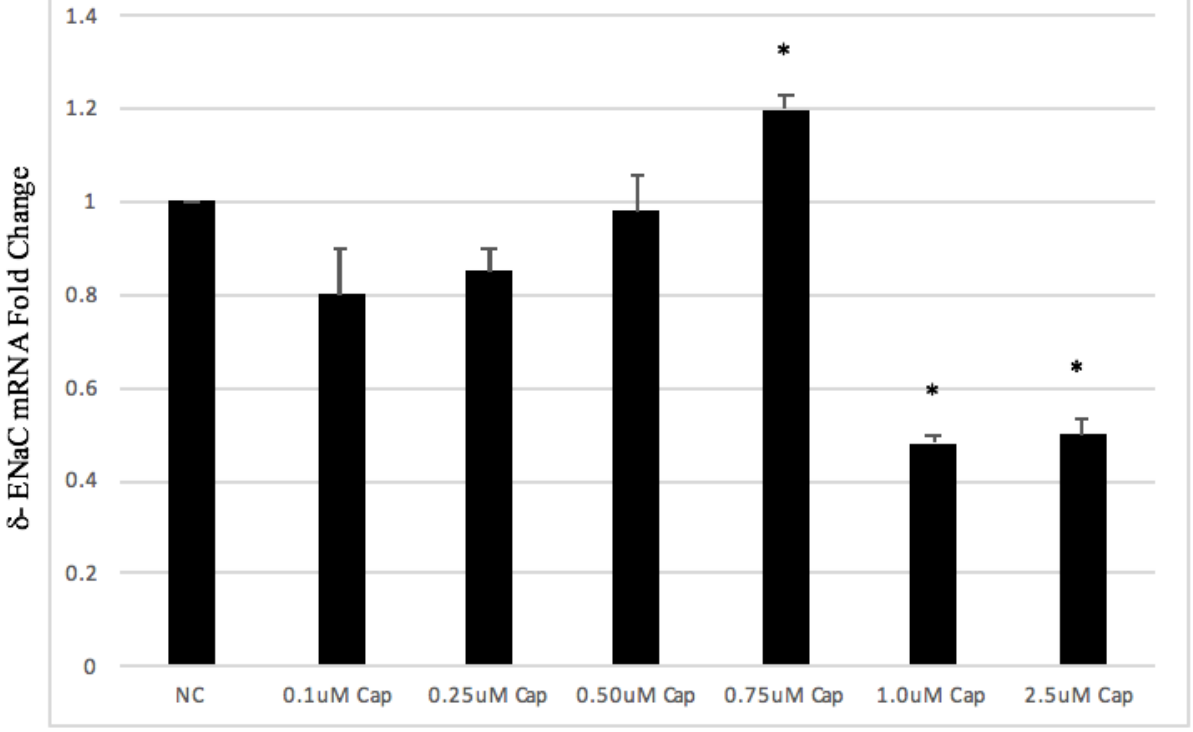


Figure 12A & 12B. The effect of capsaicin on TRPV1 subunit protein expression in HEK Cells: This data shows HEK cells which were treated with varying amounts of capsaicin for 7h and the expression level of TRPV1 was measured using Western Blot analysis. The asterisk represents a significant decrease [p = 0.016] in TRPV1 subunit protein expression while 0.75 μM and 1.0 μM capsaicin showed a significant increase [p = 0.011 and 0.032 respectively]. The values are presented as the mean ± SEM and the protein expression was normalized to β –actin.

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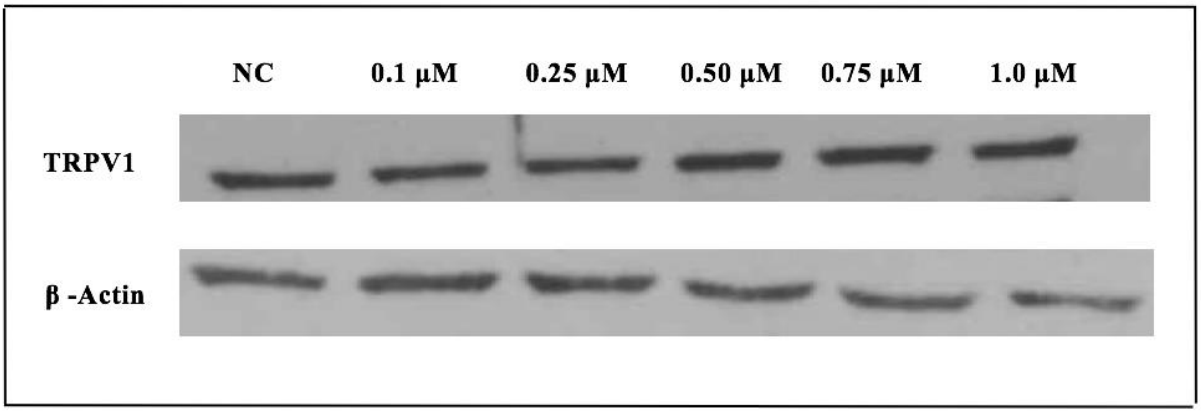
12A



12B

	Control	0.1uM Cap	0.25uM Cap	0.50uM Cap	0.75uM Cap	1.0uM Cap
Fold Change	1	0.676	0.802	1.125	1.282	1.597

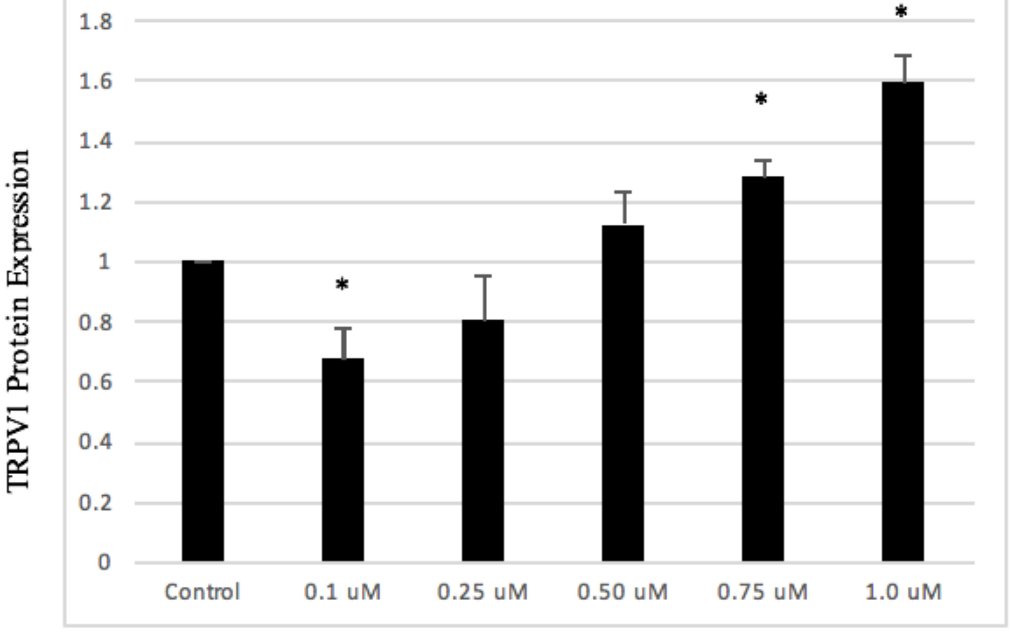
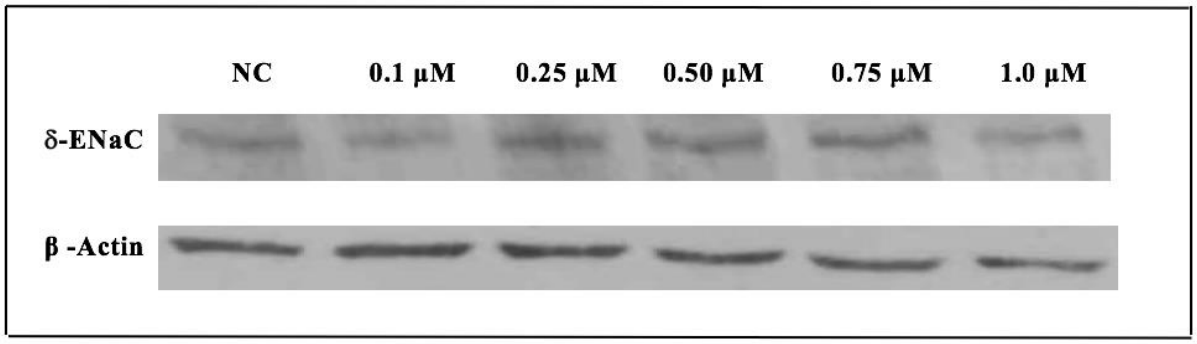


Figure 13A & 13B. The effect of Capsaicin on δ-ENaC subunit protein expression in HEK Cells: This data shows HEK cells which were treated with varying amounts of capsaicin for 7h and the expression level of δ-ENaC was measured using Western Blot analysis. The asterisk represents a significant decrease [p = 0.037 and 0.007 respectively] in expression at 0.1 μM and 1.0 μM capsaicin. The values are presented as the mean ± SEM and the protein expressions are normalized to β-Actin.

13A



13B

	Control	0.1uM Cap	0.25uM Cap	0.50uM Cap	0.75uM Cap	1.0uM Cap
Fold Change	1	0.625	1.021	1.009	1.070	0.805

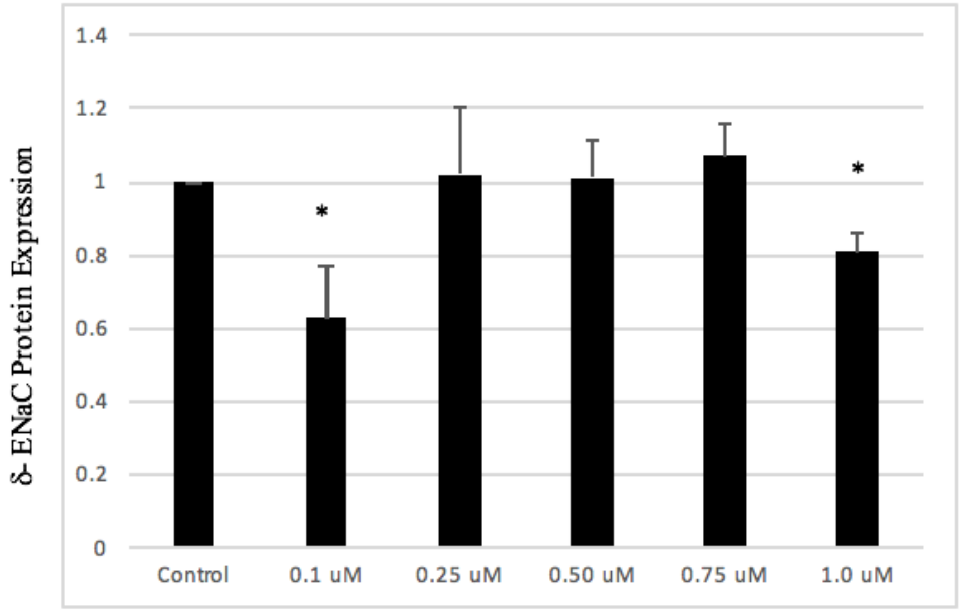
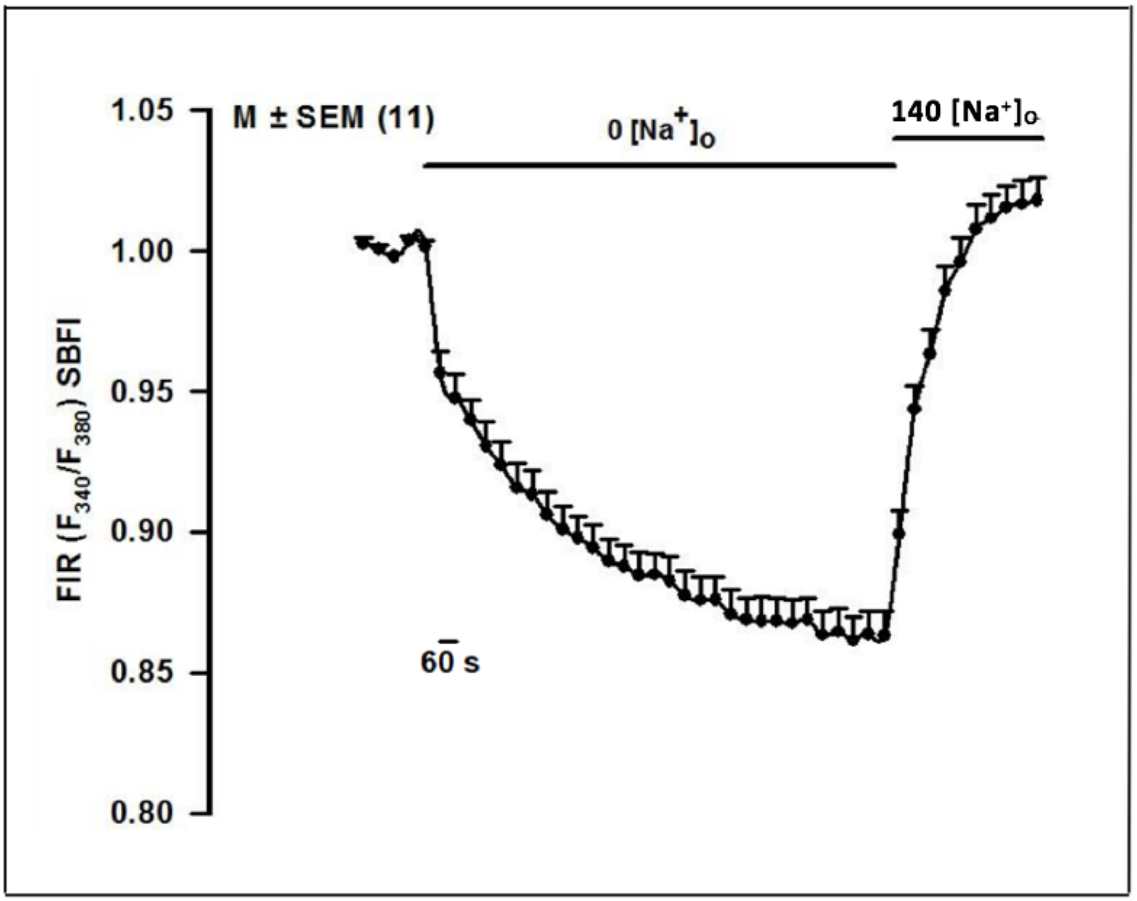


Figure 14A & 14B. Na^+ Measurement in HEK Cells: Temporal changes in FIR (F_{340}/F_{380}) were monitored in HEK cells incubated with 2.5 μM capsaicin for 7h with (14B) and without (14A) capsaicin. Relative changes in ($[Na^+]_i$) are presented as changes in FIR relative to apical 0 Na^+. HEK cells treated with 2.5 μM Cap Na^+ efflux, Na^+ influx and V_{max} are significantly lower ($p = 0.039$, 0.028, and 0.0124 respectively) relative to control. In individual cells, the data were presented as means ± SEM of N, where N represents the number of regions of interest (ROIs) within the cell.

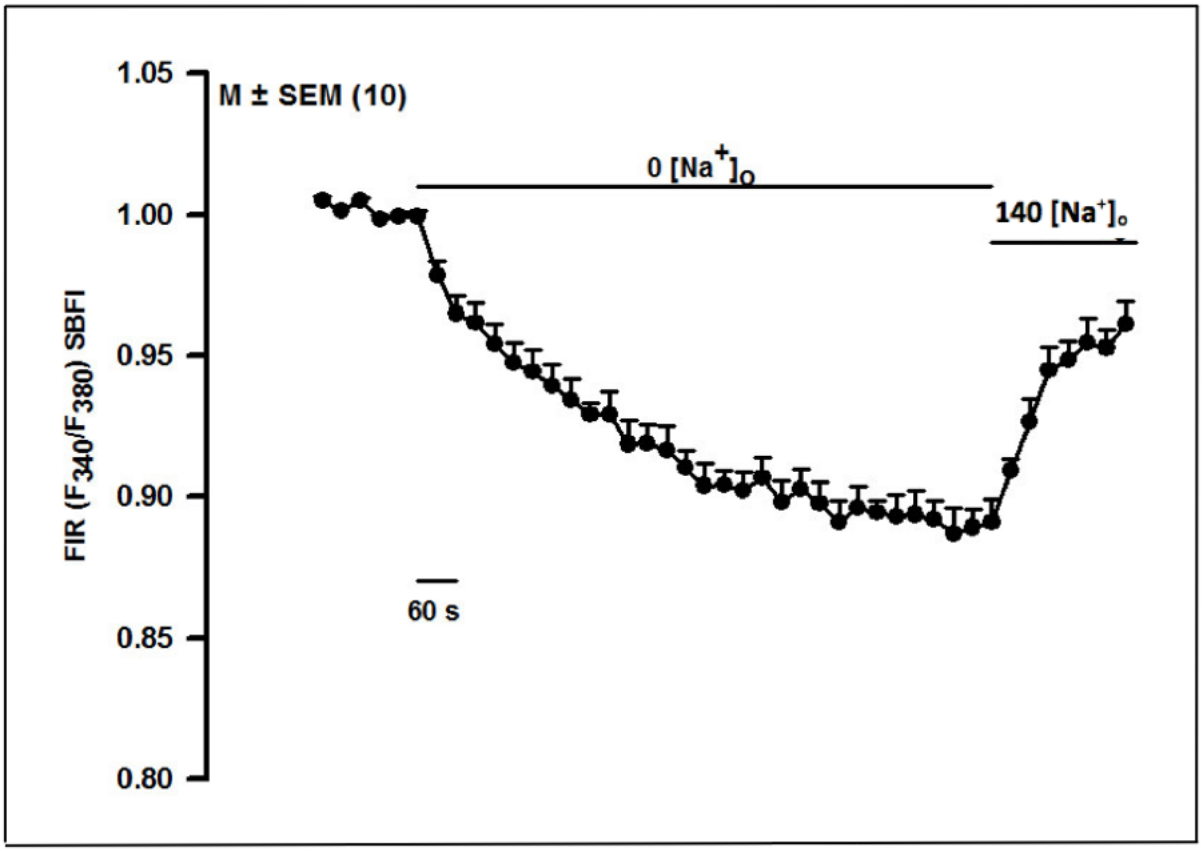
14A

	Efflux (140 Na^+ -> 0 Na^+)	Influx (0 Na^+ -> 140 Na^+)
Slope ± SEM	$-1.870 \times 10^{-4} \pm 1.38 \times 10^{-5}$	$7.355 \times 10^{-4} \pm 4.310 \times 10^{-5}$
R^2	0.9035	0.9181
V_{max}	0.1530 ± 0.0271	



14B

	Efflux (140 Na^+ -> 0 Na^+)	Influx (0 Na^+ -> 140 Na^+)
Slope ± SEM	$-1.49x10^{-4} \pm 3.34x10^{-5}$	$4.959x10^{-4} \pm 1.557x10^{-4}$
R^2	0.939	0.9439
V_{max}	0.0682 ± 0.0121	



Chapter 4: Discussion

The TRPV1 cation channel is a polymodal non-selective cation channel which is constitutively closed, however, it is opened in the presence of capsaicin, a pungent ingredient found in chili peppers (30). In addition to capsaicin, other pungent and non-pungent compounds modulate TRPV1 activity (13, 28). The presence of TRPV1 mRNA was first demonstrated in human cultured taste cells (24). Using immunofluorescence (Fig. 10), we confirmed the expression of TRPV1 protein in HBO cells. Although, we did not investigate TRPV1 channel properties directly in HBO cells, we demonstrated that capsaicin can open endogenous TRPV1 channels expressed in HEK293 cells. This is consistent with the effects of capsaicin on TRPV1 channels endogenously expressed in nociceptive neurons and in heterologous cell lines in which TRPV1 has been over expressed by TRPV1 mRNA injections (30). The opening of endogenous TRPV1 channels in TBCs in the presence of capsaicin provides a pathway for Na^+ entry (34). TRPV1 is not blocked by amiloride, therefore, Na^+ influx across the apical membrane of TBCs via TRPV1 channels contributes to the amiloride-insensitive, cation non-selective component of the salt taste in humans. In this regard, TRPV1 agonists, NGCC and Maillard Reacted Peptides produced biphasic effects on human salt taste perception (13, 28). At low concentrations, they enhance, and at high concentrations, they inhibit, salt taste perception (13, 28). In addition to Na^+ salts, humans can taste K^+, NH_4^+ and Ca^{2+} salts as salty. Thus, it is likely that TRPV1 could represent a cation non-specific pathway involved in the detection of salty taste associated with K^+, NH_4^+ and Ca^{2+} salts.

However, at present, it is not clear how long term exposure to capsaicin in food alters TRPV1 activity in HBO cells. Sensitization may arise following phosphorylation of TRPV1 by PKC. TRPV1 interacts with signal transduction pathways that activate G-protein-coupled

receptors which trigger the release of $G\alpha_q$ subunits from the heterotrimeric G-proteins, activating phospholipase C-β (PLC β) (12). PLCβ hydrolyzes PIP_2 into the second messengers IP_3 and DAG. DAG then activates PKC, which in turn phosphorylates serine and threonine in the N-terminal region of TRPV1 (12). Phosphorylation subsequently leads to the elevation of cytosolic Ca^{2+} and the subsequent release of neuropeptides such as substance P and CGRP by exocytosis (12). Substance P is found in nociceptive sensory fibers that express TRPV1 and in sensory fibers near taste buds of several mammalian species which are important in the maintenance of pain and inflammation (12, 46).

The amiloride-sensitive epithelial Na^+ channel, ENaC, is a member of the ENaC/degenerin family of non-voltage-gated ions channels (27). Unlike TRPV1, it is constitutively active and is highly selective for Na^+. It is localized in the apical membranes of tight epithelia, including the anterior fungiform taste papillae (27). In rodents, the amiloride-sensitive ENaC composed of αβγ subunits accounts for 60-70% of Na^+-specific CT response. In behavioral studies, it contributes to increase the intake of appetitive NaCl concentrations (22). A smaller component (~30%), is the amiloride-insensitive and cation-non-selective channel (22). In contrast to rodents, amiloride produces a maximal decrease of 21% in salt perception in some individuals (14). This suggests that unlike rodents, more than 80% of salt taste perception in humans is due to apical Na^+ influx in TBCs via the amiloride-insensitive cation non-selective pathway(s). Humans differ from rodents in that they express an additional ENaC subunit, the δ-subunit, which is expressed in epithelial tissues (19). The δ-subunit combines with βγ subunits to form δβγ-ENaC. Relative to αβγ-ENaC, δβγ-ENaC is about 10-fold less sensitive to inhibition by amiloride and benzamil (19). Thus, both amiloride sensitive Na^+ selective and amiloride-insensitive cation non-selective pathways contribute to human salt taste.

To date, the interactions between the ENaC-dependent and ENaC-independent pathways has not been investigated. The possibility of interaction between these two pathways arises because in our immunofluorescence studies TRPV1 was co-localized in HBO cells that also express the δ-ENaC subunit (Fig. 10). In support of this hypothesis, prior research showed that TRPV1 and α-ENaC are co-expressed in mouse cortical collecting duct cells and a cortical collecting duct cell line (30). Functionally, long term TRPV1 activation by dietary capsaicin increased urinary sodium excretion by inhibiting α-ENaC activity (30). In addition, in the above study, dietary capsaicin ameliorated the effects of a high salt (HS) diet through activation of TPRV1 (30).

In our studies, we used HEK293 cells to investigate the effect of TRPV1 activation by capsaicin on the δ-ENaC subunit expression, a human specific component of δβγ-ENaC. Using imaging studies, we demonstrated that HEK293 cells express functional TRPV1 channels and ENaC (Figs. 7 and 9). Subsequently, we investigated the effect of 7h capsaicin treatment on δ-ENaC mRNA and protein expression in HEK293 cells. Our results show that capsaicin produced a dose dependent effect on δ-ENaC mRNA and protein expression (Figs. 11B and 13B). Our most consistent findings are that 1.0 and 2.5 μM capsaicin inhibits δ-ENaC mRNA and protein expression. We hypothesize that in addition to δ-ENaC subunit, capsaicin at these concentrations may also inhibit the expression of other ENaC subunits.

The underlying mechanism of TRPV1's inhibition on ENaC subunit expression is not known. Studies in cortical collecting duct cells suggest that TRPV1 activation via capsaicin inhibits ENaC activity. It is suggested that ENaC is regulated by the Lysine Deficient Protein Kinase 1 (WNK1) and Serine/Threonine-protein Kinase 1 (SGK1) pathway in mouse CCD *in vivo* (30). The flux of Na^+ occurs by SGK1 stimulating sodium transport by increasing the

amount of functional ENaC channels in the apical cell membrane. WNK1 via the interaction with Pyruvate Dehydrogenase Kinase 1 (PDK1) fully activates SGK1 (51). SGK1 interacts with Nedd4-2 and phosphorylates it, which reduces the interaction between Nedd4-2 and ENaC and leads to elevated ENaC cell-surface expression (51). The activation and regulation of the TRPV1 channel through Protein Kinase C (PKC) and phospholipase C (PLC) increases intracellular calcium level (30). Studies showed increased ENaC activity in PKC α-KO mice, suggesting that the effect of TRPV1 on ENaC activity might be associated with activation of the PLC/PKC signaling pathway (30).

The penultimate effect of capsaicin-induced inhibition of ENaC subunit expression is a decrease in ENaC activity in HEK293 cells. In our studies, following 7h treatment of HEK293 cells with capsaicin, Na^+ transport measured as Na^+ efflux or Na^+ influx was significantly inhibited relative to untreated cells (Figs. 14A and 14B). This indicates that a capsaicin-induced decrease in ENaC subunit expression is directly correlated with ENaC function.

Although these studies were performed in HEK cells, we hypothesize that long-term exposure to high concentrations of capsaicin in food may also inhibit ENaC subunit expression in human TBCs. A lower ENaC expression in human TBCs will decrease the sensitivity of ENaC to detect lower concentrations of salt in the diet, which are normally appetitive. This may have a negative consequence of increasing salt taste by increasing the threshold at which salt is appetitive. In rats, upregulating ENaC activity in TBCs lowered the concentration at which they demonstrate maximum salt intake from 140 mM to 100 mM NaCl (42). On the other hand, inhibiting or eliminating ENaC from TBCs in mice, decreases or abolishes their responses to appetitive NaCl concentrations (6).

Several studies show the benefits of chili pepper in humans (32). In a cross sectional study, long-term consumption of a diet high in chilies has been shown to improve arterial function as well as decrease blood pressure in humans (31). Evidence has suggested that consumption of spicy foods improves energy expenditure, increases fat oxidation and subdues orexigenic sensations (32). However, the effects of capsaicin on ENaC expression and functions poses a conundrum. On the one hand, long term high dietary intake of capsaicin ameliorates the effects of a high salt (HS) diet through activation of TPRV1 and subsequent inhibition of ENaC activity in the renal cortical collecting ducts of mice *in vivo* (30). On the other hand, by inhibiting ENaC activity in TBCs, capsaicin may lead to decreased salt taste sensitivity and increase in salt intake. However, since ENaC does not seem to be the predominant salt taste receptor in humans, capsaicin's effect on ENaC may not affect salt taste perception or lead to increased salt intake. Thus, the beneficial effects of capsaicin may occur via its predominant action on ENaC activity in renal cortical collecting ducts.

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