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Dissertation

# TRANSDUCTION MECHANISMS OF MONOSODIUM GLUTAMATE IN TASTE CELLS OF RAT FUNGIFORM PAPILLAE

Submitted by

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In partial fulfillment of the requirements

for the Degree of Doctorate of Philosophy

Colorado State University

Fort Collins, Colorado

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY WEIHONG LIN ENTITLED *TRANSDUCTION MECHANISMS OF MONOSODIUM GLUTAMATE IN TASTE CELLS OF RAT FUNGIFORM PAPILLAE* BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTORATE OF PHILOSOPHY.

Committee on Graduate Work

Adviser

Department Head

ABSTRACT OF DISSERTATION

# TRANSDUCTION MECHANISMS OF MONOSODIUM GLUTAMATE IN TASTE CELLS OF RAT FUNGIFORM PAPILLAE

Monosodium glutamate (MSG) is a potent umami taste stimulus and a widely used flavor enhancer. Previous studies revealed roles of both $Na^+$ and glutamate in umami taste, but transduction mechanisms for MSG are largely unknown. This dissertation focuses on the mechanisms used by taste cells for transducting MSG and initiating sensory signals for umami taste.

Amiloride-sensitive $Na^+$ channels mediate $Na^+$ salt transduction in rodents. Their molecular identity, localization and regulation by aldosterone are not clearly defined. Using subunit-specific antibodies against the epithelial $Na^+$ channel, rENaC, we observed immunoreactivity for $\alpha$, $\beta$, and $\gamma$ subunits in taste cells. The intensity of labeling for $\beta$ and $\gamma$ was significantly lower in vallate than in fungiform papillae. Increasing blood aldosterone levels enhanced the apical immunolabeling for $\beta$ and $\gamma$ subunits, concomitant with an increase in number of amiloride-sensitive cells and in the amplitude of amiloride-sensitive current in whole-cell recordings. These results demonstrate that rENaC is the basis for $Na^+$ taste transduction.

Studies of glutamate transduction focused on the identity of receptors involved and their intracellular signaling pathways. In whole-cell recordings with a holding

potential of –80 mV, glutamate elicited a decrease in holding current (Type I), an increase in holding current (Type II), and a biphasic response involving a Type II response followed by a Type I response. Type II responses were mimicked by the NMDA receptor agonist NMDA. These responses were potentiated by glycine and suppressed by AP5. NMDA depolarized taste cells and increased the frequency of spontaneous action potentials. Type I responses were suppressed by GDP-β-S, suggesting the involvement of G protein-coupled receptors. These responses were mimicked by the mGluR4 agonist L-AP4 and suppressed by 8-bromo-cAMP and the mGluR4 antagonist CPPG. Some cells were hyperpolarized by L-AP4. These data suggest that both ionotropic and metabotropic glutamate receptors contribute to the transduction of MSG in fungiform taste cells.

Responses to glutamate occurred preferentially in taste cells that express functional rENaCs. Although separate pathways mediate $Na^+$ and glutamate transduction, the co-occurrence of glutamate receptors and rENaCs provides the potential for integration of $Na^+$ and glutamate pathways in single taste cells.

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Many thanks to all whom helped.

# Dedication

This dissertation is dedicated to my father and mother, and to my family for their trust and for their support of my long-term education.

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# Chapter 1

# INTRODUCTION

Early in this century Ikeda, a Japanese food chemist, discovered that monosodium glutamate (MSG) elicits a characteristic taste that is responsible for the special flavor of many seafood and meats. He considered the taste induced by MSG to be another basic taste and named it "umami", meaning delicious or savory (Ikeda, 1909). Today, while people worldwide enjoy the unique flavor, scientists have made little progress in understanding the mechanisms by which MSG imparts its unique flavor to food.

## General features of taste sensation

The ability to detect soluble chemical stimuli in the environment is critical to the survival of all animals, including humans. The chemical sense of taste is important for locating food, avoiding harmful chemicals, and maintaining nutritional balance. Although a vast array of chemicals induces taste sensation, the basic taste modalities of humans are classified into only five broad groups: salty, sour, bitter, sweet and umami. Salty taste is elicited by NaCl, LiCl, and other salt compounds. Most animals prefer salts in their diet because of their essential role in maintaining the osmotic balance of body fluids. Sucrose and other carbohydrates, which are indispensable for providing energy,

elicit a sweet taste. Bitter compounds are a diverse group of chemicals that are often harmful; thus the bitter taste likely is evolved as a means to detect potentially toxic chemicals in foods. Umami taste is elicited primarily by two groups of chemicals: monosodium salts of amino acids, such as glutamic acid and aspartic acid; and disodium salts of 5'-ribonucleotides, such as guanosine 5'- monophosphate (5'-GMP) and inosine 5'-monophosphate (5'-IMP). The taste of these savory chemicals signals the presence of proteins and polynucleotides in foods and also provides a hedonic sensation or pleasant feeling during food intake.

Taste receptor cells, specialized epithelial cells that are clustered into pear-shaped end organs called taste buds, mediate the sensation of taste. In rodents, most taste buds are housed in three different papillae located at the dorsal surface of the tongue (for review, see Kinnamon, 1987). Fungiform papillae are small eminencies usually containing one to two taste buds (Miller and Smith, 1984). These papillae are located on the anterior two-thirds of the tongue, with the greatest density near the tip of the tongue. Foliate papillae consist of several parallel grooves on the posterior-lateral surface of the tongue, with taste buds lining the walls of the grooves. The vallate papilla is a horseshoe-shaped trench located in the posterior region of the tongue. Hundreds of taste buds line the walls of the trench, similar to the foliate papillae. Taste buds located in these papillae are innervated by two cranial nerves. The chorda tympani nerve (CT; a branch of cranial nerve VII) innervates taste buds in fungiform papillae. The glossopharyngeal nerve (GL; cranial nerve IX) innervates taste buds in the vallate papilla. Branches of both cranial nerves innervate foliate papillae.

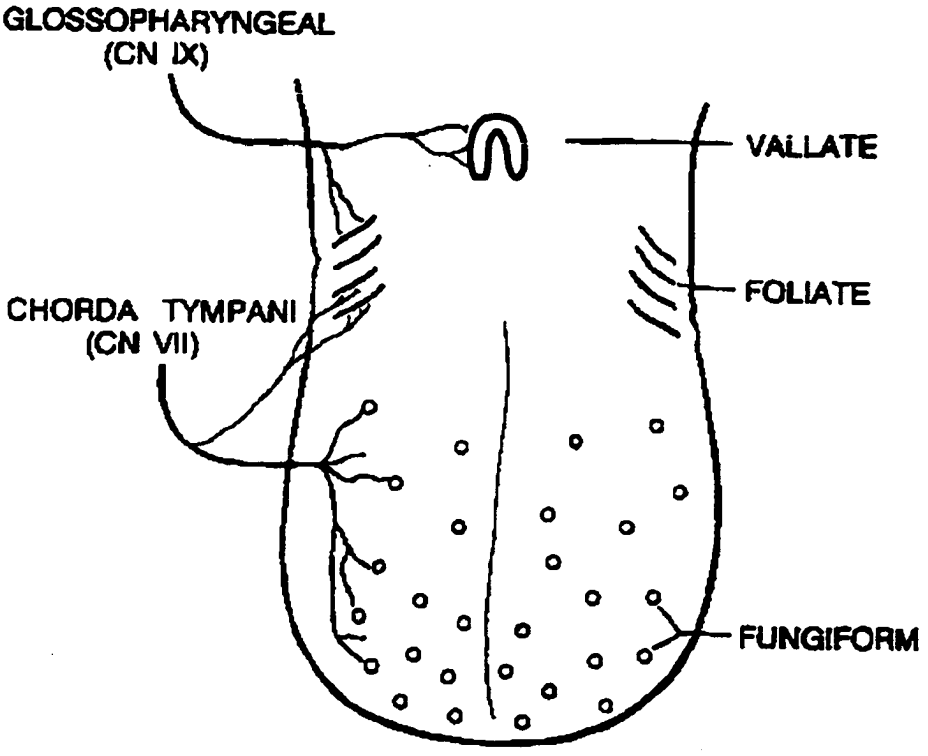



Fig. 1.1. A schematic model for the location and innervation of taste buds in the dorsal surface of a rat tongue.

Several types of cells within a taste bud have been identified (for review, see Kinnamon, 1987). Basal cells are oval in shape and reside in the basal portion of the taste bud. In mammals, basal cells are undifferentiated stem cells and are not chemosensitive. The remaining cells, generally considered to be the taste receptor cells, are elongated spindle-shaped cells that extend from the basal lamina to the surface of the tongue. These have been classified further by ultrastructural criteria as dark (type I), intermediate, and light (type II) cells. Both dark and light cells possess well-defined synapses with afferent nerve fibers (Farbman, 1965; Murray, 1973; Delay et al., 1986;

Kinnamon et al., 1988; Roper, 1989). It has not been determined if all taste cells serve as receptor cells, or if different types of cells are specialized to detect specific categories of taste stimuli.

All taste receptor cells originate from local epithelium (Stone et al, 1994). Thus, they possess many characteristics typical of epithelia, including tight junctions between adjacent cells and the presence of epithelial $Na^+$ channels, which mediate $Na^+$ salt transduction. Tight junctions separate the taste cell membrane into two functional compartments, an apical chemosensitive portion and a basolateral portion, which forms chemical synapses with primary afferent nerve fibers. The tight junctions also serve to restrict most chemicals to the apical membrane, although small ions such as $Cl^-$ and $Na^+$ may pass through tight junctions and contact the basolateral membrane (Simon et al., 1993b; Ye et al., 1993). Despite their epithelial origin, taste cells possess several properties typical of neurons, including the presence of voltage-gated $Na^+$, $K^+$ and $Ca^{2+}$ channels. These channels allow taste cells to generate action potentials in response to chemical stimuli (Kashiwayanagi et al., 1983; Roper, 1983; Kinnamon and Roper, 1988; Béhé et al., 1990; Avenet and Lindemann, 1991; Cummings et al., 1993). The role of action potentials in regulating transmitter release is not known.

In general, the sensation of taste begins when chemicals in the external environment contact the apical microvilli of taste receptor cells. This contact triggers a sequence of cellular events that ultimately results in the release of transmitter from taste cells and the change of afferent nerve firing rates. In mammals, these afferent nerves synapse with neurons in the nucleus tractus solitarius (NTS), a primary gustatory nucleus. Information from the NTS is relayed via the thalamic gustatory relay to the primary

gustatory cortex and higher order gustatory cortical zones that are located in somatosensery cortex, insular cortex and orbitofrontal cortex (for review, see Finger, 1987).

The mechanisms by which taste cells convert chemical stimuli into intracellular signals that result in the release of transmitter are defined as transduction. Taste cells utilize several different types of mechanisms for transduction. Taste stimuli may (1) interact directly with ion channels, (2) bind to receptors linked to ion channels or to G-proteins involved in second messenger cascades, or (3) diffuse through the plasma membrane and bind to intracellular targets. Ionic stimuli are usually transduced by influx through ion channels in the membrane. For example, influx of $Na^+$ through apically located epithelial $Na^+$ channels depolarizes taste cells and initiates the $Na^+$ salt taste (DeSimone et al., 1981; Avenet and Lindemann, 1988; Ye et al., 1994). Protons, which elicit a sour taste, can pass through epithelial $Na^+$ channels (Gilbertson et al., 1992, 1993) or can block $K^+$ efflux through apically-located $K^+$ channels (Kinnamon et al., 1988; Cummings and Kinnamon, 1992; Kinnamon, 1992). In addition, recent studies suggest that protons may also gate nonselective cation channels to depolarize taste cells (Ugawa et al., 1998). Some hydrophopic molecules, such as caffeine and quinine, may diffuse through the plasma membrane to act directly on intracellular targets, initiating a bitter taste (Spielman et al., 1992; Naim et al., 1994). Large impermeant molecules are believed to bind to specific membrane receptors that are usually coupled to G proteins and second messenger pathways, such as the $IP_3$ and cAMP pathways (Striem et al., 1989; Caprio et al., 1993; Bernhardt et al., 1996; Ogura et al., 1997). Recently, an $\alpha$ subunit of the taste cell specific G-protein, gustducin, was cloned and shown to be

involved in both sweet and bitter tastes (Margolskee, 1993; Ruiz-Avila et al., 1995; Wong et al., 1996), but the role of gustducin in the transduction process has not been elucidated. Overall, the molecular identity of receptors and channels involved in taste transduction has not been defined clearly.

Although taste cells in all three papillae respond to similar taste stimuli, the responses are considerably different between chorda tympani and glossopharyngeal nerves, and between taste cells of vallate and fungiform papillae. In chorda tympani nerve of the rhesus monkey, more fibers respond to NaCl, umami and sweet stimuli than to sour and bitter stimuli, while in glossopharyngeal nerve, more fibers respond to bitter than to other taste stimuli (Hellekant et al., 1997). The differences are species- and strain-dependent (Ninomiya et al., 1991; Ninomiya et al., 1992). Interestingly, taste cells from both vallate and fungiform papillae respond to NaCl, however only cells from fungiform papillae are sensitive to amiloride, a specific blocker of epithelial $Na^+$ channels (Doolin and Gilbertson, 1996). Fungiform taste buds are generally believed to be primarily involved in the initial identification and acceptance of appetitive stimuli, while those of vallate and foliate papillae are likely to be more important for the rejection of aversive chemicals (Gilbertson and Kinnamon, 1996).

Umami taste

The most common umami stimuli are MSG and the disodium salts of 5'-GMP and 5'-IMP. These compounds can be found in many foods, especially seafood, meats, milk and their by-products, mushrooms and some vegetables. Other umami taste stimuli include salts of aspartic acid, ibotenic acid, homocysteine, xanthosine 5'-monophosphate,

bi and tripeptides of glutamic and aspartic acids, and an octapeptide that contains residues of glutamic and aspartic acids (Yamasaki and Maekawa, 1978, 1980; Maga, 1983; Spanier et al., 1995). Many of these compounds were first discovered as active taste ingredients in natural flavor-enhancing products. For example, the taste of MSG was first identified from the extract of sea tangle (Ikeda, 1909); and the taste of 5'-IMP and 5'-GMP was first identified from dried bonito tuna (Kodama, 1913) and shiitake mushrooms (Nakajima et al., 1961; Shimazono, 1964). Considerable research has indicated that these compounds are responsible for the umami taste of many seafood and meat products (for review, see Maga, 1983; Yamaguchi, 1987). Synthetic compounds are now used widely as flavor enhancers in the food industry to increase food palatability.

The properties of umami taste have been studied intensively. Many believe that umami taste is independent from that of other basic tastes. As revealed by psychophysical and behavioral studies, the characteristics of umami taste can not be fully described by the adjectives sweet, sour, salty and bitter that are used for the other four basic tastes (Yamaguchi and Kimizuka, 1979; Schiffman and Gill, 1987). The differences between umami and the other basic tastes were further demonstrated with a technique termed multidimensional scaling (MDS), a computer-based mathematical analysis that allows researchers to judge the similarity of different compounds based on a map of their spatial location in taste space. Results obtained with MDS show that the taste induced by MSG, 5'-IMP and 5'-GMP fell outside that of the other four basic tastes, which suggests that the umami taste is different (Yamaguchi and Kimizuka, 1979; Schiffman and Gill, 1987). Similar conclusions were reached from animal studies. For example, behavioral studies and nerve recordings demonstrated that mice can

discriminate MSG from other basic tastes (Ninomiya and Funakoshi, 1989a and b). In alert macaque monkeys, MSG and IMP are effective taste stimuli. Although closely associated with NaCl-sensitive neurons in the primary gustatory cortex, the umami taste achieves independence at higher order synapses. In secondary gustatory regions, such as the ventral forebrain and amygdala, the response profile evoked by MSG is disparate from those of NaCl, sucrose, HCl and quinine, implying independence of the umami taste (Scott et al., 1993). The ability to discriminate the umami taste from other basic tastes is species-dependent. In general, carnivores are more sensitive to umami tastants than herbivores. In fact, hamsters may not be able to discriminate MSG from NaCl, since the behavioral response to MSG is similar to NaCl (Yamamoto et al., 1988).

Another important feature of umami taste is a synergy between MSG and 5'-ribonucleotides. A synergy occurs when the effect induced by a combination of two compounds is greater than the sum of the two effects induced individually. In humans, the threshold and intensity of umami taste are dramatically changed when both MSG and 5'-ribonucleotides are present. In the presence of 5'-IMP or 5'-GMP, the threshold for MSG can be lowered 100-fold (Yamaguchi and Kimizuka, 1979). The synergistic effects on taste intensity depend on the ratio of MSG and 5'-ribonucleotides in the mixture and on which 5'-ribonucleotide is used. The combination of MSG-GMP is more effective than the combination of MSG-IMP at the same concentration (Yamaguchi, 1967). Because of this synergy, adding umami substances to foods which usually contain small amounts of MSG or 5'-ribonucleotides strongly potentiates the intensity of the umami taste (Yoshii et al., 1986).

A synergy between MSG and 5'-ribonucleotides can be found in several species, including rats (Hiji and Sato, 1967; Sato et al., 1970), cats (Adachi and Aoyama, 1991; Rolls et al., 1996), mice (Ninomiya and Funakoshi, 1989), dogs (Nakamura and Kurihara, 1991), monkeys (Scott et al., 1993), and chimpanzees (Hellekant and Ninomiya 1991). The degree of synergy differs considerably among species, strains (Ninomiya et al., 1992) and individual animals (Nakamura and Kurihara, 1991). Usually only a subset of nerve fibers and neurons of the central gustatory system responds to umami compounds, and only some of these fibers show synergistic responses. In the chorda tympani nerve of mice, almost all fibers showing synergistic responses to MSG and GMP are sensitive to sucrose (Ninomiya and Funakoshi, 1989), In contrast, synergistic responses in chimpanzees occur in some fibers that respond best to NaCl as well as other fibers that respond best to sucrose. Similar results were obtained from NTS neurons in rats and cats, in which some of the sucrose- or NaCl-best neurons respond to MSG and IMP synergistically (Adachi and Aoyama, 1991; Nakamura and Norgren, 1993).

Several hypotheses have been proposed to explain the synergistic effects of GMP on glutamate responses (Torii and Cagan, 1980; Schiffman and Gill, 1987). Based on observations that 5'-ribonucleotides increase the binding of glutamate to membranes of bovine vallate papillae, Torri and Cagan (1983) proposed that the synergy results from an increase in the number of glutamate binding sites. However, Torri and Cagan's model can not explain the fact that 5'-ribonuleotides alone are potent taste stimuli, that responses to MSG and 5'-ribonuleotides do not always occur in the same nerve fibers, and that many fibers or neurons respond to MSG and 5'-ribonuleotides without synergy (Ninomiya and Funakoshi, 1989; Adachi and Aoyama, 1991; Hellekant and Ninomiya,

1991; Hellekant et al., 1997). Clearly, more information is needed about the identity of the receptors involved and the transduction of 5'-ribonucleitides in taste receptor cells.

The interaction between umami and the four basic tastes of sweet, sour, salty and bitter has also been studied. At low concentrations (< 5 mM) MSG does not significantly influence the taste thresholds for any of the other four basic tastes, nor is it enhanced by them (Yamaguchi and Kimizuka, 1979). However, suprathreshold concentrations of MSG (> 10 mM) suppress the intensity of sweet, bitter, and sour stimuli, but enhance the response to NaCl (Yamaguchi, 1987; Kemp and Beauchamp, 1994).

Because MSG is the $Na^+$ salt of glutamic acid, it is possible that both $Na^+$ and glutamate could contribute to its unique taste quality. Sodium ion alone, however, induces a salty taste, so that differences between the taste of MSG and NaCl are due to the presence of the glutamate anion. Glutamic acid in its free form tastes sour. When titrated with NaOH, the sour taste is replaced by the umami taste (Kuramitsu et al., 1996); the umami taste is most pronounced over a pH range of 5.5 to 8 (Fagerson, 1954). Several studies suggest that $Na^+$ does contribute significantly to the unique taste quality of umami. The detection threshold for MSG is similar to the detection threshold for NaCl (Yamaguchi, 1991). Recordings from chorda tympani nerve and neurons in the nucleus tractus solitarius suggest that the umami taste induced by MSG is more closely related to the salty taste induced by NaCl than to other tastes (Yamamoto et al., 1988; Giza and Scott, 1991). Some single fibers of the chorda tympani nerve respond to both MSG and NaCl (Ninomiya and Funakoshi, 1989a, b; Kumazawa et al., 1991; Nakamura and Kurihara, 1991; Hellankant et al., 1991, 1997), and high concentrations of MSG (0.1M) can not be discriminated from NaCl in behavioral studies using conditioned taste aversion

(Yamamoto et al., 1988). Furthermore, the epithelial sodium channel blocker amiloride suppresses the response to both MSG and NaCl and shifts the dose-response curves for MSG to a higher concentration (Nakamura and Kurihara, 1991). Finally, NaCl potentiates the responses to MSG in CT nerve recordings (Ugawa and Kurihara, 1994) and the umami taste of the beefy meaty peptide (BMP) as well as lowering the recognition thresholds of BMP (Wang et al., 1996). Overall, these studies reveal a close correlation between the tastes of MSG and NaCl. However, they also raise the question of whether the pathways for glutamate and $Na^+$ are separate in taste receptor cells. The presence of glutamate receptors and separate transduction pathways for $Na^+$ and glutamate would provide strong evidence for the uniqueness of the taste of MSG or umami.

There is evidence for the presence of both metabotropic and ionotropic glutamate receptors in taste buds. Ion channel activity directly gated by glutamate and NMDA was observed in lipid bilayers containing epithelial membranes from vallate and foliate taste regions (Brand et al., 1991; Teeter et al., 1992). Molecular cloning has provided evidence for metabotropic glutamate receptors in taste cells. Chaudhari et al. (1996) used reverse transcriptase polymerase chain reaction (RT-PCR) to clone the metabotropic glutamate receptor mGluR4 from mRNA derived from foliate and vallate papillae in the rat. *In situ* hybridization showed that mGluR4 is specifically located in taste buds and is absent from the surrounding epithelium. Behavioral studies demonstrated that the specific agonist of mGluR4, L-AP4, mimics the taste of MSG, suggesting that this receptor may be involved in the transduction of glutamate (Chaudhari et al., 1996). Furthermore, L-AP4 alters intracellular $Ca^{2+}$ levels and mimics the electorphysiological

response to MSG in isolated vallate and foliate taste cells (Hayashi et al., 1996; Bigiani et al., 1997).

Despite these studies, precise electrophysiological and pharmacological information about glutamate receptors and their related intracellular pathways in taste cells is still missing. There are no published accounts of glutamate receptors in taste cells of fungiform papillae. This is surprising, given that fungiform taste cells are considered to be the receptor cells involved in the initial identification and acceptance of appetitive stimuli.

Hypotheses and specific aims

The general hypothesis for this dissertation is: The metabotropic glutamate receptor mGluR4 plays a central role in the transduction of glutamate; the epithelial $Na^+$ channel rENaC mediates the transduction of $Na^+$; and these distinct pathways are integrated in single taste cells of fungiform papillae to produce the taste of MSG. The specific aims are: (1) To determine the molecular identity, location and regulation of amiloride-sensitive channels for $Na^+$ salt transduction; (2) To identify glutamate receptors that are expressed in fungiform taste cells; (3) To examine the involvement of the intracellular cAMP pathways in glutamate transduction; (4) To examine the synergy between MSG and 5'-GMP; and (5) To determine if $Na^+$ and glutamate pathways co-exist in single taste cells.

Organization of the dissertation

The dissertation is divided into 6 chapters. Chapters 1 and 6 are Introduction and Summary, respectively. Chapter 2 is a paper entitled "Epithelial $Na^+$ channel subunits in rat taste cells: localization and regulation by aldosterone", which has been accepted by the Journal of Comparative Neurology. This paper addresses Specific Aim 1; authorship is W. Lin, T.E. Finger, B. Rossier and S.C. Kinnamon. Chapter 3 is a paper in the final stages of editing to be submitted to the Journal of Neuroscience. Authorship will be W. Lin and S.C. Kinnamon; the manuscript addresses Specific Aims 2 and 3. Chapter 4 presents the results of a study that addresses Specific Aim 4. A portion of this information was published as a short manuscript in the Proceedings of the XII International Symposium on Olfaction and Taste; authorship is W. Lin and S.C. Kinnamon. Chapter 5 is a short paper entitled "Co-existence of epithelial sodium channels and glutamate receptors in single taste cells of rat fungiform papillae". This paper addresses Specific Aim 5 and will be submitted to a special issue of "biological signals and receptors"; authorship is W. Lin and S.C. Kinnamon.

# Chapter 2

# EPITHELIAL $NA^+$ CHANNEL SUBUNITS IN RAT TASTE CELLS: LOCALIZATION AND REGULATION BY ALDOSTERONE


## Abstract

Amiloride-sensitive $Na^+$ channels play an important role in transducing $Na^+$ salt taste. Previous studies revealed that in rodent taste cells, the channel shares electrophysiological and pharmacological properties with the epithelial $Na^+$ channel, ENaC. Using subunit-specific antibodies directed against $\alpha$, $\beta$ and $\gamma$ subunits of rat ENaC (rENaC), we observed cytoplasmic immunoreactivity for all three subunits in nearly all taste cells of fungiform papillae, and in about half of the taste cells in foliate and vallate papillae. The intensity of labeling in cells of vallate papillae was significantly lower than that of fungiform papillae, especially for $\beta$ and $\gamma$ subunits. Dual localization experiments showed that immunoreactivity for the taste cell-specific G protein, gustducin, occurs in a subset of rENaC positive taste cells.

Aldosterone is known to increase the amiloride sensitivity of the NaCl taste response. In our study, increases in blood aldosterone levels enhanced the intensity of apical immunoreactivity for $\beta$ and $\gamma$ rENaC in taste cells of all papillae. In addition,

whole cell recordings from isolated taste cells showed that in fungiform papillae, aldosterone increased the number of amiloride-sensitive taste cells and enhanced the current amplitude. In vallate taste cells, which are normally unresponsive to amiloride, aldosterone treatment induced an amiloride sensitive current in about half of the cells.

Immunoreactivity for rENaC subunits also was present in non-sensory epithelial cells, especially in the anterior portion of the tongue. In addition, immunoreactivity for all subunits, but especially β and γ, was associated with some nerve fibers innervating taste papillae. These extra-gustatory sites of rENaC expression may indicate a role for this channel in paracellular transduction of sodium ions.

## Introduction

The production of a "salty" taste by application of NaCl to the tongue involves multiple transduction mechanisms. In rodents, the bulk of transduction in the anterior tongue, e.g., fungiform papillae, appears attributable to direct influx of $Na^+$ through apical amiloride-sensitive sodium channels in taste receptor cells. A portion of the NaCl response is, however, amiloride-insensitive and is thought to involve a paracellular pathway in which $Na^+$ penetrates the epithelial surface via the tight junctions and enters taste receptor cells through the basolateral membrane. In the posterior tongue, i.e., vallate papillae, the bulk of $Na^+$ transduction is not sensitive to amiloride, but the mechanisms involved have not been elucidated. They may involve apical $Na^+$ channels that lack amiloride sensitivity, or they may involve the paracellular pathway, similar to the amiloride-insensitive NaCl response in fungiform papillae (for review, see Lindemann, et al., 1999). The molecular basis for $Na^+$ transduction in the tongue is not known.

The importance of the amiloride-sensitive $Na^+$ channel in $Na^+$ taste transduction was first demonstrated by the finding that amiloride, a well known epithelial $Na^+$ channel blocker (Benos, 1982; Kleyman and Cragoe, 1988), significantly suppresses both the neural response and the transepithelial current when NaCl is applied to the anterior tongue surface (DeSimone et al., 1984; Heck et al., 1984; Simon and Garvin, 1985, Simon et al.,1988, 1993a, b; Mierson et al., 1985). These studies demonstrated that the NaCl response arises from the inward flux of $Na^+$ via a cellular pathway that is sensitive to amiloride. Single cell patch clamp recordings provided direct evidence that the amiloride-sensitive $Na^+$ channel exists in taste receptor cells (Avenet and Lindemann, 1988, 1991; Gilbertson et al., 1992, 1993; Doolin and Gilbertson, 1996). In mammalian taste cells, amiloride-sensitive $Na^+$ channels share electrophysiological and pharmacological properties with amiloride-sensitive $Na^+$ channels in kidney epithelial cells, including a low single channel conductance (near 5 pS; Avenet, 1992), high sensitivity to amiloride (Gilbertson et al., 1993; Doolin and Gilbertson, 1996), high selectivity for $Na^+$ over $K^+$ (Lin and Kinnamon, unpublished data), significant permeability to protons (Gilbertson et al., 1992, 1993), and self-inhibition by extracellular $Na^+$ (Gilbertson and Zhang, 1998a). In addition, these channels are regulated by the hormones aldosterone and vasopressin, both of which increase the number of channels in apical membrane (Gibertson et al., 1993; Herness, 1992).

Recently, a highly $Na^+$ selective, amiloride-sensitive epithelial sodium channel (rENaC) was cloned (Canessa et al.,1993, 1994; Lingueglia et al., 1993). The channel comprises three homologous subunits: $\alpha$, $\beta$ and $\gamma$. Although small amiloride-sensitive currents can be obtained by expression of $\alpha$ subunit alone, co-expression of all three subunits greatly enhances the magnitude of the current. The channel has high sensitivity

to amiloride ($K_i$ about 0.1 μM), low single channel conductance (5-7 pS) and no significant $K^+$ conductance (Canessa et al., 1994). The channel is expressed in tight epithelia such as colon, kidney and lung (Duc et al., 1994), and plays an important role in $Na^+$ transport (Rossier et al., 1994).

In rodents, taste receptor cells occur in three distinct papillae: fungiform, on anterior tongue, and foliate and vallate on posterior tongue. Approximately 65% of the fungiform taste cells exhibit functional amiloride-sensitive $Na^+$ currents, while only 35% of the foliate taste cells are amiloride-sensitive. In contrast, taste cells of vallate papillae are completely amiloride-insensitive (Doolin and Gilbertson, 1996), although α rENaC mRNA (Li et al., 1994) and immunoreactivity to the purified amiloride-sensitive $Na^+$ channel protein (Simon et al., 1993b) have been detected in those cells. Therefore, it is important to examine the expression of each of the three rENaC subunits in taste cells, and to determine if differences in expression patterns between vallate and fungiform papillae can account for the observed functional differences.

Apically-applied amiloride does not suppress all of the NaCl response, even in anterior tongue (Heck et al., 1984). Since amiloride can only access and block the apical $Na^+$ channels, $Na^+$ may have other actions which are unaffected by apically-applied amiloride. An amiloride-sensitive $Na^+$ channel might also exist at the basolateral membrane of taste cells where it could allow sodium influx from extracellular spaces (DeSimone et al., 1995, Simon et al 1993b; Stewart et al., 1995; Ye et al., 1993; Mierson et al., 1996). There are two possible ways that Na ions in the oral cavity might reach extracellular spaces. The ions might pass through the tight junction between cells (DeSimone et al., 1984; Simon et al 1993b; Ye et al., 1993), or through channels in non-sensory epithelial cells. Experiments with isolated lingual epithelium suggest that a

significant portion of the NaCl-induced transepithelial current is due to the influx of $Na^+$ though non-sensory epithelium (DeSimone et al., 1981, 1984; Heck et al., 1984; Mierson et al., 1985; Simon and Garvin, 1985, Simon et al.,1988, 1993b), and that this $Na^+$ transport is sensitive to amiloride (Simon et al., 1993b). Similarly, molecular studies demonstrate that α rENaC mRNA is expressed in non-sensory epithelial cells, suggesting a role of epithelial cells in $Na^+$ transduction (Li et al., 1994).

Using subunit-specific antibodies against the three subunits of rENaC in immunocytochemistry, we intended to investigate: (1) Are all three of the rENaC subunits in taste cells of all lingual papillae? (2) Is there any difference in the expression of the three rENaC subunits between taste cells of fungiform and vallate papillae and is this expression altered in response to increased aldosterone? (3) Are the three rENaC subunits also expressed in non-sensory epithelial cells? Preliminary results of these studies have been reported in abstract form (Lin et al 1997a, b).

## Materials and Methods

### Animals

Adult Sprague-Dawley male or female rats were used in this study. Most rats (25) were maintained on a normal $Na^+$ diet. Fourteen rats were subjected to treatments designed to raise plasma aldosterone levels, which in other tissues, increases the abundance of $Na^+$ channels in aldosterone target cells (Asher et al., 1996; Duc et al., 1994; Lingueglia et al., 1994). These rats were placed either on a low $Na^+$ diet (0.3% $Na^+$, Purina Mills Inc, St. Louis, MO; six rats) for 2 weeks or injected i.p. with

aldosterone (200-250 μg/Kg ; Sigma, St. Louis, MO; A-6628; two rats) 24 and 48 hours before killing. Six rats were subjected to both dietary $Na^+$ deprivation and aldosterone injection. There were no apparent differences observed among the different treatments designed to raise aldosterone levels. All animal handling procedures were carried out under the supervision and approval of Institutional Animal Care and Use Committees.

Antibodies:

1) Affinity-purified polyclonal antibodies against subunits of rENaC were raised in rabbit against Glutathione S-Transferase (GST)-fusion proteins from the $NH_2$ terminus of α rENaC (amino acids E10 to E77), the COOH terminus of β rENaC (amino acid G559 to E636), and of γ rENaC (amino acid A570 to L650) (for detailed characteristics of the antibodies, see Duc et al., 1994). For immunocytochemistry, affinity-purified antibodies were used in the dilution range of 1:50 - 1:200.

2) Affinity-purified rabbit polyclonal anti-gustducin antibody, Gα-gust (1-20) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA; Cat# SC-395). The working dilution was 1:1000.

3) Monoclonal mouse anti-β-tubulin Isotype III was obtained from Sigma (Product No: T-8660). The antibody specifically recognizes a neural form of β-tubulin (Isotype III). Working dilution was 1:400.

4) All secondary antibodies were obtained from Jackson ImmnoResearch Laboratories, Inc. (West Grove, PA). These include: Lissamine rhodamine (LRSC)-conjugated Affinipure™ goat anti-rabbit IgG (Code No: 111-085-144); Fluorescein (FITC)-conjugated Affinipure™ Fab Fragment goat anti-rabbit IgG (Code No: 111-097-

003); Lissamine rhodamine(LRSC)-conjugated Affinipure™ Fab Fragment goat anti-rabbit IgG (Code No:111-087-003); Affinipure™ Fab fragment goat anti-rabbit IgG (Code No: 111-007-003; dilution 1:10-1:50); Fluorescein (FITC)-conjugated Affinipure™ donkey anti-mouse IgG (Code No: 715-095-151). Lissamine rhodamine-conjugated Affinipure™ donkey anti-mouse IgG (Code No: 715-085-151). Final working dilution for all conjugated secondary antibodies was 1:100.

Tissue preparation

The rats were anesthetized with sodium pentobarbital (40 mg/Kg) and perfused transcardially with a modified Paraformaldehyde Lysine Periodate (PLP) fixative (McLean and Nakane, 1974) containing 0.019 M L-lysine-monohydrochloride, 3% paraformaldehyde and 0.23% sodium periodate in 0.1 M phosphate buffered saline (PBS), pH 7.2. Tongue and kidney, and in some experiments brain, were removed and postfixed for 2 hours before being transferred into PBS with 30% sucrose overnight. The tissues were frozen and cut with a cryostat into free-floating 25-30-μm-thick sections.

Immunocytochemistry

Single label for rENaC subunits

Sections containing taste buds from vallate, foliate and fungiform papillae as well as control sections of kidney were selected and rinsed with 0.1 M PBS. The sections then were incubated for 1.5 hours in blocking solution containing 2% normal goat serum, 0.3% Triton X-100 and 1% bovine serum albumin in PBS. This step was followed directly by incubation overnight or 2 days in primary antiserum diluted 1:50-1:200 in

blocking solution. After incubation of the primary antibodies, the sections were washed 3x in PBS and incubated for 1 hour at room temperature with 1:100 dilution of secondary antibody, goat-anti-rabbit IgG conjugated with LRSC. Sections were then washed in PBS and mounted on slides with Fluoromount-G (Fisher Biotech, Birmingham, AL). Positive controls consisted of kidney tissues which produced immunostaining as reported previously (Duc et al., 1994); negative controls, all of which lacked significant reactivity, involved either replacing the primary antiserum with blocking solution, or pre-incubation of the primary antiserum with GST-fusion protein attached to agarose beads.

Preparation of GST-fusion proteins

Constructs consisted of the pGEX vector encoding glutathione-s-transferase (GST) fused to the specific sequence from either the α, β, or γ subunit of rENaC. Constructs were grown up in Xl-1 blue *E. coli* cells cultured on LB (Luria Broth base, Gibco BRL, Grand Island, NY) plates containing 20 μg / ml ampicillin. Individual colonies were then transferred to LB medium containing 100 μg/ml ampicillin. GST-fusion protein expression was induced with 0.1 mM Isopropyl-β-D-thio-galactoside (IPTG, Boehringer Mannheim, Indianapolis, IN). Following cell lysis and centrifugation, the fusion proteins were isolated from the bacterial lysate with a slurry of glutathione agarose beads (Glutathione sepharose 4B, Pharmacia Biotech, Piscataway, NJ). For 200 ml bacteria cultures, 400 μl of slurry were used. Following incubation at 4° C, the fusion proteins were attached to the glutathione agarose beads. Half of the resultant slurry (200 μl) for each fusion protein was rinsed, mixed with 200 μl of 1:100 diluted primary

antiserum, and incubated overnight at 4° C. The antiserum-bead mixtures were then centrifuged 16,000 X g for 1minute at 4° C and the supernatants were applied to tissue.

To check the purity and specificity of peptide production, the remaining beads containing either GST-fusion protein or fusion protein plus the primary antibody were eluted with reduced L-glutathione (Sigma, G-1404). The supernatants then underwent electrophoresis on SDS-polyacrylamide mini gels (ready made, 15% Tris-Glycine gel, with 4% stacking gel, Bio-Rad, Hercules, CA, Cat. No: 161-0908). The GST-fusion proteins from each of the three subunits have bands around 33 kD. Primary antibodies attached to the GST-fusion proteins shifted bands to higher molecular weights.

Propidium iodide counterstaining

In order to estimate the percentage of immunoreactive cells in taste buds of different papillae, sections from three papillae first were labeled with antibodies against rENaC subunits as described above except the secondary antibody was conjugated with FITC. Since propidium iodide also binds to cytoplasmic RNA, sections were pre-treated with 500 μg/ml RNase A (Boehringer) at the temperature of 30 to 35° C for 30 minutes. The RNase was pre-boiled 5 minute to inactivate residual DNase. Sections then were rinsed briefly in PBS and treated with propidium iodide (5 μg / ml PBS) for 1 minute, rinsed and mounted as above.

Double labeling with β-tubulin

Since rENaC and β-tubulin antibodies were raised in different hosts, a combination of two primary antibodies could be applied simultaneously to sections, and a

solution of secondary goat-anti rabbit antibody conjugated with LRSC and donkey-anti-mouse conjugated with FITC was used to visualize the immunoreactivity. Controls consisted of single label with each primary antibody followed by the combination of two secondary antibodies thereby testing for inappropriate cross-reactivity of the secondary antisera. None was observed. Brain and kidney tissues served as positive and negative controls for the two primary antisera.

Double labeling of rENaC subunits and gustducin

Since the antibodies against these antigens are both raised in rabbits, we utilized a labeling procedure that relies on labeled Fab fragments as secondary antibodies (Carl et al., 1993; Negoescu et al., 1994). Briefly, sections were exposed to an anti-rENaC subunit antiserum followed, after washing, by reaction with a secondary Fab fragment antibody conjugated with LRSC. The tissue then was exposed to an excess of unconjugated anti-rabbit Fab fragment (1:10 to 1:50) for 4 hours in room temperature to cover all the sites recognized by anti-rabbit sera. Sections then were rinsed and incubated in blocking solution again for one and half hours, followed by incubation overnight in the primary antiserum against gustducin. The sections then were rinsed and incubated for one hour with anti-rabbit Fab fragment conjugated with FITC.

Controls consisted of omitting the primary antisera; of changing the sequence of primary antibody application, e.g., labeling gustducin first; and the sequence of application of the two secondary Fab fragments. Also, we used sections of kidney, which exhibit strong rENaC immunoreactivity, as a negative control for gustducin immunoreactivity. For these double-label experiments, it is essential to control for the

possibility of residual recognition of the first antiserum (e.g., anti-rENaC) by the second-applied labeled Fab fragment despite previous exposure to excess unlabeled Fab. Accordingly, some sections were exposed only to the first primary antiserum, followed by incubation in the sequence of labeled and unlabeled Fab fragments as for double labeling. All controls were negative for inappropriate labeling, establishing the specificity of the reaction for the particular antisera and Fab fragments employed in these studies.

Electronic images were composed in Photoshop ™. Adjustments were made only to contrast, brightness and color balance unless otherwise specified.

Semiquantitative immunocytochemistry

To evaluate quantitatively the difference of immunoreactivity for subunits of rENaC between taste cells of fungiform, foliate and vallate papillae, for each subunit we processed the sections from fungiform, foliate and vallate papillae simultaneously under identical conditions in terms of antiserum dilution and incubation conditions. The results were captured on film with a fixed exposure that had been determined empirically not to saturate any area of the film. The negatives were scanned into a film scanner (Nikon Coolscan) using fixed brightness and contrast settings. The digitized images then were analyzed for average pixel intensity in individual taste cells outlined by hand in NIH Image Software (version 1.61). The average level of non-specific fluorescence in each image was measured by selecting areas of the image containing connective tissue only. A one-way analysis of variance (ANOVA) was used to determine statistical differences in the intensity of immunolabel in taste cells of different papillae. Fisher's Least

Significant Difference (LSD) was used to compare the intensity of immunolabel for each subunit in taste cells between papillae. Values of $p<0.01$ were considered significant.

Cell counting

In some animals, 25μm cross sections were obtained from foliate and vallate papillae and immunoreacted with antibodies against the three subunits of rENaC. To evaluate the number of immunoreactive cell profiles in each taste bud, each labeled profile was counted without regard to cell diameter. Ten taste buds were randomly selected for each subunit and the numbers were averaged to obtain the mean cell number.

Electrophysiology

*Isolation of taste buds.* Taste buds were isolated according to previously published methods (Béhé et al., 1990). Briefly, rats were killed with $CO_2$. The tongue was excised and was injected under the epithelium with an enzyme mixture containing 3 mg dispase (Grade II; Boehringer Mannheim) 1 mg trypsin inhibitor (Type I-s; Sigma Chemical Corp.) and 0.7 mg collagenase B (Boehringer Mannheim) in 1 ml standard bath solution (Tyrode's). The anterior part of the tongue housing fungiform papillae was incubated in $Ca^{2+}$ and $Mg^{2+}$-free oxygenated Tyrode's for 30 minutes. The posterior tongue, which houses the vallate papilla, was incubated for 1 hour or until the epithelium could be gently separated from the underlying connective tissue. Individual taste buds were removed from papillae by suction with a fire-polished pipette and plated onto glass slides coated with Cell-Tak (Collaborative Research, Bedford, MA).

*Solutions.* Standard bath solution consisted of 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

buffer (HEPES), 10 mM glucose and 10 mM sodium pyruvate. The pH was adjusted to 7.4 with NaOH. The $Ca^{2+}$-and $Mg^{2+}$-free Tyrode's used to isolate taste buds contained 2 mM 1,2-bis(2-Aminophenoxy)ethane-N,N,N',N'-tetraacetic Acid (BAPTA; Molecular Probes, Eugene, OR). Thirty μM amiloride was added to the bath solution to test for the presence of amiloride-sensitive $Na^+$ current. Bath solutions were gravity-fed into the 0.5 ml recording chamber. Flow rates were typically 4-5 ml/min, permitting complete solution exchange in less than 10 seconds.

The intracellular pipette solution contained 140 m M KCl or 130 mM K gluconate plus 10 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES (pH 7.2 with KOH), 11 mM EGTA, 1 mM ATP, and 0.4 mM GTP. (pH 7.2 with KOH).

*Patch-clamp recording.* The whole-cell voltage-clamp technique was used to record steady-state currents (Hamill et al., 1981). Patch recording pipettes were pulled from microhematocrit capillary tubes (Scientific Products, McGaw Park, IL) with a two-stage vertical puller (model PB-7; Narishige, Tokyo, Japan). Pipette resistance was 4-8 megohms. Membrane currents were filtered at 2 KHz and recorded with an Axopatch patch-clamp amplifier (model 200B; Axon Instruments, Foster City, CA). Voltage-gated $Na^+$ and $K^+$ currents were induced by applying voltage steps from an Indec laboratory computer system (Sunnyvale, CA) and were used to distinguish taste cells from non-sensory epithelial cells. For steady-state measurements, holding current was recorded at -80 mV and 20 mV hyperpolarizing voltage pulses were used to monitor membrane conductance. An amiloride response was defined as a reversible decrease in holding current and membrane conductance in response to bath application of amiloride.

## Results

### Immunoreactivity for rENaC subunits

#### Fungiform papillae

In fluorescent labeled sections of fungiform papillae, immunoreactivity for three subunits of rENaC, α, β and γ (α rENaC-ir, β rENaC-ir and γ rENaC-ir) was present in many spindle-shaped taste cells of each taste bud (Fig. 2.1, A, B and C). The reactivity was distributed throughout the cell including the nucleoplasm as well as the cytoplasm of both apical and basal portions of the cell. The results indicated that each of the three rENaC subunits is present in taste receptor cells. No selective labeling was detected with antisera preabsorbed with the corresponding α, β or γ subunit peptide (Fig. 2.1, D-F).

#### Foliate and vallate papillae

Immunoreactivity for α, β and γ rENaC subunits was present in approximately half of the taste cells of foliate and vallate taste buds (Fig. 2.2). As in fungiform taste cells, the labeling was present throughout the cell. However, in some taste cells in vallate and foliate taste buds reactivity for the α subunit was especially intense in a filamentous network in the supranuclear portion of the cell (Fig. 2.5D). This location and appearance is similar to that of the Golgi apparatus (Stone and Finger, unpub. obs.), although no attempt was made in the present study to confirm this. In contrast to fungiform papillae, in vallate and foliate papillae, the α rENaC-ir appeared stronger than β rENaC-ir and γ rENaC-ir. In some animals, the γ rENaC-ir in vallate papillae appeared markedly lower than α and β subunits. No selective labeling was detected with pre-absorbed antisera (data not shown).

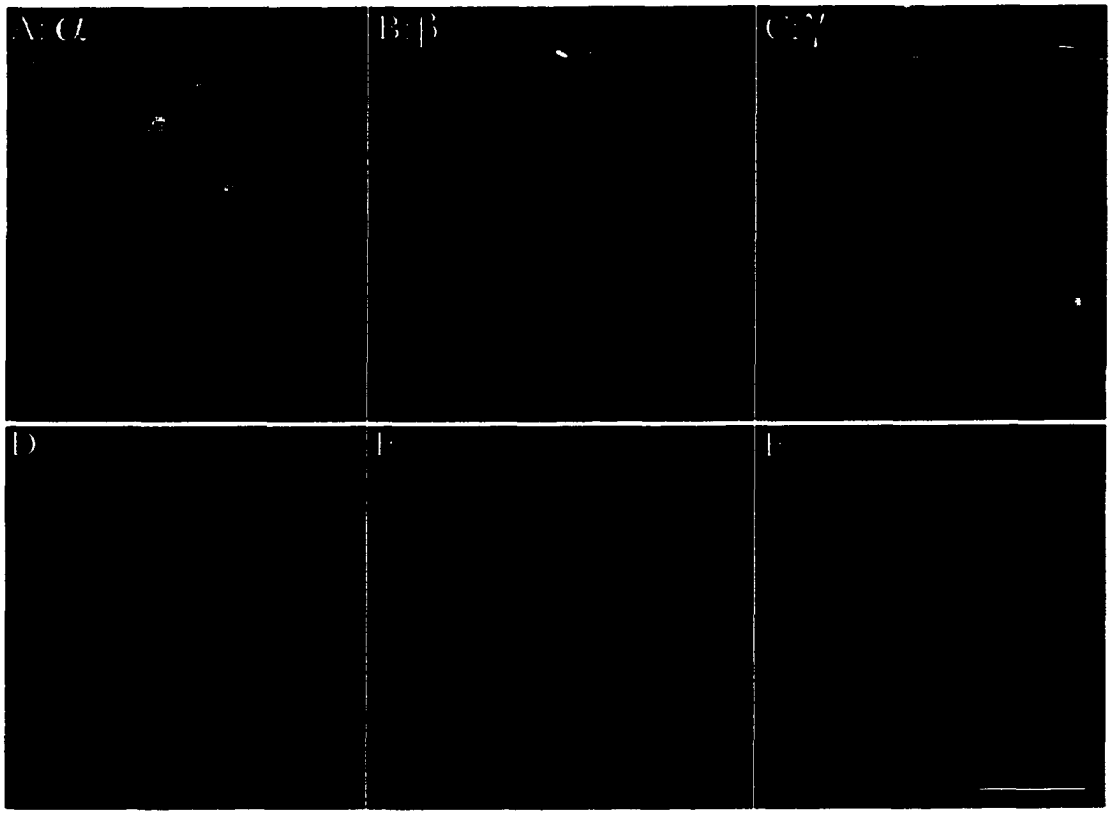


Fig. 2.1. Images of immunocytochemical localization of rat epithelial $Na^+$ channel (rENaC) subunits in taste cells of fungiform papillae. Positive labeling for α (A), β (B), γ (C) subunits of rENaC were detected with similar intensities in most taste receptor cells. Significant immunoreactivity also is present in surrounding epithelial cells. Panels D (α), E (β) and F (γ), show results with antisera pre-absorbed with the cognate peptide. No selective labeling was observed. A-C, laser scanning confocal microscope (LSCM) images; D-F, standard epifluorescent microscopy. Scale bar: 20 μm.

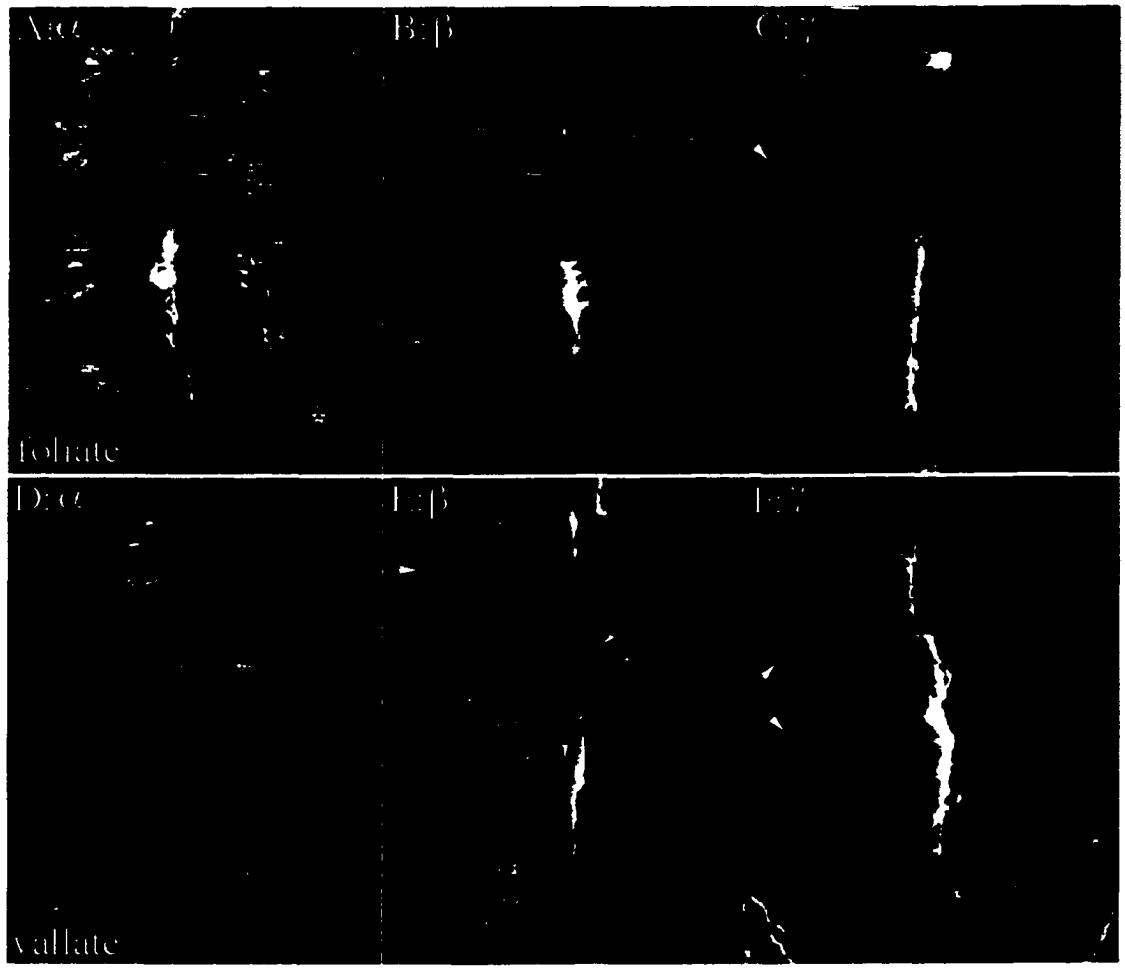
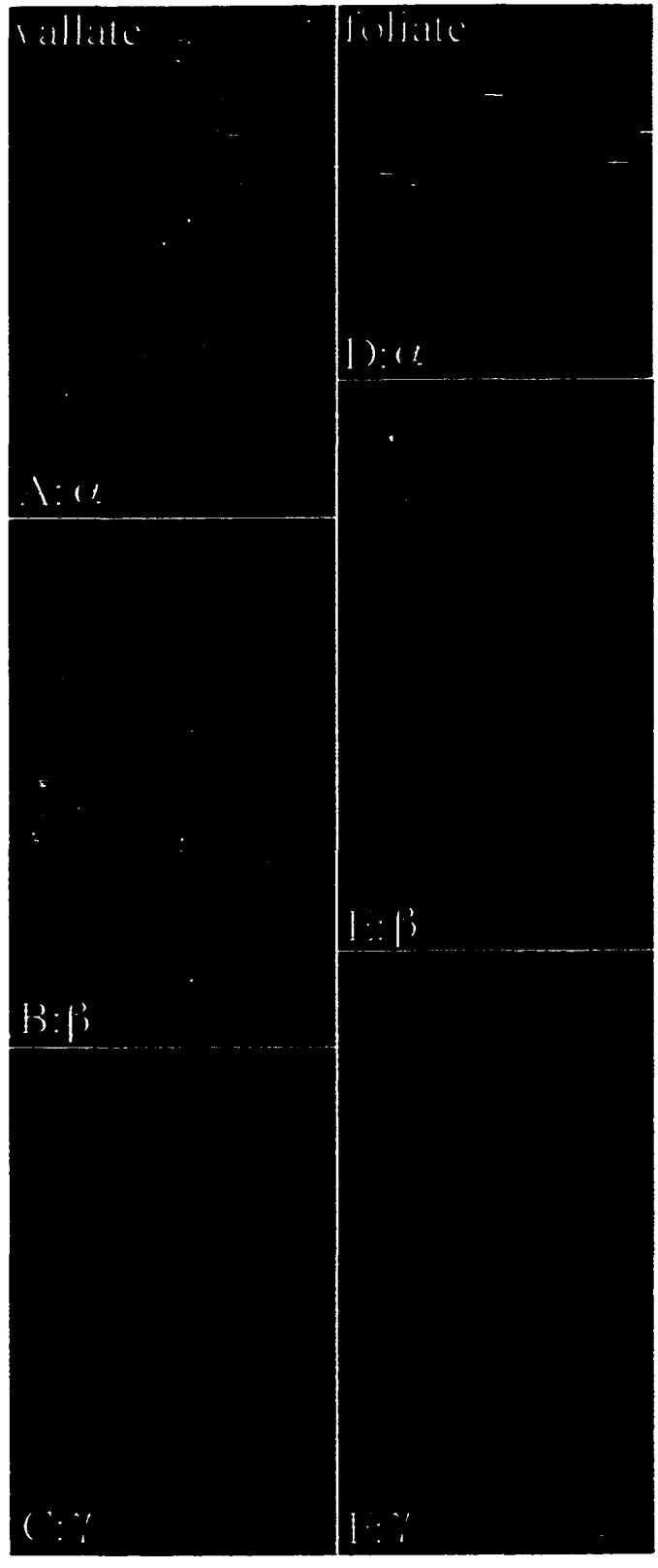



Fig. 2.2. LSCM images of immunocytolocalization of α, β and γ rENaC subunits of foliate (A-C) and vallate papillae (D-F). The immunolabeling for α rENaC was relatively stronger; the label for γ subunit was somewhat weaker. Reactivity was largely cytoplasmic including the nucleus, but some label was located in the apical region (arrowheads). Nerve fibers located at the bottom of the taste buds were labeled with antisera against β and γ subunits (arrows). Scale bar: 20 μm.

To investigate the location of immunoreactive taste cells in individual taste buds and the relative number of immunoreactive taste cells per taste bud, we obtained 25 μm-thick cross-sections through taste buds from foliate and vallate papillae and immunoreacted them with antiserum against α, β and γ subunits. As shown in figure 2.3, taste cells labeled for α, or β, or γ subunit were usually round or oval in cross section, a characteristic of type II taste cells (Pumplin et al., 1997). The number of labeled cells in each taste bud varied, as individual taste buds vary in diameter and the total number of cells. The average number of immunoreactive profiles per taste bud in foliate papillae for the different subunits was $\alpha = 13.5 \pm 4.6$, $\beta = 14.1 \pm 4.9$, and $\gamma = 13.7 \pm 2.5$. In vallate papillae, the average number of profiles per taste bud was $\alpha = 13.9 \pm 5.0$, $\beta = 13.0 \pm 3.6$, and $\gamma = 13.6 \pm 3.7$. The number of cells labeled and the location of labeled cells in individual taste buds were similar for each subunit, suggesting that the three subunits of rENaC may be co-localized in the same taste cells.

In sections counterstained with propidium iodide (Fig. 2.4), taste cells in vallate and foliate papillae that were immunoreactive for each of the rENaC subunits usually had an oval or round nucleus. Cells with irregular nuclei often were not immunoreactive (arrows, Fig. 2.4). In contrast, in fungiform taste buds, virtually all taste cell nuclei, as identified with propidium iodide staining, were situated in cells exhibiting reactivity for the rENaC subunits. Thus, these nuclei exhibited heterogeneity of shapes.

Fig. 2.3. LSCM images of immunoreactivity for rENaC subunits in taste cell cross-sections from vallate (A-C) and foliate (D-F) papillae. Most labeled cells were round or oval shaped and labeled cells were distributed throughout the width of the taste buds. On average, about 13 cells per taste bud were labeled for each subunit. There were no significant differences in the location and number of labeled cells for each subunit, suggesting that they may be co-localized. Scale bar: 20 μm.



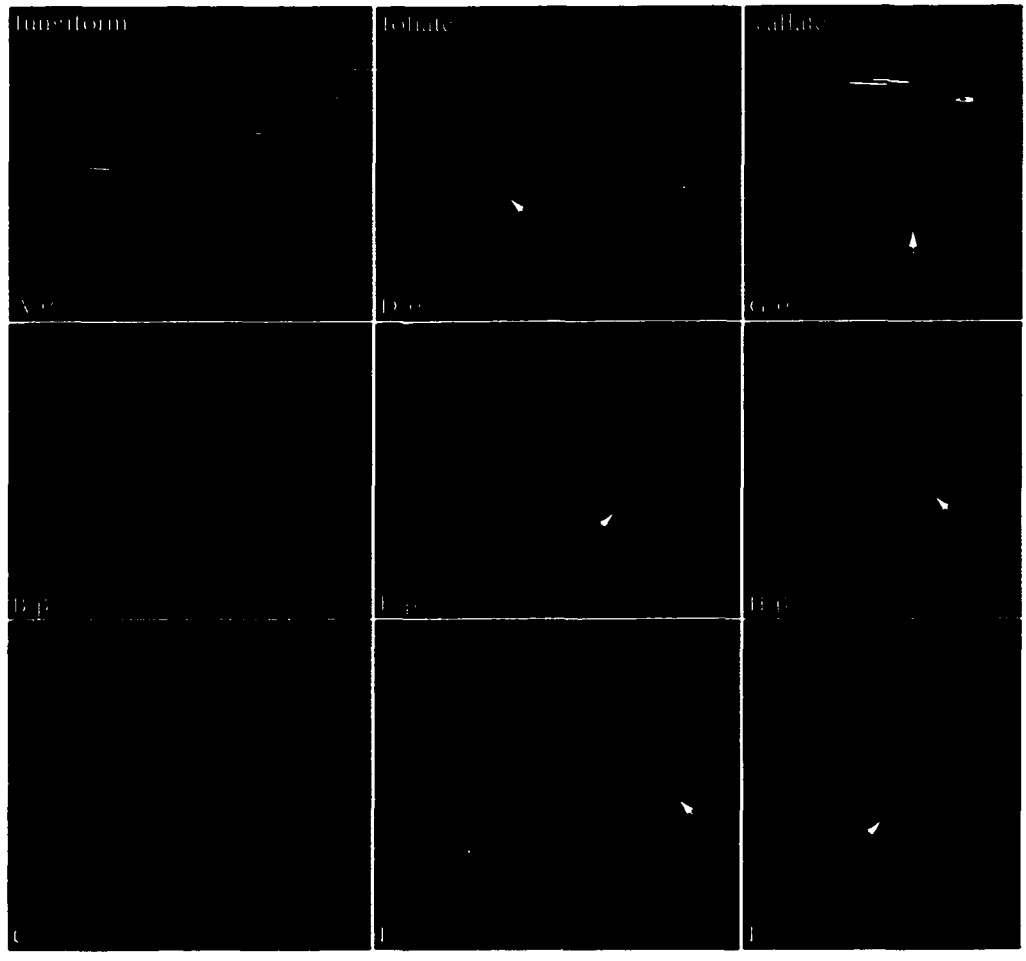


Fig. 2.4. Confocal images of taste buds counterstained with propidium iodide (red) and immunofluorescently labeled for rENaC subunits (green). The rENaC subunits are expressed in cells with oval or round nuclei. Nearly all taste cells in fungiform papillae (A-C) were double-labeled indicating that nearly all of these taste cells express rENaC. In foliate (D-F) and vallate (G-I) papillae, several nuclei can be seen labeled only with propidium iodide (arrows) indicating the presence of taste cells lacking rENaC immunoreactivity. Scale bar: 10 μm.

Relative abundance of subunits in different papillae

The results above showed that three subunits of rENaC are expressed in taste cells of fungiform, foliate and vallate papillae. Thus, the lack of amiloride sensitive current in taste cells of vallate papilla can not be explained by absence of any particular rENaC subunit. The results were, however, suggestive of quantitative differences in the abundance of each subunit in the different papillae. To assess quantitative differences in subunit expression, we compared the intensity of immunolabeling for each rENaC subunit in different papillae under standardized immunolabeling and imaging conditions. The mean values of the intensity from taste buds of different papillae are indicated in table 1 and shown in figure 2.5. For all three subunits of rENaC, the intensity of immunolabeling in taste cells of fungiform papillae was significantly higher than the intensity in cells from foliate and vallate papillae, although the differences were much more dramatic for β and γ subunits than for α subunit. For β and γ subunits, the intensity of immunolabeling was fungiform > foliate > vallate (one way ANOVA plus Fisher's LSD: β subunit: $F_{2,45}=165.5$, $p<.001$; γ subunit: $F_{2,45}=139.9$, $p<.001$). For α subunit, the intensity of immunolabeling in fungiform taste cells was significantly higher than that of either foliate or vallate (One way ANOVA plus Fisher's LSD: $F_{2,45}=19.9$, $p<0.01$). There was no significant difference in the immunolabeling intensity of α subunit in the foliate and vallate papillae.

Table. 2.1. Intensity of specific immunolabeling for each subunit in taste cells of fungiform, foliate and vallate papillae. The intensity of labeling for each subunit in taste cells was measured with NIH Image program from images obtained under fixed labeling and acquisition conditions, as in Fig. 2.5. Non-specific fluorescence was measured over connective tissue and subtracted from the raw intensity measured over individual taste cells to obtain the specific fluorescence. On average, the intensity values for non-specific fluorescence and absolute background were 60.8 $\pm$ 0.7 and 10.0 $\pm$ 0.1, respectively (n=24 images). There was no significant difference in nonspecific labeling or background fluorescence in the different images. Data from each taste bud represent the mean value obtained from four individual taste cells. The intensity of specific immunolabeling for all three subunits in fungiform taste buds was significantly higher than for foliate or vallate taste buds ($p<0.01$). The differences were, however, more dramatic for the $\beta$ and $\gamma$ subunits than for $\alpha$ subunit, with fungiform>foliate>vallate ($p<0.01$).

Table 1: Intensity of immunoreactivity for subunits of rENaC

| | | α–rENaC | β–rENaC | γ–rENaC |
|---|---|---|---|---|
| fungiform | bud1 | 142.0 ± 5.0 | 157.0 ± 3.8 | 149.1± 2.4 |
| | bud2 | 157.1 ± 2.7 | 155.2 ± 2.4 | 149.5 ± 4.1 |
| | bud3 | 156.9 ± 2.0 | 147.3 ± 2.1 | 145.8 ± 5.1 |
| | bud4 | 157.3 ± 3.9 | 150.0 ± 2.3 | 155.6 ± 3.3 |
| | mean ± sem | 153.3 ± 2.3 | 152.4 ± 1.6 | 150.0 ± 2.0 |
| foliate | bud1 | 138.4 ± 5.0 | 111.9 ± 4.2 | 115.3 ± 3.9 |
| | bud2 | 140.6 ± 4.4 | 116.6 ± 1.5 | 120.5 ± 3.7 |
| | bud3 | 124.1 ± 2.5 | 117.2 ± 3.2 | 108.7 ± 3.2 |
| | bud4 | 145.6 ± 3.9 | 135.4 ± 3.3 | 109.8 ± 3.4 |
| | mean ± sem | 137.2 ± 2.7 | 120.3 ± 2.8 | 113.6 ± 2.1 |
| | foliate vs. fungiform | 0.90 | 0.79 | 0.76 |
| vallate | bud1 | 135.2 ± 2.0 | 99.8 ± 3.2 | 92.3 ± 3.6 |
| | bud2 | 130.7 ± 1.1 | 94.5 ± 5.9 | 110.1 ± 3.8 |
| | bud3 | 139.3 ± 1.5 | 87.5 ± 3.4 | 101.7 ± 4.0 |
| | bud4 | 138.7 ± 2.0 | 99.2 ± 3.1 | 104.9 ± 3.3 |
| | mean ± sem | 136.0 ± 1.2 | 95.3 ± 2.2 | 102.3 ± 2.4 |
| | vallate vs. fungiform | 0.89 | 0.63 | 0.68 |

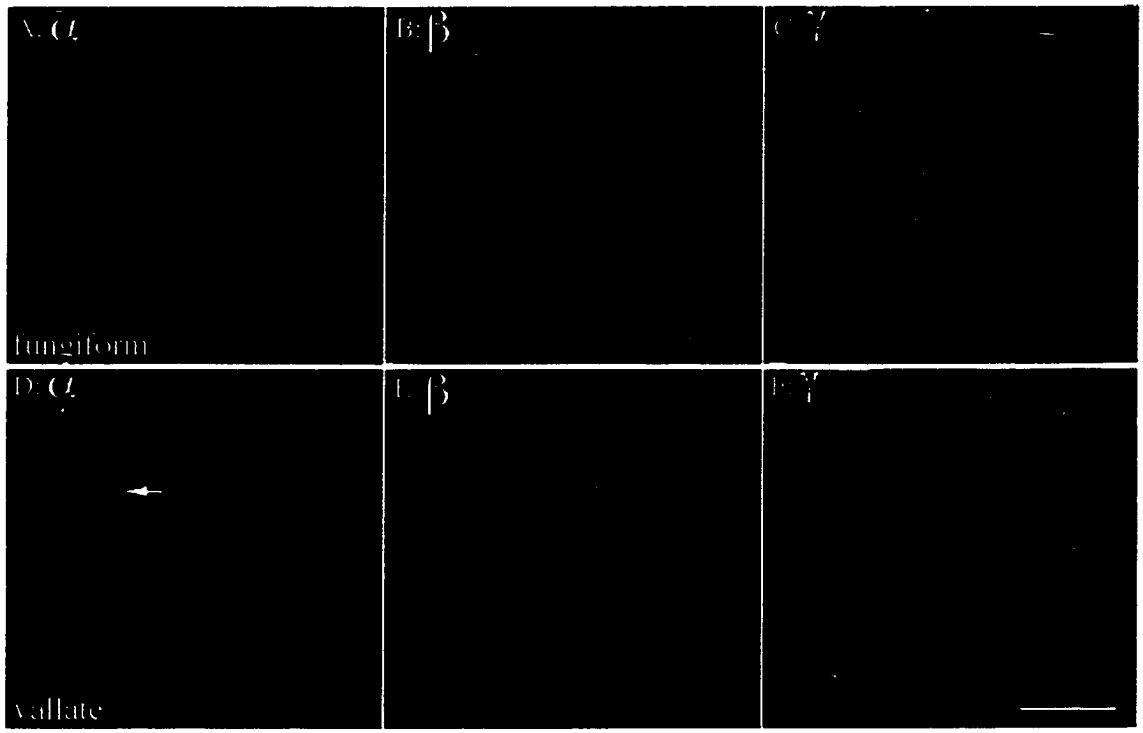


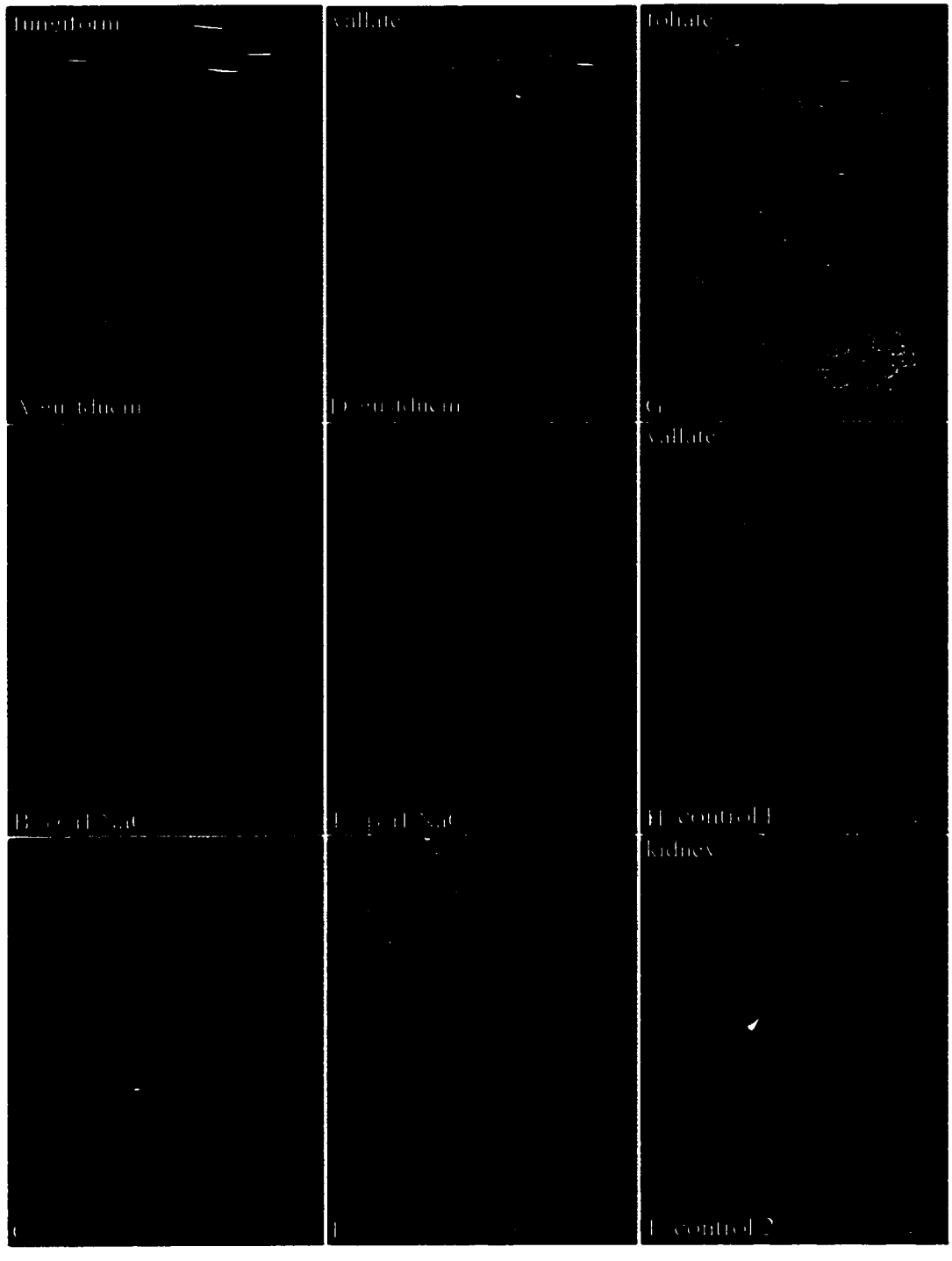

Fig. 2.5. Semiquantitative comparison of immunoreactivity for each subunit of rENaC in fungiform, foliate and vallate papillae under conditions of fixed exposure and handling conditions for each antiserum. The immunoreactivity for each of the three different subunits was significantly stronger in taste cells of fungiform papillae (A-C) than in vallate (D-F) and foliate (not shown). Among three papillae, the labels for β and γ subunits was weakest in vallate taste buds. Note the punctate supranuclear reactivity for the α rENaC in the vallate taste bud (Panel D, arrow). Scale bar: 20 μm.

Co-localization of subunits of rENaC and gustducin in taste cells

Gustducin is a taste cell specific G-protein α subunit expressed in a subset of Type II (light) taste cells in each papilla (Mclaughlin et al., 1992; Tabata et al., 1995; Boughter et al., 1997). We double-labeled the taste tissue with antisera against subunits of rENaC and gustducin to determine if gustducin is co-localized with rENaC. In taste buds of all papillae, more cells were labeled with antibodies to subunits of rENaC than gustducin. All gustducin-immunoreactive taste cells also exhibit some degree of immunoreactivity to each rENaC subunit, even though a few double reacting cells had relatively weaker labeling for a subunit of rENaC compared to other cells in the same taste bud. The double labeling is easily detected since gustducin immunoreactivity is excluded from the nucleus whereas rENaC immunoreactivity is present in the nuclear compartment. The co-localization was found with each subunit of rENaC, and was found in taste cells of fungiform (Fig. 2.6C), foliate (Fig. 2.6, G) and vallate (Fig. 2.6F) papillae.

As a control for inappropriate cross-reactivity of the secondary antibody, kidney sections also were double labeled with antisera against each subunit of rENaC and gustducin. Because gustducin is not expressed in kidney, only subunits of rENaC were labeled (Fig. 2.6I: β subunit); no labeling with the inappropriate secondary antiserum was detected even in this strongly labeled tissue. In addition, when the gustducin primary antibody was eliminated, no cross-reactivity of the FITC-labeled secondary was detected in lingual papillae (Fig. 2.6H).

Fig. 2.6. LSCM images of co-localization of subunits of rENaC and gustducin in taste cells of the different gustatory papillae. The immunoreactivity of rENaC subunits is shown in red (B: α and E: β); immunoreaction of gustducin is shown in green (A and D). The gustducin-labeled cells are a subset of those reacting with a subunit of rENaC, showing yellow (C, F and G). C and F are color composites of pairs A, B and D, E, respectively. G: Cross-section of taste cells from foliate papilla, reacted with gustducin and α rENaC. Note more cells reacted for a subunit of rENaC than gustducin. H: control section from vallate papilla, the primary antibody against gustducin was omitted, showing the primary antibody against α rENaC does not react with the secondary antibody conjugated with FITC. I: Sections from kidney were double-labeled with β rENaC and gustducin. Since distal convoluted tubule cells do not express gustducin, only β rENaC labeling is present, showing red only (arrow). Scale bars: 20 μm.



Immunoreactivity in Non-gustatory Epithelial Cells and Sensory Nerve Fibers

In our experiments of rENaC immunoreactivity in taste cells, we also observed significant immunoreactivity for the three subunits in lingual epithelial cells with the intensity appearing slightly weaker than the intensity in taste cells (Fig. 2.1, 2, 4 and 5). Stronger labeling was present in epithelial cells located in the anterior portion of the tongue. The keratinized layer of the epithelium did not appear to be labeled.

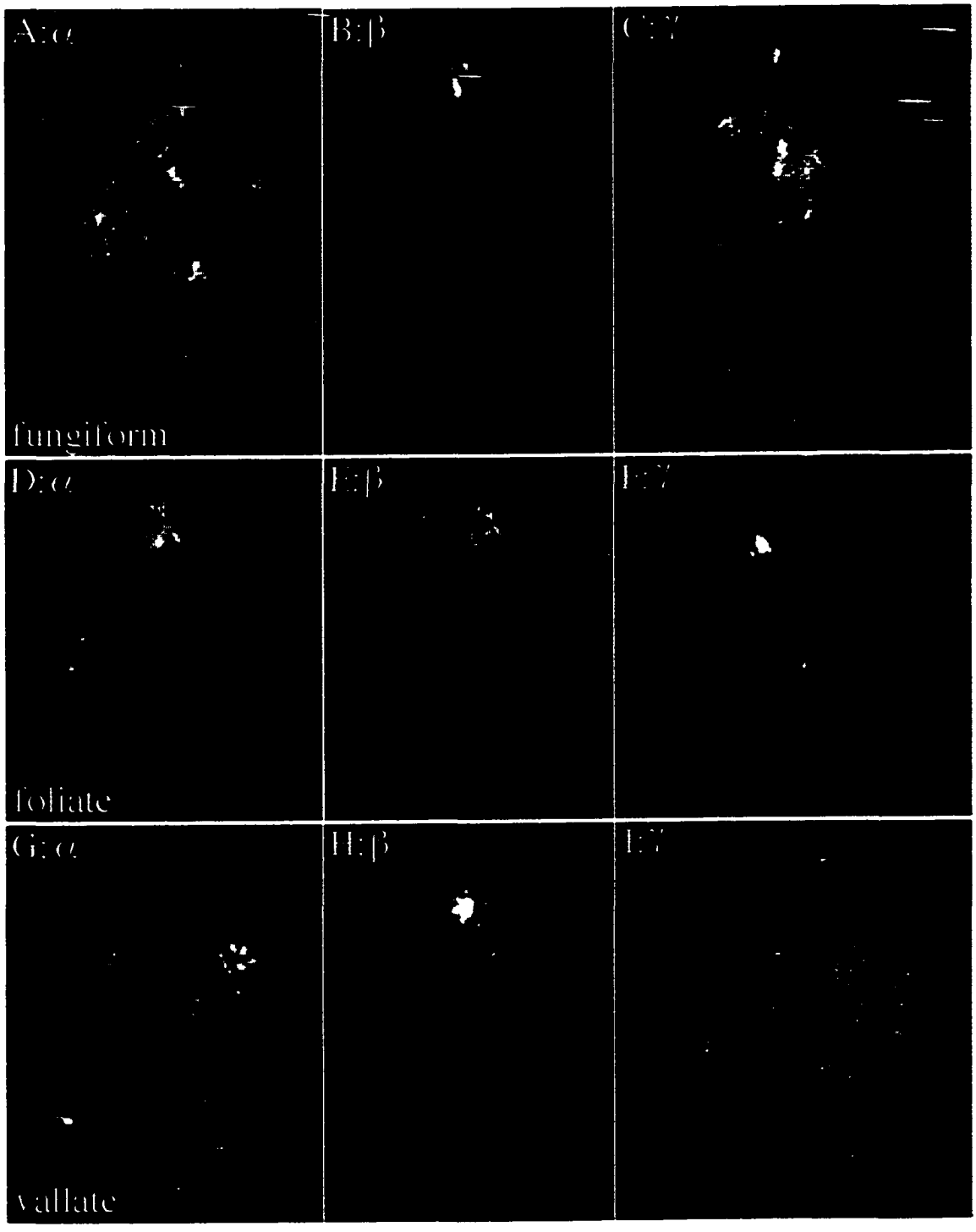
Both β rENaC-ir and γ rENaC-ir was associated with some nerve fibers in the vicinity of the taste buds, especially in the nerve plexus below the taste buds in fungiform papillae (Fig. 2.2 and 2.5C). Reactivity for α subunit in fibrous processes below the epithelium was occasionally present but was more equivocal. To further examine if the labeling for β rENaC and γ rENaC is located in nerve fibers, we double-labeled tongue sections with antisera against a subunit of rENaC and β-tubulin type III, a neuron specific isotype (Banerjee, et al., 1990). The results are shown in Fig. 2.7. The diameter of the profiles immunoreactive for β-tubulin often was less than those reactive for β rENaC. In addition, the coarse fiber-like β rENaC immunoreactivity often stops below the base of the taste bud (Fig. 2.7). These findings are consonant with the interpretation that β rENaC immunoreactivity is associated with the myelin sheath and not just the axonal process. However, in some cases we did observe labeling of fibers contained within taste buds, suggesting that the nerve fibers themselves may be labeled as well. We did not undertake ultrastructural studies in order to resolve this issue.

Fig. 2.7. Immunolocalization β rENaC in sensory nerve fibers. Sections were double-labeled for β rENaC and β–tubulin type III in a section of fungiform papilla. Some nerve fibers were immunopositive for β rENaC and β-tubulin, showing yellow in the color composite. Some label for β rENaC appears to be associated with the myelin sheath (bottom panel). Similar results were also obtained with the double-labels of γ rENaC and β–tubulin in nerve fibers. Scale bars: 20 μm.

Apical accumulation of rENaC with high serum aldosterone

Recent studies suggest that increased serum aldosterone increases the abundance of rENaC subunits, especially the β and γ subunits (Asher et al., 1996, Lingueglia et al., 1994). We therefore investigated the effect of raising serum aldosterone on the distribution and prevalence of rENaC subunit immunolabeling in taste buds. In order to increase serum aldosterone levels, rats were treated for two weeks either by restriction to a low $Na^+$ diet, or aldosterone injection or both. The results are shown in Fig. 2.8. In general, these treatments substantially increased the immunolabeling for β and γ subunits in the apical region of the foliate and vallate taste buds (6 out of 9 treated animals). The α subunit immunoreactivity was less affected by the treatments, however, heavy labeling for this subunit was present in apical regions of some foliate taste buds (Fig. 2.8 D). In comparison, only occasional taste buds in untreated rats show somewhat stronger label at the apical (e.g., Fig. 2.2E, arrowhead) compared to the basolateral regions of the taste bud. Changes in distribution of rENaC subunit immunoreactivity were less pronounced but still noticeable in taste buds of fungiform papillae. Fungiform papillae in untreated rats often exhibit slightly stronger apical immunoreactivity for β and γ subunits. With the increase in blood aldosterone, β rENaC-ir in fungiform taste buds was more obvious at the apical region compared to untreated animals (c.f. Fig. 2.8B and 2.1B). In summary, increasing blood aldosterone levels enhanced the intensity of immunolabeling for β and γ subunits of rENaC in the apical region of taste buds.

Fig. 2.8. Effect of increased aldosterone on apical localization of rENaC subunits. Sections of fungiform, foliate and vallate papillae from low $Na^+$ diet and aldosterone pre-treated rats immunoreacted with antisera against subunits of rENaC. Heavy label for β and γ rENaC was observed in apical regions of taste buds. In some buds, the expression of β rENaC appeared so heavy at the apical membrane that the label in basolateral region seemed faint (E). The reactivity for α rENaC at the apical region of some foliate taste buds was also greatly enhanced (c.f. Figs. 1, 2). In general, the enhancement of apical reactivity in fungiform taste buds was less obvious than in foliate. A-C: fungiform; D-F: foliate; G-I: vallatetaste buds. A, D, G: α subunit; B, E, H: β subunits; C, F, I: γ subunit. Scale bar: 20 μm.



Whole cell recordings from taste cells of $Na^+$-deprived and control rats

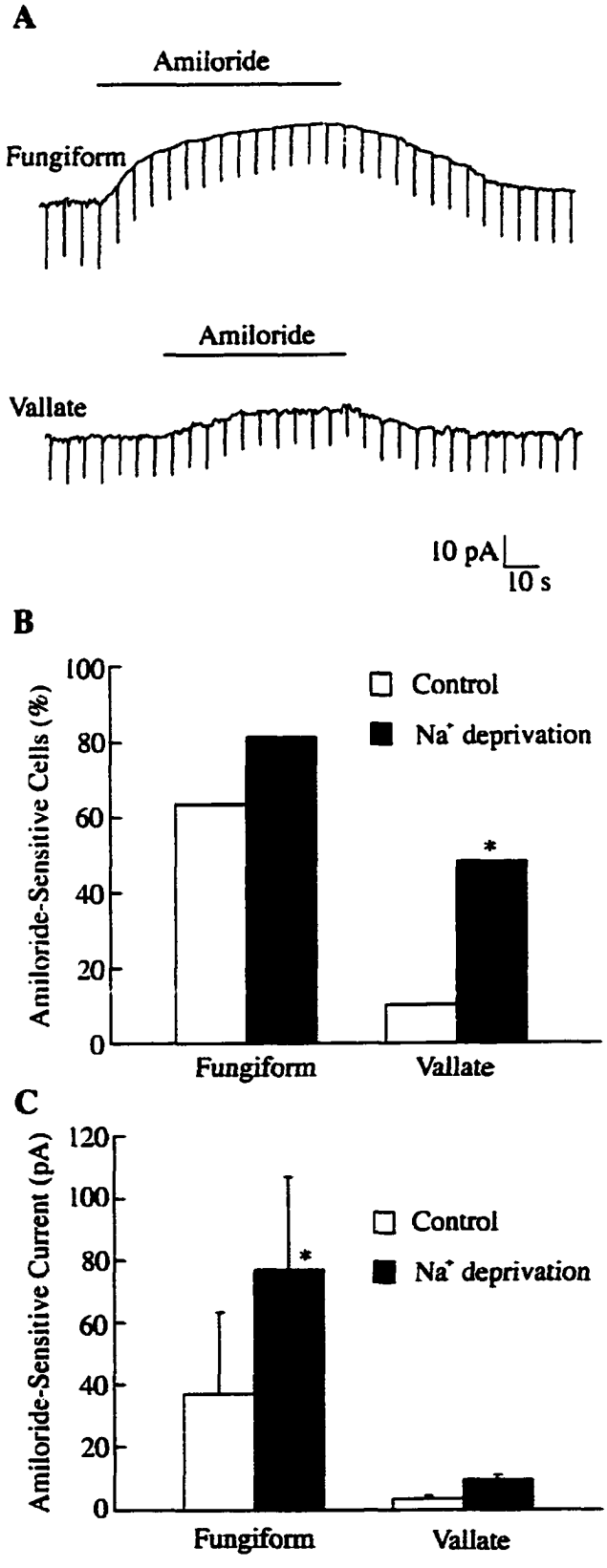
In order to test the functional correlates of the changed localization of β and γ rENaC immunoreactivity, we examined the amiloride-sensitivity of $Na^+$ currents in taste cells from control and aldosterone-treated rats. Previous studies of rats fed a normal $Na^+$ diet showed that approximately 65% of fungiform taste cells express functional amiloride-sensitive $Na^+$ currents, but vallate taste cells fail to respond to amiloride (Doolin and Gilbertson, 1996). We repeated these experiments on control rats to compare them to the diet-treated rats under the same recording conditions. Only two out of 20 vallate taste cells of control rats showed any amiloride-blockable current, and the current was <5 pA in each case. In contrast, 63.3% of the fungiform taste cells exhibited an amiloride-sensitive current (n= 30), and the current amplitude averaged 37 pA. Compared to control rats, the $Na^+$-deprived rats (n=5) showed an increase in both the amplitude of the amiloride-sensitive current and the percentage of cells exhibiting current, in both fungiform and vallate papillae (Fig. 2.9). The increase in the percentage of taste cells expressing current was most dramatic in the vallate papillae, where nearly half of the 25 taste cells tested from $Na^+$-deprived rats exhibited amiloride-sensitive current compared to only 10% (n = 20) exhibiting current in control rats (Fig. 2.9B; Chi-square Test: $\chi^2$=5.82, p<0.05). The amiloride-sensitive currents in vallate taste cells of $Na^+$-deprived rats were, however, small, averaging 10 pA compared to an average current of 77 pA in fungiform taste cells from the same animals (Figs. 2.9A, C). Sodium deprivation also increased the number of fungiform taste cells expressing amiloride-sensitive currents (81.2%, n=16; c.f. 63.3% in normal animals; Fig. 2.9B), and these currents were significantly larger in magnitude than control fungiform taste cells and vallate cells from the treated rats (Fig. 2.9C; Kruskal-Wallis Test and Dunn's Multiple

Fig. 2.9. Responses to amiloride in taste cells of vallate and fungiform papillae from rats treated with $Na^+$ deprivation and aldosterone.

A. Amiloride (30 μM) suppressed the steady-state holding current in cells from both fungiform and vallate papillae in whole-cell recordings (holding voltage = -80 mV).

B. The percentage of cells that responded to amiloride is increased in both vallate and fungiform papillae in treated rats. The increase is statistically significant in taste cells of vallate papillae (Chi-square Test: $\chi^2=5.82$, $p<0.05$). Asterisk indicates a significant difference.

C. The amplitude of the amiloride-sensitive current in fungiform taste cells of $Na^+$-deprived rats is significantly higher than that of control rats. Although there was also an increase in the amplitude of the current in the vallate taste cells of the $Na^+$-deprived rats, the increase was not analyzed statistically due to the small sample size of amiloride-sensitive cells in the control group. The vallate taste cells of the $Na^+$-deprived rats expressed amiloride-sensitive current, but the amplitude was significantly smaller than currents in fungiform taste cells. (Kruskal-Wallis Test followed by Dunn's Multiple Comparison: KW = 6.24, $P < 0.05$). Asterisk indicates significant difference.



Comparison: KW=6.24, $p<0.05$). Thus, raising serum aldosterone levels directly or by sodium-deprivation resulted in a larger percentage of taste cells expressing amiloride-sensitive $Na^+$ current and a larger magnitude of current, in both vallate and fungiform taste cells.

## Discussion

Our experiments show that all three rENaC subunits are present within many taste cells of taste buds in fungiform, foliate and vallate papilla, although the relative abundance of the subunits differs among the papillae. In addition, rENaC subunit immunoreactivity is present in non-sensory epithelial cells as well as being associated with some nerve fibers. Increases in serum aldosterone result in an increase in apical immunoreactivity for $\beta$ and $\gamma$ subunits, concomitant with an increase in the amiloride-sensitive $Na^+$ current in both vallate and fungiform taste cells.

Our results are consonant with previous immunocytochemical and molecular studies on the lingual epithelium. Li et. al. (1994) used in situ hybridization to show that the $\alpha$ subunit mRNA is present in vallate epithelium, including taste buds. More recently, Kretz et al.(1999) utilized both immunohistochemistry (with different antisera than used in our study) and semiquantitative PCR to investigate localization and abundance of rENaC subunits in lingual taste buds. These latter studies are generally consistent with the present findings in showing lesser expression of $\beta$- and $\gamma$ -subunits in foliate and vallate papillae. In contrast to the present results, these investigators were unable to detect $\gamma$-subunit immunoreactivity in vallate papilla although the mRNA was detectable with PCR.

The presence of all three rENaC subunits in taste buds of all papillae of rats fed a normal $Na^+$ diet is striking in that functional rENaC channels are present predominantly in fungiform taste buds in such animals (Doolin and Gilbertson, 1996). Consistent with these data, we find that β - and γ-subunits are most abundant in fungiform taste buds, lowest in vallate taste buds and intermediate in foliate buds (see also Kretz et al, 1999). However, these relatively subtle quantitative differences do not adequately explain the grossly different functional data. These results suggest that post-translational regulation of the subunits, either in terms of assembly or insertion into the appropriate membrane may be a key factor in regulating the presence or absence of functional channels. This hypothesis is strengthened by our results in aldosterone-treated animals in which we observe an increase in apical labeling of the three subunits, concomitant with an increase in function.

Effects of aldosterone

Numerous previous studies have demonstrated that aldosterone increases $Na^+$ transport in tight epithelia (for review, see Garty and Palmer 1997) as well as increasing the abundance of rENaC subunits (Asher et al., 1996; Lingueglia, 1994). In the taste system, aldosterone increases the amiloride-sensitivity of the gustatory neural response to NaCl in the anterior tongue of rats (Herness, 1992), but there are no published accounts of glossopharyngeal nerve responses to NaCl in aldosterone-treated rats. Our experiments show that elevated aldosterone levels result in increased immunoreactivity for β and γ subunits in apical regions of taste buds in all papillae, with the most striking effects in vallate and foliate papillae. Concomitant with the anatomical studies, we found that aldosterone treatment increased the amplitude of the amiloride-sensitive current in

taste cells of fungiform papillae and induced amiloride-sensitivity in taste cells of vallate papillae. Interestingly, the percentage of taste cells in vallate papillae expressing functional amiloride-sensitive currents after aldosterone treatment is similar to the percentage of taste cells exhibiting immunoreactivity for the three subunits of rENaC in untreated animals. These data strongly suggest that rENaC is responsible for functional amiloride-sensitive $Na^+$ currents in taste cells, but that in vallate taste cells of rats fed a normal $Na^+$ diet, it is not normally targeted to the apical membrane. Whether the aldosterone-induced apical immunoreactivity represents a redistribution of existing channel protein or a synthesis of new rENaC subunits was not determined. In other systems, however, aldosterone causes both activation of existing channels and synthesis of new rENaC subunits (Garty and Palmer 1997).

Still, examining only taste cells exhibiting functional currents, differences in amiloride-sensitivity remain between vallate and fungiform taste cells, even following aldosterone stimulation. Amiloride-sensitive $Na^+$ currents in vallate taste cells are much smaller than those in fungiform taste cells (e.g., Fig. 2.9). Alternative splicing or alternative post-translational processing of rENaC subunit transcripts may explain the differences between the immunocytochemical and functional data. Li et al. (1995) cloned alternatively spliced forms of α rENaC (α rENaC-a and α rENaC-b) from rat circumvallate papilla. These transcripts have short deletions that introduce stop codons within the extracellular loop resulting in a truncation of the COOH terminus. Despite this deletion, the truncated α rENaC-a subunit still binds phenamil, an amiloride analog (Li et al., 1995). However, unlike the normal α rENaC subunit, the truncated α rENaC-a subunit fails to generate amiloride-sensitive $Na^+$ and $Li^+$ current in Xenopus oocytes, indicating that the truncated αrENaC-a fails to form functional channels in the plasma

membrane. Since the COOH-terminus of the normal α subunit is critical for apical membrane targeting (Rotin et al., 1994), the splice variant form of the channel may remain in the Golgi apparatus or cytoplasm. Indeed, in some taste buds in the vallate papilla, immunoreactivity for the α subunit appears concentrated in a granular network surrounding the nucleus reminiscent of the distribution of the Golgi within taste cells (see Fig. 2.5D). Recently, however, Kretz et al. (1999) were unable to detect these splice variants in either vallate or fungiform tissues. Whether other splice variants may exist is unknown.

Gustducin and cell types

Taste buds contain several morphologically- and histochemically-different types of elongate taste cells (Kinnamon, 1987; Pumplin et al., 1997). The functional significance of these different cell types in mammals is not clear. We utilized dual localization techniques to test whether any rENaC subunits occurred within the same taste cells as gustducin, a taste cell specific G-protein. Gustducin occurs in a subset of so-called Type II cells that constitute about 25-50% of cells in lingual taste buds (Tabata et al., 1995; Boughter et al., 1997). In taste buds of all papillae, gustducin is expressed in a subset of the rENaC positive taste cells. Thus, rENaC immunoreactivity occurs in at least some Type II taste cells. In fungiform papillae, rENaC subunit immunoreactivity is present in virtually all the taste cells, including those exhibiting gustducin-immunoreactivity. Thus, rENaC immunoreactivity must occur in at least some Type I taste cells as well, since Type I taste cells constitute approximately half of the cells in the taste bud. Whether expression of a functional amiloride-sensitive $Na^+$ channel is correlated with a particular morphological cell type is not clear.

Gustducin knock-out mice are deficient in detection of sweet and bitter compounds (Wong et al., 1996), implicating this protein in their transduction cascade. Surprisingly, we find that all gustducin-immunoreactive taste cells also are immunoreactive for rENaC subunits, although rENaC-immunoreactivity is present in many taste cells that do not express gustducin. What the functional implications of this may be are enigmatic since gustducin does not appear to be a crucial element of the $Na^+$ transduction pathway (Wong et al., 1996). Further studies will be necessary to clarify the functional implications, if any, of the co-localization of gustducin and rENaC subunits.

Paracellular Sodium Transduction.

One of the proposed mechanisms for $Na^+$ transduction by taste buds involves sodium influx through the tight junctions at the apex of the taste buds resulting in increased extracellular $Na^+$ within the taste bud. Basolateral ion channels, including rENaC, could provide a pathway for extracellular $Na^+$ to depolarize taste cells, if $Na^+$ levels in interstitial spaces exceed the 145 mM levels normally there. Under normal dietary conditions, rENaC immunoreactivity is distributed throughout the taste cells, thus functional rENaC may be present on the basolateral membrane as suggested by others (DeSimone et al., 1995, Simon et al., 1993b; Stewart et al., 1995; Ye et al.,1993; Mierson et al., 1996). Functional experiments to test this have yielded equivocal results: Béhé et al. (1989) and Mierson et al. (1996) provide evidence for functional amiloride-sensitive $Na^+$ currents on basolateral membranes, but a recent study by Gilbertson and Zhang (1998b) failed to find any basolateral amiloride-sensitivity. Further studies will be required to resolve this issue.

The presence of rENaC in cells other than taste cells may have important functional implications. In agreement with the results of Li et al. (1994) and Kretz et al. (1999), we observed the immunoreactivity for three subunits of rENaC in non-sensory epithelial cells, especially those situated in the anterior part of the tongue. These data, and the functional data of Simon et al. (1993a), suggest that $Na^+$ ions could pass through these rENaCs to increase the $Na^+$ concentration in extracellular space, thereby directly depolarizing intragemmal and perigemmal nerve fibers. Lingual trigeminal nerve fibers, which do not receive input from taste cells, do in fact respond to moderate levels of NaCl applied to the tongue (Kawamura et al., 1968; Simon et al., 1993b). Interestingly, we observed that β and γ subunits are expressed in association with some nerve fibers in and subjacent to taste buds. We also occasionally observe immunoreactivity for α subunit in these fibers, but the intensity of immunoreactivity is much less than for β and γ subunits. Although the label often appears associated with myelin, we also observe immunolabel in fibers that penetrate taste buds, suggesting that the nerve fibers themselves may express rENaC protein. Even though little α subunit is present, the β- and γ-subunits may participate in functional amiloride-sensitive $Na^+$ channels in combination with brain homologs (δ-NaCh and BNC-1) of the peripheral rENaC (Waldmann et al., 1995; Price et al., 1996). Indeed, BNC-1 is co-expressed with β and γ-rENaC in nodose ganglia (Drummond et al., 1997). Whether the β and γ-rENaC participate in functional channels in ganglion cells providing sensory innervation to the gustatory epithelium is unclear.

# Chapter 3

# TRANSDUCTION MECHANISMS FOR GLUTAMATE IN TASTE CELLS OF RAT FUNGIFORM PAPILLAE


## Abstract

Monosodium glutamate elicits a unique taste in humans called umami. Using the whole-cell patch clamp technique, we examined the effects of glutamate and glutamate receptor agonists on membrane properties of single taste cells in isolated taste buds from rat fungiform papillae. Glutamate induced responses in 58 % of the total cells tested at a holding potential of -80 mV. Responses were classified into three types: a decrease in holding current (Type I), an increase in holding current (Type II), and a biphasic response consisting of an increase followed by a decrease in holding current (Type III). The Type I response and decreased holding current of the Type III response were abolished by inclusion of GDP-β-S in the pipet, suggesting that this response was G protein-mediated. These data suggest that taste cells express multiple types of glutamate receptors.

The glutamate receptor ionotropic agonist NMDA mimicked the Type II glutamate response in 45 % of taste cells tested. The current was potentiated by the co-agonist glycine and was suppressed by the NMDA receptor antagonist AP5. Replacement of extracellular $Na^+$ with the non-permeant cation NMDG decreased the

current amplitude, which in turn was partially recovered by substitution of $Ba^{2+}$ for NMDG. These data suggest that primarily $Na^{+}$ carries the NMDA-activated current, but that the channel is also permeable to divalent cations. In current-clamp mode, NMDA depolarized cells and increased the frequency of spontaneous action potentials.

Bath application of the mGluR4 agonist L-AP4 induced two different types of responses. In 32% of the cells tested, L-AP4 decreased membrane holding current and conductance, mimicking a Type I glutamate response. The response to L-AP4 was suppressed by the metabotropic antagonists MPPG and CPPG and by 8-bromo-cAMP, suggesting a role for cAMP in the transduction pathway. The L-AP4-induced response reversed near the $Cl^{-}$ equilibrium, suggesting that a $Cl^{-}$ conductance may be involved. Under current-clamp conditions, membrane hyperpolarization and a decrease in the firing rate of spontaneous action potentials accompanied this type of response in some sells. These data are consistent with previous studies suggesting that mGluR4 is expressed in taste cells (Chaudhari et al., 1996; Bigiani et al., 1997). In 17% of taste cells tested, L-AP4 elicited an increase in holding current, which resulted in taste cell depolarization under current-clamp conditions. Replacement of extracellular $Na^{+}$ with NMDG eliminated the current. Whether this response was mediated by mGluR4 or another receptor is not known.

Taken together, our results suggest that both NMDA and mGluR4 receptors are present in taste receptor cells of fungiform papillae. Further studies will be required to determine their precise role in the transduction of MSG.

# Introduction

Monosodium glutamate (MSG) is a natural component of many foods, including seafood, meats, milk and their by-products, mushrooms and some vegetables. For many years, both naturally occurring and synthetic MSG has been used as a flavor enhancer to increase food palatability. The taste induced by MSG is called "umami", a Japanese term meaning delicious or savory (Ikeda, 1909). It is believed that the appetitive taste of MSG and other amino acids reflects the requirement of protein in the diet of most animals.

A number of studies have characterized the properties of umami taste (for review, see Chapter 1). As a potent taste stimulus, MSG alters the activity of afferent nerve fibers and neurons in central gustatory pathways (Adachi and Aoyama, 1991; Hellekant and Ninomiya, 1991; Ninomiya et al., 1991; Oomura et al., 1991; Plata-Salaman et al., 1992; Nakamura and Norgren, 1993; Hellekant et al., 1997). In general, it is believed that glutamate is the primary stimulus for the umami taste (Yamaguchi and Kimizuka, 1979; Schiffman and Gill, 1987; Yamaguchi, 1987; Ninomiya and Funakoshi, 1989; Yamaguchi, 1991).

Glutamate is a major excitatory neurotransmitter in the central nervous system. There are two main glutamate receptor families that mediate many important neural processes. The ionotropic glutamate receptors (iGluRs) belong to the family of ligand-gated ion channel receptors and can be further subdivided into NMDA, AMPA and Kainate receptors. The metabotropic glutamate receptors (mGluRs) belong to the family of G-protein coupled receptors. The mGluRs are subdivided into three groups based on

their second messenger pathways, pharmacology and sequence homology. Group I (mGluR1 and 5) is coupled to the $IP_3$ pathway. Both group II (mGluR2 and 3) and group III (mGluR4 to 8) are negatively coupled to cAMP pathways (for review, Hollmann and Heinemann, 1994; Nakanishi and Masu, 1994; Pin and Duvoisin, 1995).

Interestingly, other ligands of brain glutamate receptors, including aspartate, ibotenate, and homocysteinate, are also potent taste stimuli. These compounds induce responses in the chorda tympani nerve (Faurion, 1991) and elicit an umami taste in humans (for review, see Maga, 1983). These data suggest that excitatory amino acid receptors in the brain may be expressed in taste cells and may participate in transducing umami taste. In fact, there is evidence for the presence of both iGluRs and mGluRs in taste receptor cells. The activity of ion channels directly gated by glutamate and NMDA was recorded from lipid bilayers into which epithelial membranes from vallate and foliate taste regions were incorporated (Brand et al., 1991; Teeter et al., 1992). Recently a metabotropic glutamate receptor, mGluR4 was cloned from lingual epithelium of rat foliate and vallate papillae, and behavioral and electrophysiological studies demonstrated that the agonist of mGluR4, L-AP4, mimicked glutamate responses (Chaudhari et al., 1996; Bigiani et al., 1997). In addition, the glutamate receptor agonists NMDA and L-AP4 affect intracellular $Ca^{2+}$ levels in mice vallate and foliate taste cells (Hayashi et al., 1996).

Despite these studies, the precise role of glutamate receptors in the transduction of umami taste stimuli is still unclear. Moreover, there are no published reports about transduction of MSG in taste cells of fungiform papillae, which are believed to be

primarily involved in the initial identification of appetitive stimuli (Gilbertson and Kinnamon, 1996).

This study focuses on glutamate transduction in taste cells of rat fungiform papillae. Using the whole-cell patch-clamp technique and pharmacological agents, we examined the following questions: (1) Are both NMDA and mGluR4 receptors present in taste cells of fungiform papillae? (2) What intracellular signaling pathways are involved? (3) Which ions are involved in responses to NMDA and L-AP4? Preliminary accounts of this work have been published in abstract form (Lin et al., 1996; Lin and Kinnamon, 1996).

## Methods

### Isolation of taste buds

Four to 12 week old male Sprague-Dawley rats were used. All experiments were performed on taste receptor cells of freshly isolated taste buds from fungiform papillae. The isolation method was adapted from Béhé et al., (1990). Briefly, rats were sacrificed with $CO_2$ and the tongue was dissected and placed into cold Tyrode's solution. Approximately 0.3 to 0.8 ml of an enzyme mixture containing 3 mg dispase, 0.7 mg collagenase B(Boehringer Mannheim, Indianapolis, IN) and 1 mg trypsin inhibitor (type I-S; Sigma Chemical Corp., St. Louis, MO) in 1.0 ml of Tyrode's was injected between the lingual epithelium and the muscle layers of the tongue. The tongue was then incubated in $Ca^{2+}$ and $Mg^{2+}$-free oxygenated Tyrode's for 30 min. After incubation, the lingual epithelium was peeled and pinned serosal side up in a Sylgard-covered petri dish.

Taste buds were removed by gentle suction with a glass pipette and plated onto Cell-Tak (Collaborative Research, Bedford, MA) coated glass slide chambers. The chambers were formed by affixing a Sylgard ring (2 mm wall thickness with an opening diameter of 1.5 cm) to the Cell-Tak-coated slide.

Solutions and chemicals

Normal Tyrode's was used as a standard bath solution, containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (HEPES), 10 mM glucose and 10 mM sodium pyruvate (pH 7.4 with NaOH). The $Ca^{2+}$-and $Mg^{2+}$-free Tyrode's for isolating taste buds contained 2 mM BAPTA (Molecular Probes, Eugene, OR). The 70 mM $Na^+$ or $Na^+$-free Tyrode's was obtained by replacing $Na^+$ with equimolar N-methyl-D-glutamine (NMDG). The pH was adjusted to 7.4 with HCl. For $Ba^{2+}$-Tyrode's, NaCl was replaced by 100 mM $BaCl_2$ and 50 mM NMDG. Low $Cl^-$ Tyrode's contained 140 mM $Na^+$ gluconate instead of NaCl. Bath solutions were gravity-fed into the 0.5 ml recording chamber. The standard intracellular pipet solution contained 140 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES (pH 7.2 with KOH), 11 mM EGTA, 1 mM ATP, and 0.4 mM GTP (pH 7.2 with KOH). Low $Cl^-$ pipette solution contained 130 mM K gluconate and 10 mM KCl in place of 140 mM KCl.

Most of the chemicals used were bath applied except GDP-β–S, which was included in the pipet solution. Monosodium glutamate, guanosine 5'-o-(2)-thiodi-phosphate, (trilithium salt, GDP-β–S) and 8-bromo adenosine 3'5'-cyclic monophosphate (8-bromo-cAMP) were from Sigma Chemical Corp. The agonists and antagonists of

glutamate receptors were obtained from Tocris Cookson (Ballwin, MO); these included N-methyl-D-aspartic acid (NMDA), MK-801, D (-)-2-amino-5-phosphonopentanoic acid (AP5), L-2-amino-4-phosphonobutyric acid (L-AP4), (R, S)-a-methyl-4-phosphonophenylglycine (MPPG), (RS)-α-cyclopropyl-4-phosphonophenylglycine (CPPG), (s)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (APMA).

Patch-clamp recordings

The whole-cell patch clamp technique was used (Hamill et al., 1981). The steady-state holding current was recorded at –80 mV, except as noted. In some experiments, changes in membrane potential were monitored under current-clamp conditions. The glass pipettes for recording were pulled from microhematocrit capillary tubes (Scientific Products, McGaw Park, IL) with a two-stage vertical puller (model PB-7; Narishige, Tokyo, Japan). Pipette resistance was 3 to 6 MΩ when filled with normal pipette solution and 4 to 8 MΩ when filled with the low $Cl^-$ pipette solution. Membrane currents were lowpass filtered at 2 KHz and recorded with an Axopatch patch-clamp amplifier (Model 200B, Axon Instruments, Foster City, CA). Voltage-activated $Na^+$ and $K^+$ currents were generated by applying depolarizing voltage steps from a holding potential of –80 mV; these were used to distinguish taste cells from non-sensory epithelial cells. Hyperpolarizing voltage pulses (20 mV) were used to monitor membrane conductance during whole-cell recording. All voltage commands were generated by an Indec laboratory computer system (Sunnyvale, CA).

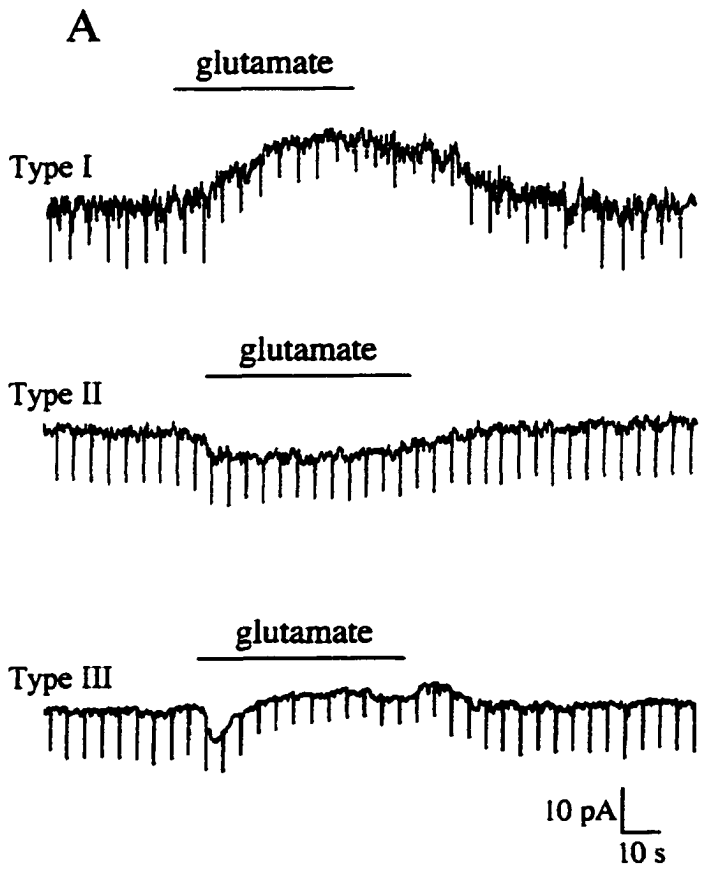
## Results

### Responses to glutamate

Taste cells from isolated taste buds were voltage-clamped at -80 mV and holding current and membrane conductance were monitored in response to bath application of MSG (1 mM). Responses were arbitrarily grouped into three types: a decrease in holding current and membrane conductance (Type I), an increase in holding current (Type II), and a biphasic response, i.e. an increase, followed by a decrease in holding current (Type III). A total of 108 cells out of the 185 tested responded to MSG. Of these, 40 cells exhibited Type I responses, 38 cells Type II responses, and 30 cells Type III responses (Fig 3.1). In general, it took more time for a Type I response to reach peak amplitude than a Type II response. The time taken to reach half of the response amplitude ($T_{1/2}$ value) for Type I and II responses are 22.5 ± 1.1 and 2.7 ± 0.5 seconds respectively ($n=16$, $p<0.001$). These responses were considered to be induced primarily by glutamate, since the addition of 1mM MSG has a negligible effect on bath $Na^+$ concentration. These results are consistent with a previous study of vallate taste cells that showed both increases and decreases of holding current in response to MSG (Bigiani et al., 1996).

The mGluRs in brain are coupled to G-protein-mediated intracellular pathways; activation of receptors in mGluR group III inhibits the activity of adenylate cyclases and decrease the intracellular cAMP level (Pin and Duvoisin, 1995; Fig. 3.2). To determine if both iGluRs and mGluRs are expressed in fungiform taste cells, we added a non-hydrolyzable GDP analog, GDP-β-S (0.2 mM) to the recording pipet to inhibit G protein-mediated pathways. With GDP-β-S in the pipette solution, Type I responses were

Fig. 3.1: Responses to glutamate. A. Bath application of 1 mM glutamate induced three different types responses: a decrease in holding current and membrane conductance (top trace, Type I); an increase in holding current (middle trace, Type II) and a biphasic response with an increase followed by a decrease in holding current (bottom trace, Type III). B. Number and percentage of taste cells exhibiting each type of response.



abolished after 15-30 min. of whole cell recording (n=4). In addition, Type III responses were converted to type II responses (n=4; Fig. 3.3A), while Type II responses remained unchanged (n=9). We next examined if intracellular cAMP pathways are involved in responses to glutamate. Bath application of a membrane permeable cAMP analog, 8-bromo-cAMP (1 mM) suppressed Type I responses (7 out of 10 cells; Fig. 3.3B) and the outward component of the Type III response (n=3), while having no significant effect on Type II responses (n=6). The results indicate that both iGluRs and mGluRs may be present in fungiform taste cells; and Type I responses may be coupled to G protein-mediated intracellular cAMP pathways. Based on the results above, we utilized specific glutamate receptor agonists and antagonists in further experiments to identify possible subtypes of glutamate receptors in taste cells.

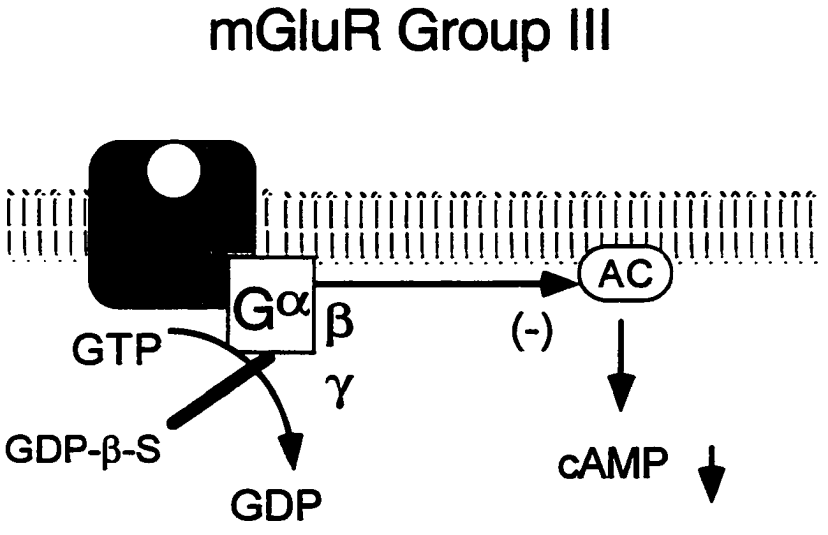
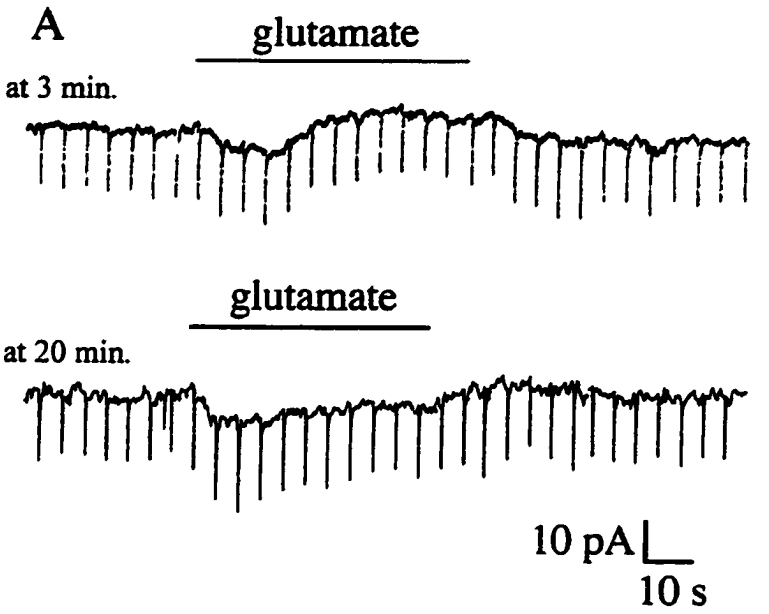
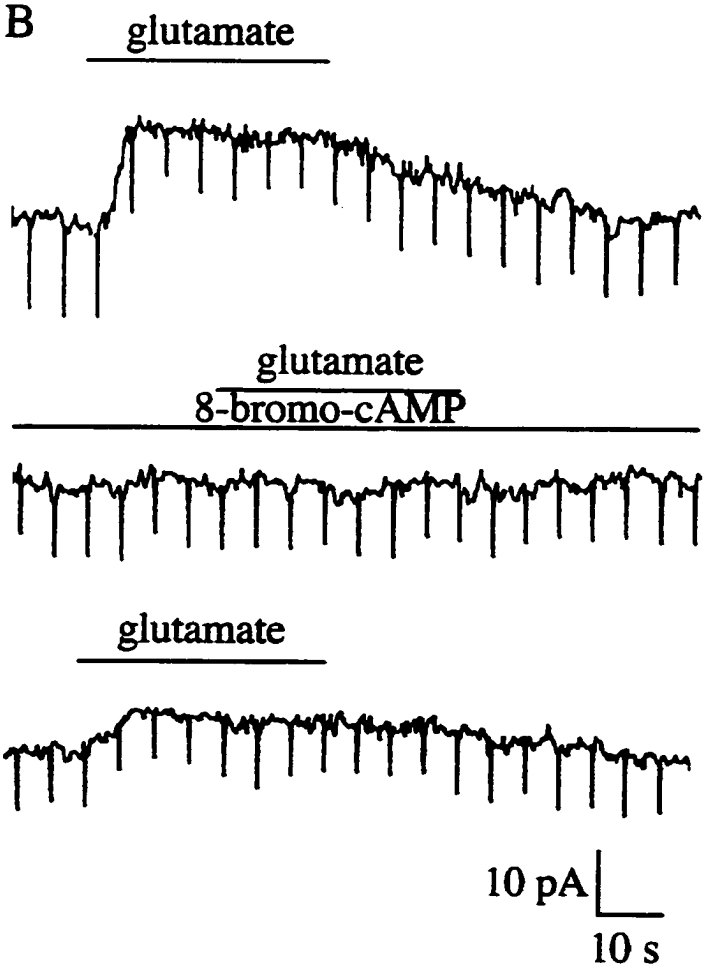



Fig. 3.2. A schematic model of the signaling pathway mediated by glutamate receptors in mGluR Group III. Activation of these receptors inhibits adenylate cyclases, resulting in a decrease in the intracellular cAMP level via G proteins. GDP-β-S blocks the pathway by inhibiting the activation of G proteins.

Fig. 3.3. The Type I response to glutamate is mediated by G protein-coupled intracellular cAMP pathways. A. A biphasic response (top trace) is converted to a Type II response (lower trace) by intracellular perfusion with GDP-β-S. Note that the GDP-β-S suppressed the outward component (Type I) of the biphasic response with time. B. The Type I response is suppressed by bath application of 1 mM 8-bromo-cAMP.



Responses to NMDA

Properties of the brain NMDA receptors are shown in Fig. 3.4. Both NMDA and glycine are required for full activation of the receptor channel. Extracellular $Mg^{2+}$ blocks the channel at negative membrane potentials, resulting in voltage-dependent activation of the channel. The channel is permeable to $Na^{+}$, $Ca^{2+}$ and $K^{+}$(Mayer et al., 1984; Nowak et al., 1984; Johnson and Ascher, 1987; Watkins, 1994). We examined whether NMDA receptors are present in fungiform taste cells and if the receptor is similar to those in brain. Bath application of NMDA (1 mM) alone increased holding current in about 45% of cells tested when cells were voltage-clamped at -80 mV (n=111). The response generally mimicked Type II responses to glutamate. In the presence of 10 μM glycine, the amplitude of NMDA sensitive-currents increased from 5.2 ± 1.1 pA to 7.7 ± 1.0 pA (n=10, Fig. 3.5A). The above experiments suggest that glycine is a co-factor for the taste cell NMDA receptor; thus it was added to all solutions which contained NMDA. Glycine (10 μM) applied alone usually did not change holding current.

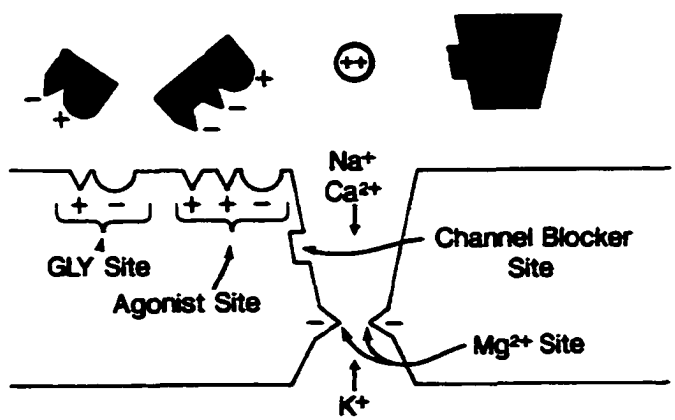
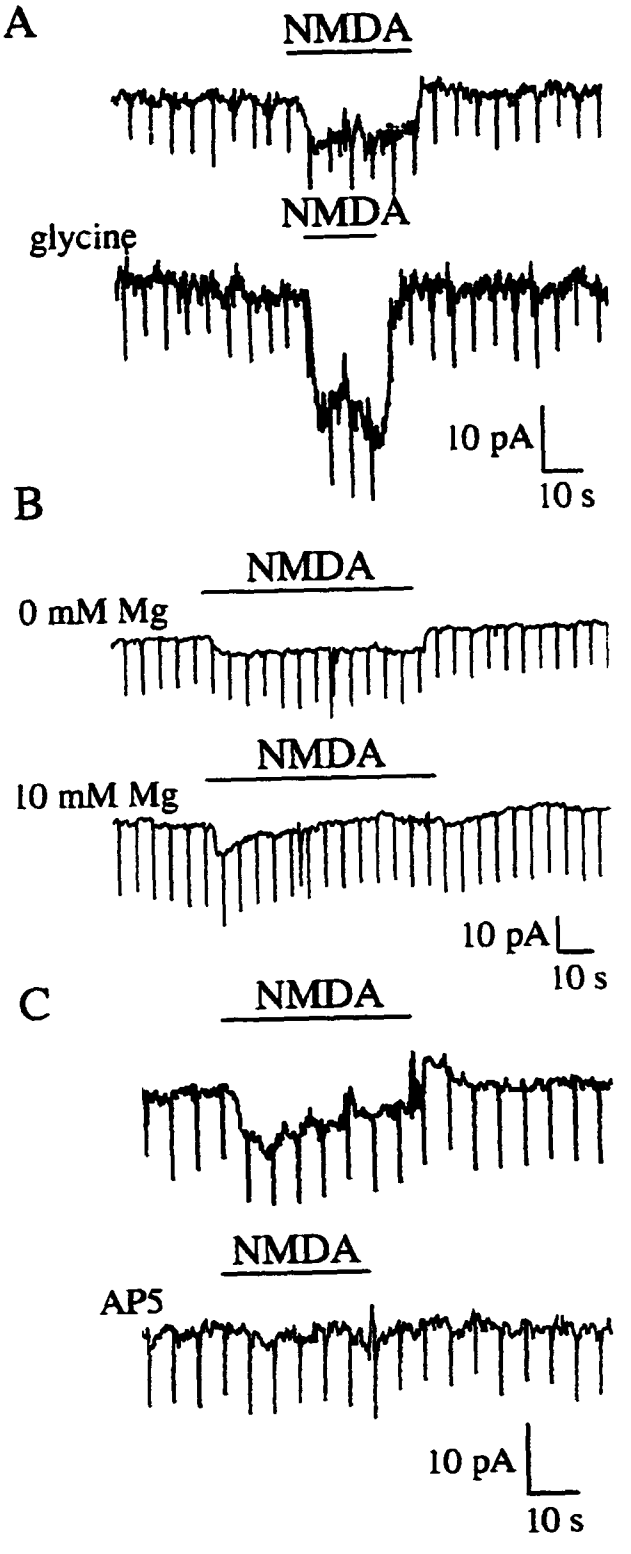



Fig. 3.4. Schematic representation of the NMDA receptor complex. The symbols above the receptor represent (from left): glycine-; glutamate- or NMDA; $Mg^{2+}$; and PCP-like agents (Watkins, 1994).

Fig. 3.5. A. The presence of glycine (1 μM) in the bath potentiates the response to NMDA. B. The response to NMDA is desensitized more slowly in $Mg^{2+}$-free bath solution than in 10 mM $Mg^{2+}$. C. The antagonist AP5 suppresses the responses to NMDA. Glycine was present in both bath and NMDA solutions in B and C.



Next, we tested blockage by extracellular $Mg^{2+}$. Since membrane depolarization removes the $Mg^{2+}$ block, we examined the NMDA-sensitive current at two holding potentials: -80 and -40 mV. Only four out of ten cells tested showed greater current amplitude at -40 mV than at -80 mV, while in most cells, the peak currents induced at -80 mV were bigger than those at -40 mV (8.6 ± 0.8 pA and 5.9 ± 0.6 pA respectively, n=7 $t<0.05$). Similar results were obtained from experiments conducted in extracellular $Mg^{2+}$-free solution, in which only three out nine cells showed an increase in the peak current. However, when cells were bathed in $Mg^{2+}$ free solution, the NMDA-induced current appeared to desensitize slower in six out nine cells tested. In contrast, when cells were bathed in 10 mM $Mg^{2+}$ solution, the current desensitized much faster, though the peak current did not decrease (n=3). These data suggested that extracellular $Mg^{2+}$ normally does not block the receptor channel, but once it opens, $Mg^{2+}$ can partially occlude the channel (Fig. 3.5B). These results are consistent with the effect of MK-801, an open channel blocker. MK-801 blocked the current partially (data not shown), while 50 μM AP5, a specific antagonist of the channel, suppressed most of the current (Fig 3.5C, n=4). In addition, we tested the effect of cAMP on the NMDA induced current. Similar to Type II responses to glutamate, 8-bromo-cAMP did not suppress the NMDA-sensitive current (n=3, data not shown).

In order to determine the reversal potential of the NMDA-sensitive current, we recorded the current at different holding potentials (Fig. 3.6A). Unlike the NMDA-sensitive current in the brain, the current in taste cells did not reverse at 0 mV, but reversed at potentials considerably more positive than 0 mV (n=7). Due to the instability of recording at positive potentials, we could not obtain the actual reversal potential for

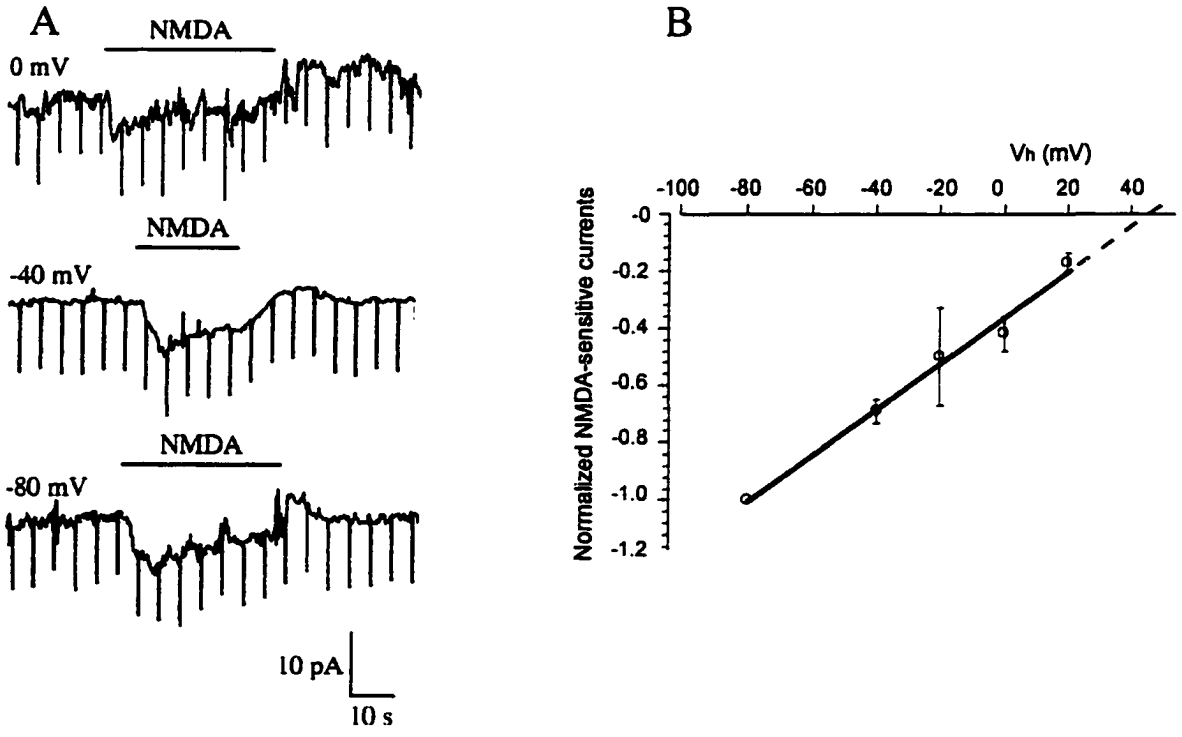



Fig. 3.6. The NMDA-sensitive current reverses at positive potentials. A. NMDA responses at different holding potentials on the presence of 10 μm glycine. B. The current-voltage relationships of NMDA responses. NMDA-sensitive currents were normalized to those elicited at –80 mV. Each point represents average (mean ± sem) of two to seven cells. The extrapolated reversal potential is about 46mV.

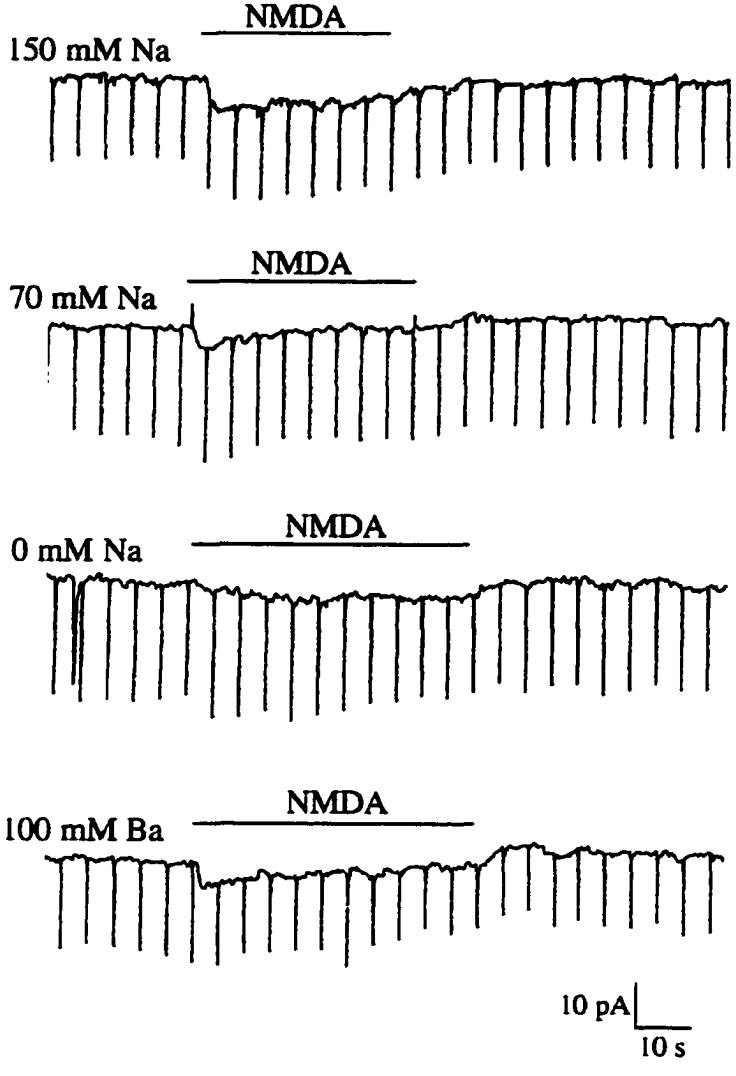



Fig. 3.7. Ionic selectivity of the NMDA-induced current in taste cells. Lowering the $Na^+$ concentration in the bath reduces responses to NMDA; the responses are partially recoverable by replacing the NMDG with 100 mM $Ba^{2+}$, which suggests that the receptors are permeant to both $Na^+$ and divalent cations.

the NMDA-sensitive current. Our results, however, suggest that the reversal potential is near the equilibrium potentials of $Na^+$ and $Ca^{2+}$. To further test the involvement of $Na^+$ and $Ca^{2+}$, we replaced bath $Na^+$ with the impermeant cation NMDG. The peak current induced by NMDA was reduced in 70 mM $Na^+$ solution (from 6.42 ± 2.1 pA to 3.6 ±1.1 pA; n=3). However, the current was not eliminated, even in $Na^+$ free solution. When 100 mM NMDG was replaced with 100 mM $Ba^{2+}$ in the bath solution, the current was partially recovered (Fig 3.7), suggesting that the NMDA-sensitive current is carried by both $Na^+$ and divalent cations.

Finally, we examined the effect of NMDA on membrane potentials with the current-clamp configuration. When cells were clamped with the standard pipette solution containing 140 mM KCl and bathed in the normal Tyrode's solution, resting membrane potentials ranged from -20 mV to -70 mV. Only a few cells had resting potentials more negative than -50 mV, and some of these fired action potentials spontaneously. Spontaneously active cells usually had relatively large voltage-gated $Na^+$ currents (peak current ~ 2000 pA) and small voltage-gated $K^+$ currents. NMDA depolarized these cells and increased the frequency of action potentials (Fig. 3.8A, n=3). In cells that were not spontaneously active at rest, NMDA caused membrane depolarization, but action potentials were not elicited (Fig. 3.8B; n=6). In the particular cell shown in Fig. 3.8B, replacing normal bath solution to a $Mg^{2+}$ free Tyrode's dramatically increased responses to NMDA. Depolarizations induced by NMDA were often followed by a small hyperpolarization during wash out of NMDA. Similar wash effects were often observed with Type II and Type III glutamate responses under voltage-clamp.

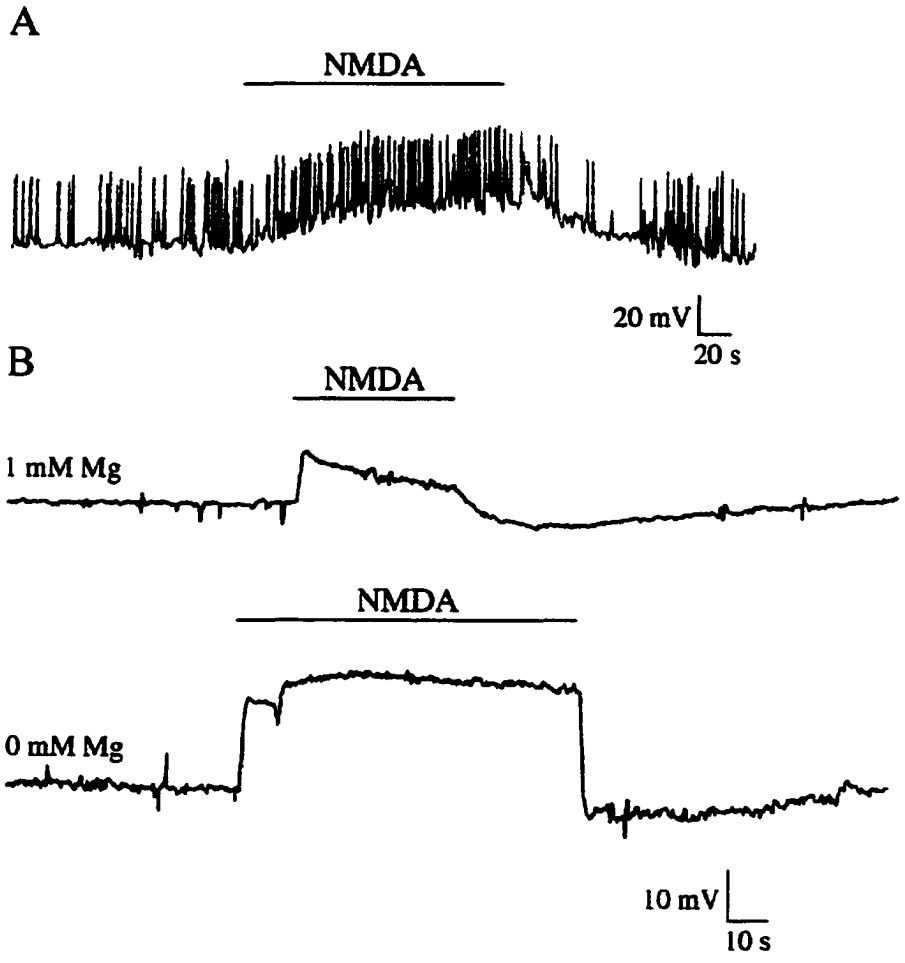



Fig. 3.8. A. In current-clamp configuration, bath application of NMDA results in membrane depolarization and increased frequency of spontaneous action potentials in a few cells. B. NMDA depolarizes a taste cell that does not fire action potentials spontaneously. In this particular cell, the response is much larger in $Mg^{2+}$ free solution (the resting potential: -65 mV).

Responses to L-AP4

L-AP4 is a specific agonist for the metabotropic group III receptors (for review, see Thomsen 1997). Previous studies showed that mGluR4 is expressed in vallate taste cells and L-AP4 mimics responses to glutamate both physiologically (Bigiani et al., 1997) and behaviorally (Chaudhari et al., 1996). Thus, mGluR4 also may be expressed in taste cells of fungiform papillae. Bath application of L-AP4 (10 to 20 μM) decreased the holding current in 30 out of 93 cells tested, mimicking the type I response to glutamate. The presence of 8-bromo-cAMP suppressed the response (Fig. 3.9A). In addition, concomitant with this type of response, L-AP4 induced either a hyperpolarization or had no effect on membrane potentials for most cells tested in current-clamp mode. In a few cells, L-AP4 decreased the firing rate of spontaneous action potentials. The mGluR4 antagonists MPPG 500 μM and CPPG 100 μM suppressed responses to L-AP4 (n=4 and 3 respectively; Fig. 3.9B). To determine the reversal potential of this L-AP4 sensitive current, we recorded the responses at different holding potentials. When the pipet solution contained 140 mM KCl, the current reversed about 0 mV (n=2, data not shown). However, when the pipette solution contained 130 mM K gluconate and 10 mM KCl the current then reversed at about -40 mV, a potential near the equilibrium potential of $Cl^-$ (Fig. 3.10), suggesting that L-AP4 may suppress a $Cl^-$ conductance. Further experiments are required to address the issue thoroughly.

In general, the results above are consistent with previous studies on effects of L-AP4 in vallate taste cells (Bigiani et al., 1997). However, we recorded an *increase* in holding current in response to L-AP4 in 16 out of 93 cells (Fig. 3.11A). Four of these cells responded to L-AP4 but not to glutamate. Unlike the effect of L-AP4 described

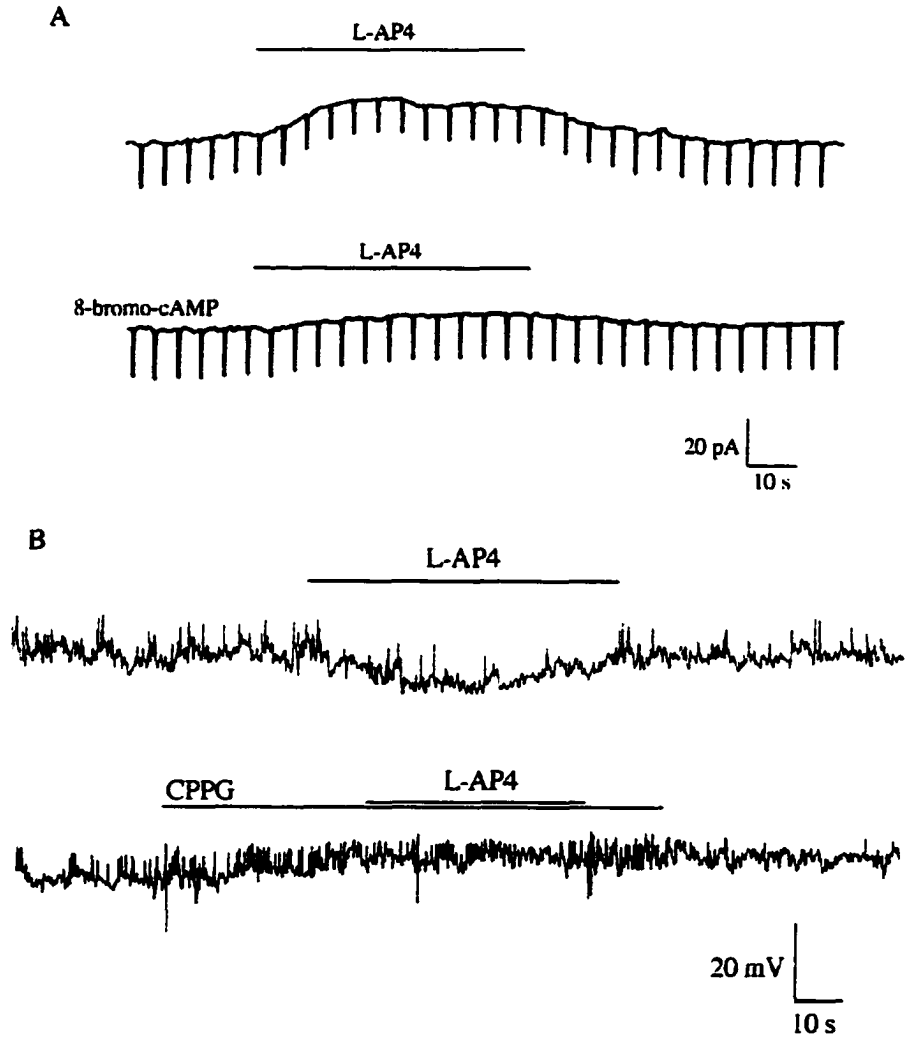



Fig. 3.9. Most responses to L-AP4 mimic the type I response to glutamate. A. The decrease in holding current is suppressed by 8-bromo-cAMP. B. In current-clamp mode, L-AP4 hyperpolarizes the cells, and the metabotropic antagonist CPPG suppresses the effect (the resting potential: -67 mV).

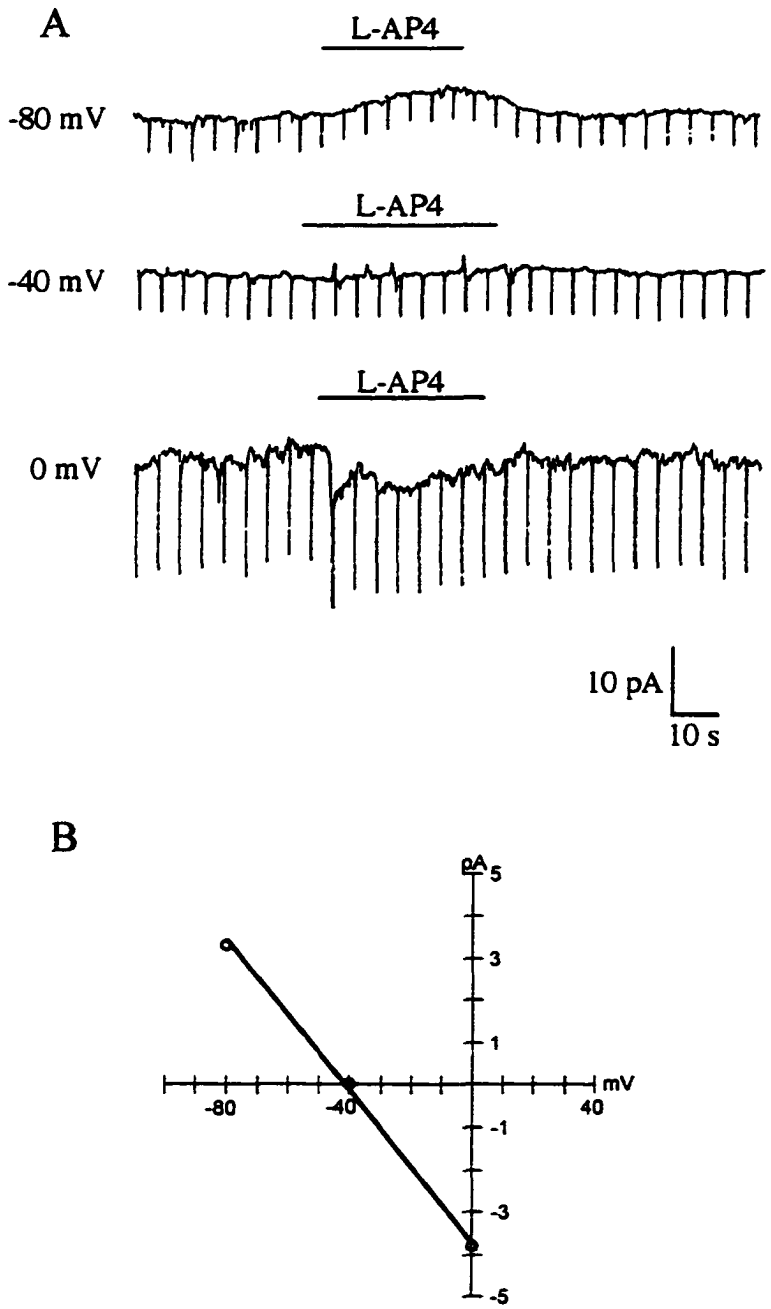



Fig. 3.10. A. The L-AP4-sensitive current reverses about -40 mV, near the $Cl^-$ equilibrium potential, when the pipet is filled with 130 mM K gluconate and 10 mM KCl. B. The I-V plot of the L-AP4-sensitive currents shown in A.

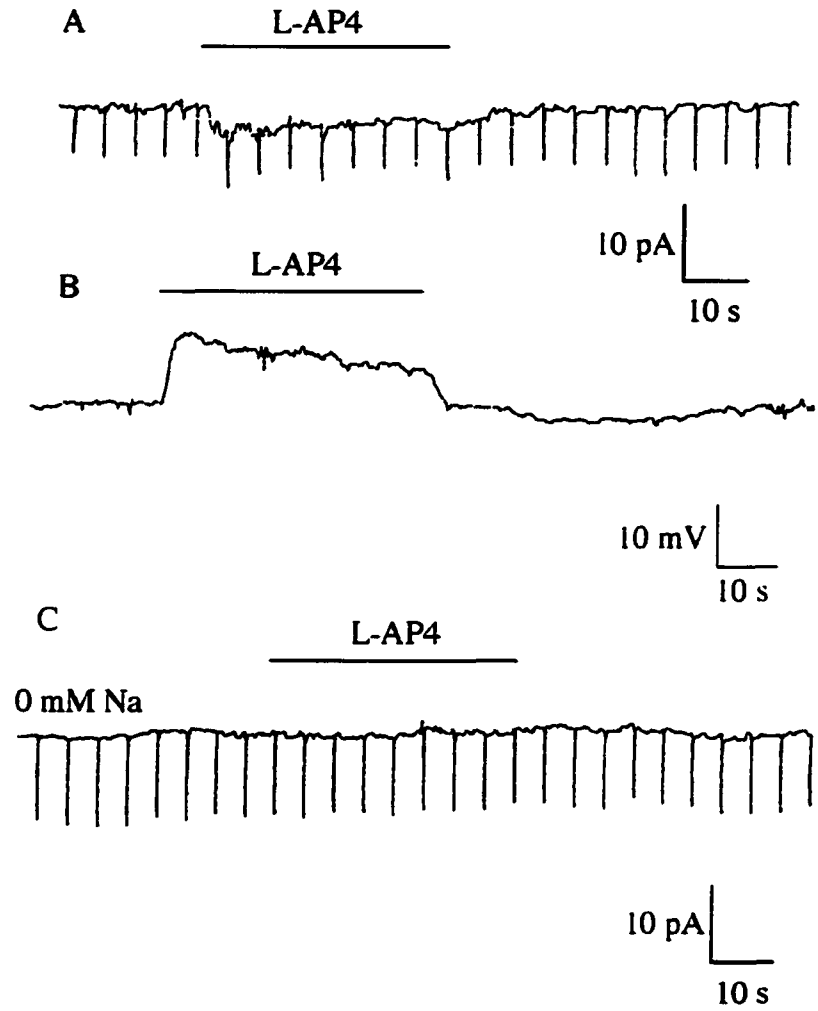



Fig. 3.9. In some cells, L-AP4 induces an increase in holding current and membrane depolarization. A. Voltage-clamp recording in response to 0.1 mM L-AP4. B. Current-clamp recording in response to 1 mM L-AP4. C. The response is eliminated by lowering the extracellular $Na^+$ concentration; same cell as A.

above, the amplitude of this response increased with increasing concentrations of L-AP4 in the bath. These data are consistent with the dual effects of L-AP4 reported previously in vallate taste cells. Although most L-AP4-sensitive taste cells showed a decrease in intracellular $Ca^{2+}$, a few cells showed increases in intracellular $Ca^{2+}$ in response to L-AP4 (Hayashi, et al., 1996). The inward currents elicited by L-AP4 were not significantly suppressed by the metabotropic antagonist CPPG (100 μM; n=2). In addition, this type of L-AP4 response usually was accompanied by membrane depolarization (n=3, Fig. 3.9B), and replacement of extracellular $Na^+$ with NMDG eliminated the response (Fig. 3.9C). Further experiments will be required to examine this effect of L-AP4 thoroughly.

In order to determine if both mGluR4 and the NMDA receptor channel are located in single taste cells, we applied glutamate, L-AP4 and NMDA to the same taste cells respectively. Data were pooled from all types of glutamate responses and are shown in Figure 3.12. In a total of 32 cells that responded to glutamate, 11 cells (34%) responded to both L-AP4 and NMDA, eight cells (25%) responded to NMDA only, nine cells (28%) responded to L-AP4 only, and four cells (13%) responded to neither NMDA nor L-AP4. The lack of response to both NMDA and L-AP4 suggests that other types of glutamate receptors may be expressed in taste cells. Therefore, we also tested some taste cells for responses to AMPA, a specific agonist of the AMPA receptor. Bath application of AMPA (100 μM) induced responses similar to the Type II response of glutamate, i.e., an increase in holding current in a small subset of cells. Further study is needed to examine the role of AMPA receptors in taste transduction.

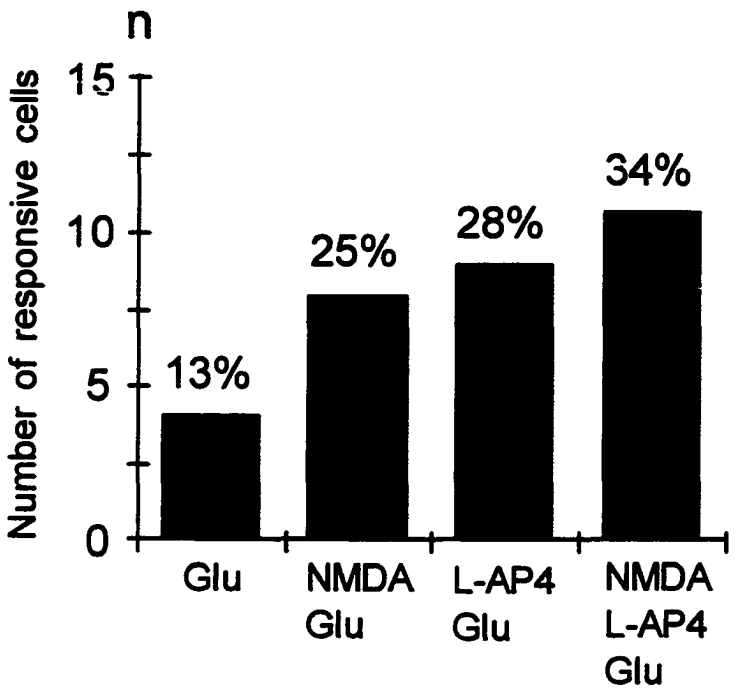



Fig. 3.12. Number and percentage of cells that respond to NMDA and L-AP4 in these glutamate (Glu)-sensitive cells.

## Discussion

This study focused on the transduction mechanisms of glutamate in isolated taste cells of fungiform papillae. Using the whole-cell patch-clamp technique, we examined the effects of glutamate and the glutamate receptor agonists, NMDA and L-AP4 on membrane properties, ion conductance and intracellular signaling pathways. We provided the first electrophysiological evidence for the presence of both NMDA receptor channels and mGluR4 in fungiform taste cells, suggesting that these receptors may both participate in the transduction of glutamate taste.

Responses to glutamate

In our study, glutamate induced three different types of responses. Since these responses were pooled from all taste cells tested, we could not rule out the possibility that the multiple responses occur selectively in different cell types. However, we did obtain results from single taste cells that responded to both NMDA and L-AP4, suggesting that both receptors are often present in the same cells. We suggest that Type III responses represent compound responses that involve co-activation of NMDA and mGluR4 receptors. Several observations are consistent with this interpretation. First, cells exhibiting Type III responses usually responded to both NMDA and L-AP4, suggesting that both types of receptors are present in these cells. Second, the time to half-maximal activation was considerably longer for responses elicited by L-AP4 than for those elicited by NMDA; thus, the inward current should precede the outward current. Finally, GDP-β-S and 8-bromo-cAMP suppressed only the glutamate-activated outward current in cells expressing Type III responses. These data are consistent with the studies of Hayashi et al. (1996) and Bigiani et al. (1997), in which both increases or decreases in intracellular $Ca^{2+}$ or holding currents in response to glutamate were shown in vallate taste cells of mice and rats.

The data presented in this study showed that the percentage of cells responding biphasically (28%, Fig. 3.1B) is lower than the percentage of cells that respond to both L-AP4 and NMDA (34%, Fig. 3.12). This difference could be due to the fact that some of the responses to L-AP4 elicited increases in holding current. Taste cells could express receptors for this novel response of L-AP4 in addition to NMDA receptors. Such a combination would not likely result in a biphasic response to glutamate.

Responses to NMDA

In vallate taste cells from mice, NMDA depolarized the taste cells and increased intracellular $Ca^{2+}$ levels (Hayashi et al., 1996). Our data are in agreement with this study. Our data on fungiform taste receptor cells differ, however, from those of Bigiani et al. (1997) who showed that only a small fraction of vallate taste cells in rats showed increases in holding current in response to glutamate. In our study, most glutamate-sensitive taste cells exhibited some inward current in response to glutamate and many also responded to NMDA. It appears that there may be differences in glutamate transduction in different species and even among papillae of the same species.

Our study extended the findings of previous studies to show that NMDA receptors in taste cells share many properties with brain NMDA receptors, such as co-activation with glycine and suppression by AP5 (Ascher and Johnson, 1994; Gibb et al., 1994). However, the effect of extracellular $Mg^{2+}$ on the NMDA-sensitive current is somewhat different in taste cells than in brain. We observed blockage by $Mg^{2+}$ in some cells, while in most of the cells tested, the amplitude of the peak current was not reduced in the presence of $Mg^{2+}$, though the desensitization of the channel was much faster. In the brain, blockage of NMDA receptor channels by $Mg^{2+}$ depends on the subunit composition of the receptors. The amino acid residue glutamine in transmembrane segment M2 is responsible the blockage by $Mg^{2+}$ (Mori et al., 1992). Since the NMDA receptor in taste cells has not been cloned, we cannot explain the different effects of $Mg^{2+}$ block on taste cell NMDA receptors. It is believed that extracellular $Mg^{2+}$ blocks the NMDA receptor channel only at membrane potentials more negative than -40 mV (Mayer et al., 1994). Since resting potentials of most taste cells in fungiform papillae are in the

range of -30 to -60 mV (Furue and Yoshii, 1997), $Mg^{2+}$ would not be expected to have the same blocking effect in taste cells *in vivo*. Another difference between the NMDA receptor in taste cells and those in the brain is that the current in taste cells is carried primarily by $Na^{+}$ and $Ca^{2+}$, while the current in brain is fairly non-selective. The reason for lack of $K^{+}$ permeability in NMDA receptors of taste cells is not known.

Responses to L-AP4

Most of the responses to L-AP4 involved a decrease in holding current and membrane conductance, concomitant with membrane hyperpolarization in some cells. These results are consistent with previous findings in taste cells of vallate and foliate papillae (Hayashi et al., 1996; Bigiani et al., 1997). We showed further that the response is suppressed by cAMP and by the metabotropic antagonist CPPG, supporting the idea that mGluR4 is expressed in rat taste cells (Chaudhari et al., 1996). Suppression by cAMP is to be expected, since activation of mGluR4 decreases intracellular cAMP levels in brain (Pin and Duvoisin, 1995). In addition, MSG decreases cAMP levels in tissue from rat vallate and foliate papillae (Zhou and Chaudhari, 1997).

In contrast to the above-described effects of L-AP4, we observed that some cells responded to L-AP4 with an increase in holding current and membrane depolarization. This response was not reported in taste cells of rat vallate papillae (Bigiani et al., 1997), although in taste cells of mice vallate papillae, L-AP4 could either decrease or increase the intracellular $Ca^{2+}$ level (Hayashi et al., 1996). Whether mGluR4 also mediates the response is not yet determined. Interestingly, taste cells express two different forms of mGluR4; a long form that is similar to mGluR4 in the brain, and a short form in which a

significant portion of the extracellular N-terminus has been truncated. This short form may be specific to taste cells (Chaudhari et al., 1997). It will be interesting to determine if the short form of mGluR4 has similar properties to the long form.

The present study suggests that NMDA receptors and mGluR4 may be co-activated to depolarize taste cells. An important caveat in this interpretation is that glutamate was applied to the entire taste cell membrane, rather than selectively to the apical membrane as occurs *in vivo*. It is possible that some responses to glutamate observed in the present study were mediated by receptors located on the basolateral membrane rather than the apical membrane. Indeed, glutamate has been found in afferent nerve fibers of mudpuppy and could be released onto taste cells (Lu and Roper, 1993). Further studies will be required to determine the location of NMDA receptors and mGluR4 in taste cell membranes. However, L-AP4 elicits primarily a decrease in holding current and membrane hyperpolarization, while most cells respond to glutamate with an increase in intracellular $Ca^{2+}$ and membrane depolarization (Hayashi et al., 1996). Therefore, the most parsimonious interpretation is that co-activation of NMDA receptors and mGluR4 is required for the umami taste response.

# Chapter 4

# SYNERGISTIC RESPONSES TO GLUTAMATE AND GUANOSINE 5'-MONOPHOSPHATE IN TASTE CELLS OF RAT FUNGIFORM PAPILLAE


## Abstract

Synergy between monosodium glutamate (MSG) and 5'-ribonucleotides is an important feature of the umami taste. Previous studies showed that the umami taste is greatly potentiated when both guanosine 5'-monophosphate (5'-GMP) and MSG are present (Yamaguchi and kimizuka, 1979). The transduction mechanism for 5'-GMP and its synergy with MSG is not known. Using the whole-cell patch-clamp technique, we examined responses to 5'-GMP, MSG, and a combination of 5'-GMP and MSG in taste receptor cells of rat fungiform papillae. Our results showed that 5'-GMP induces responses that are similar to those of glutamate, e.g., a decrease in holding current, an increase in holding current, or a biphasic response. Interestingly, responses to 5'-GMP and glutamate do not always occur in the same taste cells, indicating that they likely activate different receptors. A subset of cells showed synergistic responses to the mixture of 5'-GMP and MSG. Most responses to 5'-GMP, as well as the synergy between

5'-GMP and MSG, were suppressed by 8-bromo-cAMP. These data suggest that the synergy involves metabotropic receptors negatively coupled to the cAMP pathway.

## Introduction

Monosodium glutamate (MSG) and guanosine 5'-monophosphate (5'-GMP) both elicit the umami taste quality, and these compounds often co-exist naturally in foods (for review, see Maga, 1983; Fuke and Konosu, 1991). Taste responses to 5'-GMP have been recorded from single fibers of both chorda tympani and glossopharyngeal nerves, and from neurons in central gustatory system (Ninomiya and Funakoshi, 1989; Adachi and Aoyama, 1991; Hellekant and Ninomiya, 1991; Scott et al., 1993). Interestingly, there is a strong synergy between MSG and 5'-GMP; that is, the response induced by the mixture of these compounds is greater than the sum of the individual responses. In humans, the umami taste threshold can be lowered 100-fold and the intensity of the taste can be increased dramatically when both MSG and 5'-GMP are present (Yamaguchi and Kimizuka, 1979).

A few studies have examined the mechanisms involved in the synergistic response. A biochemical study showed that the presence of 5'-ribonucleotides increased the binding of glutamate to membranes of bovine vallate papillae, which suggests that the synergy is due to an increase in the number of binding sites for glutamate (Torii and Cagan, 1980). This hypothesis was supported by a study showing that 5'-GMP potentiated the response to glutamate in lipid bilayers containing membranes from mouse vallate papillae (Brand et al., 1991). These studies suggest that the synergy occurs at the

membrane surface, possibly by altering the conformation of the glutamate receptor. However, it is also possible that the synergy could involve an amplification of intracellular pathways, particularly since recent behavioral studies suggest that the synergy occurs only with metabotropic glutamate agonists (E. Delay and S. Roper, unpublished observations).

We have shown that both NMDA receptors and mGluR4 may mediate the transduction of glutamate in fungiform taste cells of the rat (see chapter 3). In addition, we demonstrated that responses to L-AP4, a specific agonist of mGluR4, are suppressed by 8-bromo-cAMP, suggesting that mGluR4 is negatively coupled to the cAMP pathway, as also occurs in brain. Thus, it is possible that the synergy between 5'-GMP and glutamate also involves intracellular cAMP pathways.

This study focused on the transduction mechanism of 5'-GMP and its synergy with glutamate in taste cells of rat fungiform papillae. Using the whole-cell patch-clamp technique, we examined responses to 5'-GMP alone and in combination with MSG to examine the following questions: (1) Do responses to MSG and 5'-GMP always occur in the same taste cells? (2) Do all taste cells that respond to both MSG and 5'-GMP exhibit synergy to the mixture? (3) What is the role of the cAMP pathway in the transduction of 5'-GMP and its synergy with MSG?

## Methods

### Isolation of taste buds

Male Sprague-Dawley 4-12 week old rats were used. All experiments were performed on taste receptor cells of freshly isolated taste buds from fungiform papillae.

The isolation method was adapted from Béhé et al. (1990). Briefly, rats were killed with $CO_2$ and the tongue was dissected and placed into cold Tyrode's solution. Approximately 0.3 to 0.8 ml of an enzyme solution containing 3 mg dispase, 0.7mg collagenase B (Boehringer Mannheim, Indianapolis, IN) and 1 mg trypsin inhibitor (type I-S; Sigma Chemical Corp., St. Louis, MO) in 1.0 ml of Tyrode's was injected beneath the lingual epithelium. The tongue was then incubated in $Ca^{2+}$- and $Mg^{2+}$-free oxygenated Tyrode's for 30 min. After incubation, the lingual epithelium was peeled gently and pinned serosal side up in a Sylgard-covered petri dish. Taste buds were removed from fungiform papillae by gentle suction with a glass pipette and plated onto Cell-Tak (Collaborative Research, Bedford, MA) coated glass slides, on which a recording chamber was formed previously with a silicon ring about 2 mm thick and 1.5 cm in diameter.

Solutions and chemicals

Normal Tyrode's was used as a standard bath solution, containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (HEPES), 10 mM glucose and 10 mM sodium pyruvate (pH 7.4 with NaOH). The $Ca^{2+}$-and $Mg^{2+}$-free Tyrode's for isolating taste buds consisted of 2 mM BAPTA (Molecular Probes, Eugene, OR). The intracellular pipette solution contained 140 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES (pH 7.2 with KOH). 11 mM EGTA, 1 mM ATP, and 0.4 mM GTP (pH 7.2 with KOH). Most of the chemicals used were bath applied. Monosodium glutamate, guanosine

5'-monophosphate and 8-bromo adenosine 3':5'-cyclic monophosphate (8-bromo-cAMP) were from Sigma Chemical Corp., St. Louis, MO.

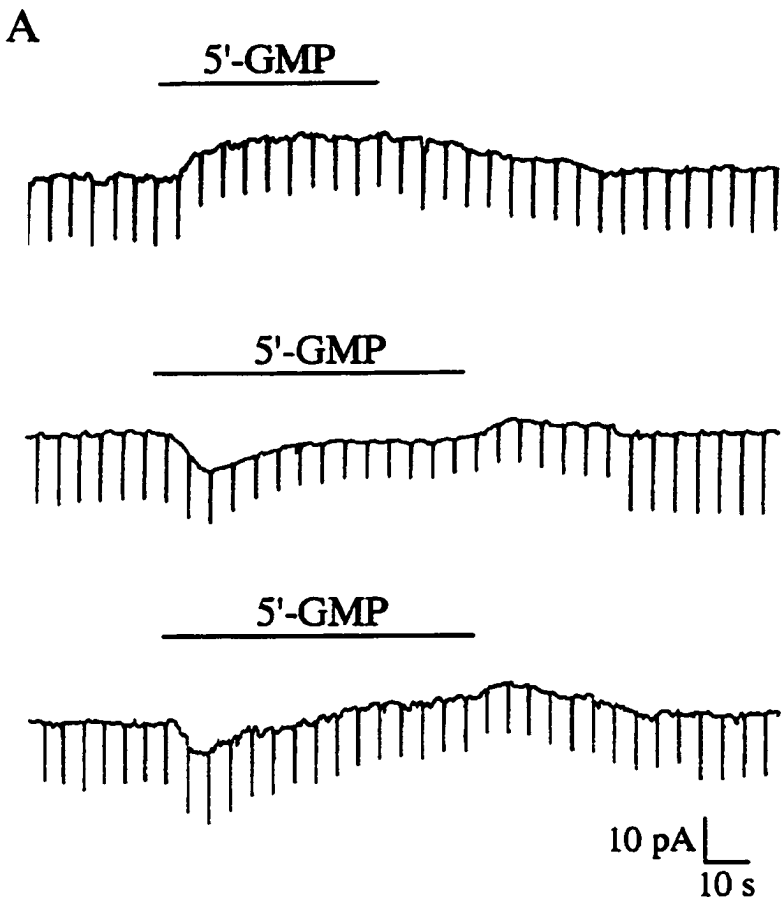
Patch-clamp recordings

The whole-cell patch-clamp technique was used (Hamill, et al., 1981). The steady-state current was recorded with the voltage-clamp configuration. Glass pipettes for patch recording were pulled from microhematocrit capillary tubes (Scientific Products, McGaw Park, IL) with a two-stage vertical puller (model PB-7; Narishige, Tokyo, Japan). The pipette resistance was 3 to 6 MΩ. Membrane currents were lowpass filtered at 2 KHz and recorded with an Axopatch patch-clamp amplifier (Model 200B, Axon Instruments, Foster City, CA). Voltage-gated $Na^+$ and $K^+$ currents were induced by applying voltage steps from an Indec laboratory computer system (Sunnyvale, CA) and were used to distinguish taste cells from non-sensory epithelial cells. Taste cells were held at -80 mV and 20 mV hyperpolarizing voltage pulses were used to monitor the membrane conductance.

## Results

Responses to 5'-GMP

5'-GMP (100μM) applied to the bath solution elicited three types of responses: a decrease in holding current (type I), an increase in holding current (type II), or a biphasic response, characterized by an increase followed by a decrease in holding current (type III; Fig.4.1A). In a total 127 cells tested, 72 cells responded to 5'-GMP. Among them,



28 cells showed type I responses, with the mean current amplitude of 15.3 ± 3.8 pA (n=15). Nineteen cells showed type II responses, with a mean current amplitude of 10.4 ± 3.3 pA. Twenty-five cells showed type III responses (Fig 4.1B). In general, the types of responses elicited by 5'-GMP were similar to those of glutamate (see Chapter 3); although in a single cell, responses to 5'-GMP and glutamate were not always of the same type. Of these 127 cells, 54 cells responded to both 5'-GMP and glutamate. Interestingly, 18 cells responded to 5'-GMP only, while 17 cells responded to glutamate only (Fig 4.2). These results suggest that 5'-GMP may activate receptors distinct from those of glutamate.

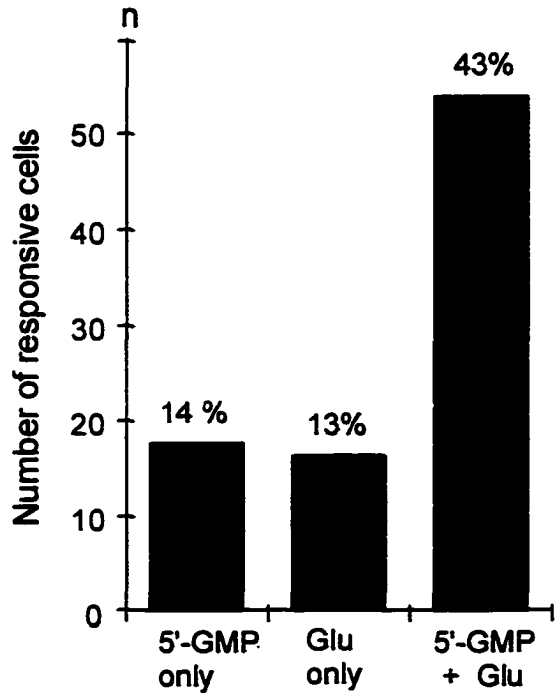



Fig. 4.2. Number and percentage of cells that responded to 5'-GMP and glutamate. Most cells that responded to glutamate also responded to 5'-GMP. Note that some cells only responded to 5'-GMP or glutamate (n=127, 38 cells had no response to neither compound).

Synergistic responses to 5'-GMP and glutamate

Synergy between 5'-GMP and glutamate is an important feature of the umami taste. 5'-GMP (0.1 mM) and MSG (1 mM ) applied simultaneously to the bath induced synergistic responses in 13 out of 48 cells tested. Most synergistic responses occurred in cells that responded to both 5'-GMP and glutamate. A few occurred in cells that did not have measurable responses to glutamate. Both increases and decreases in holding current could be potentiated (n=5 and 8 respectively). The synergy was estimated by a ratio in which the response to the mixture of 5'-GMP and glutamate was divided by the sum of individual responses to 5'-GMP and glutamate. The mean ratios for these synergistic responses were 1.3 ± 0.1 and 2.5 ± 0.8 respectively. Therefore, synergistic responses showing decreases in holding current were usually greater than those showing increases in holding current (Fig. 4.3).

Effect of intracellular cAMP

Increases in intracellular cAMP have been shown to antagonize most type I responses to glutamate (see Chapter 3). To determine if cAMP is also involved in the transduction of 5'-GMP, a membrane-permeable cAMP analog, 8-bromo-cAMP (1 mM) was added to the bath. In four out of five cells tested, cAMP eliminated or reduced responses to 5'-GMP (Fig. 4.4). Interestingly, 8-bromo-cAMP also eliminated or suppressed the synergistic response to the mixture of 5'-GMP and MSG. In five out of seven cells tested, the response was reversibly reduced by 8-bromo-cAMP (Fig. 4.5). These data suggest that the receptors involved in the transduction of 5'-GMP and the synergism between 5'-GMP and MSG are negatively coupled to the cAMP pathway.

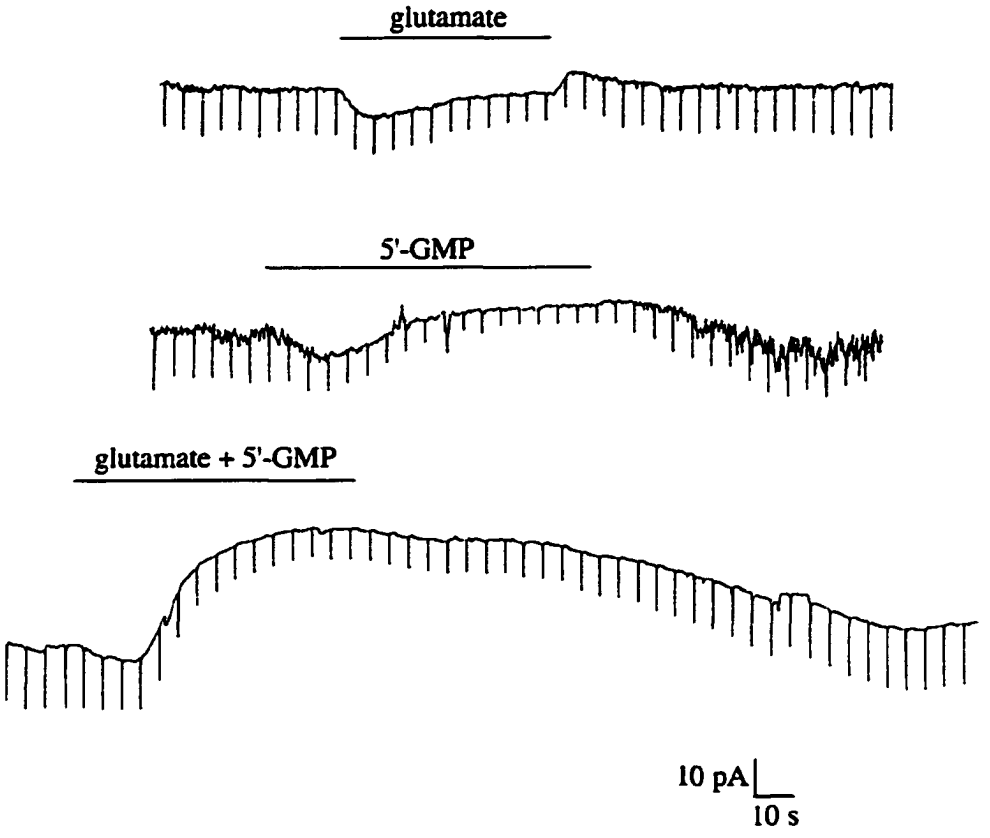



Fig. 4.3. A typical synergistic response to 5'-GMP (0.1 mM) and glutamate (1 mM) showing a potentiated decrease in holding current. Similar results were obtained from 8 out of 48 cells. Five other cells showed a potentiated increase in holding current, but the potentiation was not as dramatic (data not shown).

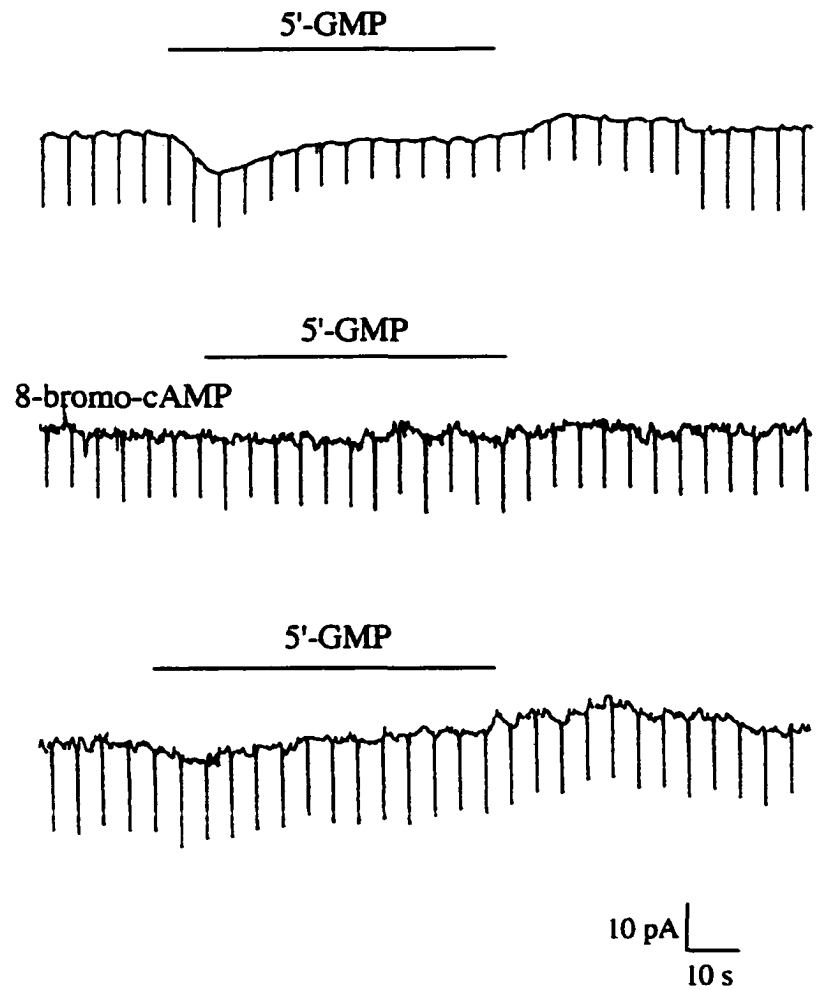



Fig. 4.4. Effect of intracellular cAMP on responses to 5'-GMP. 8-bromo-cAMP (1 mM) added to the bath eliminated the response to 5'-GMP. The effect was partially reversible (bottom trace).

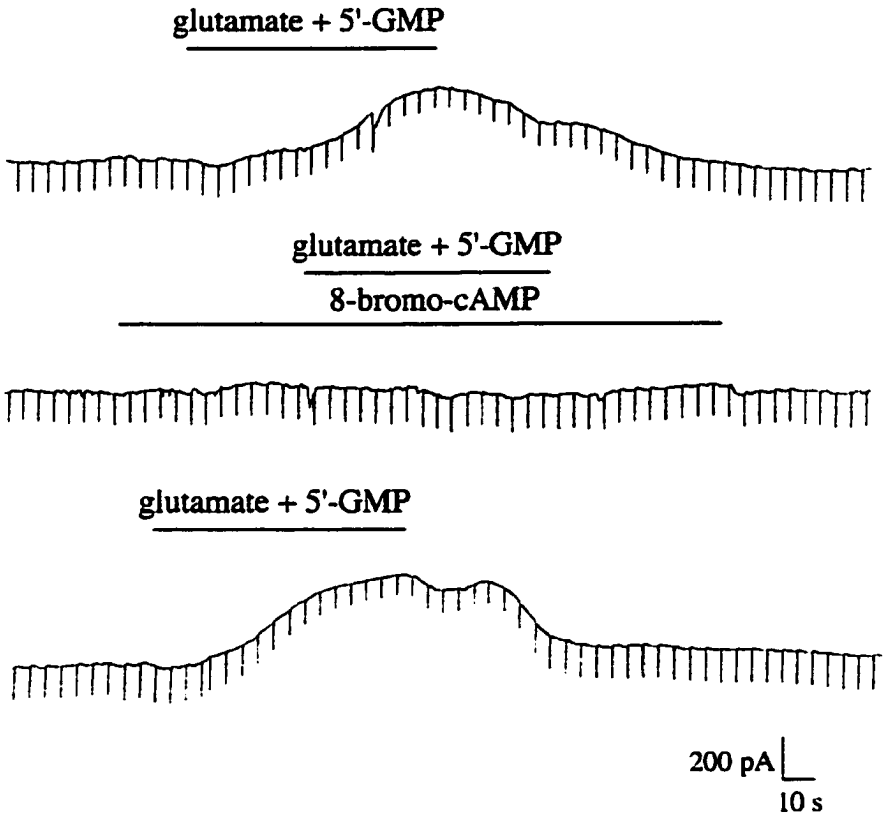



Fig. 4.5. Effect of intracellular cAMP on the synergistic response. 8-bromo-cAMP (1 mM) added to the bath eliminated the synergistic response to 5'-GMP and glutamate. The effect was reversible (bottom trace).

## Discussion

This study is the first to examine electrophysiological recordings of responses to 5'-GMP and glutamate in intact taste receptor cells. We demonstrated that most cells respond to both glutamate and 5'-GMP; however, only a subset of cells respond synergistically to the mixture of 5'-GMP and glutamate. In addition, our data suggest that the cAMP pathway may be involved in the transduction of 5'-GMP and its synergy with glutamate

### Response to 5'-GMP

Our results are compatible with previous recordings from the chorda tympani nerve. In these studies, 5'-GMP and other 5'-ribonucleotides such as inosine 5'-monophosphate induced measurable responses; most fibers and neurons that respond to MSG also respond to 5'-GMP, while in some fibers and neurons, these responses are independent (Hiji and Sato, 1967; Sato et al., 1970; Nakamura and Norgren, 1991; Yamamoto et al., 1991; Ninomiya et al., 1992; Ugawa and Kurihara, 1994). Interestingly, our study also revealed that some taste cells respond independently to 5'-GMP and glutamate. These results suggest that the transduction of 5'-GMP and glutamate may be mediated by different receptors.

We observed that 5'-GMP, like glutamate, induced three types of responses: a Type I response, a Type II response, and a biphasic Type III response. However, unlike Type II responses induced by glutamate, Type II responses to 5'-GMP were suppressed by cAMP. Thus, it is not likely that the Type II response to 5'-GMP is mediated by the NMDA receptor. The Type I response to 5'-GMP strongly resembles the Type I response to glutamate, which is mediated by mGluR4 (see chapter 3). Thus, it is possible

that 5'-GMP binds to either mGluR4 or to another receptor negatively coupled to the cAMP pathway. The requirement for second messengers may explain why bilayers did not respond to 5'-GMP, where only fractions of membrane from vallate and foliate papillae were incorporated (Brand et al., 1991). Still, the receptors that mediate responses to 5'-GMP have not been identified. It is possible that 5'-GMP activates its own receptors, as well as binding to metabotropic glutamate receptors. It will be interesting to determine if antagonists of glutamate receptors suppress responses to 5'-GMP.

Synergy between 5'-GMP and glutamate

Synergy between 5-GMP and MSG is a characteristic phenomenon of the umami taste. Our results show that at least some cells respond to 5'-GMP and glutamate synergistically. Responses involving both an increase and a decrease in holding current could be potentiated. These data are consistent with other studies. 5'-ribonuleotides potentiate the increase in membrane conductance induced by glutamate (Brand et al., 1991) and increase the number of cells that show increased intracellular $Ca^{2+}$ concentration in response to glutamate (Hayashi et al., 1996). Recently, a behavioral study showed that synergy occurs between 5'-GMP and L-AP4, suggesting that the synergism may involve mGluR4 (E. Delay and S. Roper, unpublished observations). Our data support this hypothesis, since increases in intracellular cAMP suppressed most synergistic responses that involved a decrease in holding current. However, it is not clear if the synergy occurs at the receptor level or by integration of intracellular signaling pathways. It is possible that both mechanisms contribute to the synergy. Although our

data support separate receptors for 5'-GMP and glutamate, it is possible that 5'-GMP also binds to glutamate receptors, causing an allosteric change in receptor conformation. This could increase the binding sites for glutamate, as proposed originally by Torii and Cagan (1980). In any case, more studies on synergy are required to address why single nerve fibers (Ninomiya and Funakoshi, 1989; Hellekant and Ninomiya, 1991; Hellekant et al., 1997) and neurons in the central gustatory system (Adachi and Aoyama, 1991) can respond to both 5'-GMP and glutamate, but without synergy.

# Chapter 5

# CO-LOCALIZATION OF EPITHELIAL SODIUM CHANNELS AND GLUTAMATE RECEPTORS IN SINGLE TASTE CELLS OF FUNGIFORM PAPILLAE


## Abstract

Umami taste is elicited by monosodium glutamate (MSG), a compound consisting of two potent taste stimuli, $Na^+$ and glutamate. In rat fungiform taste cells, amiloride-sensitive epithelial sodium channels (rENaCs) mediate $Na^+$ transduction, while glutamate is transduced by a combination of iontropic and metabotropic glutamate receptors. Several studies revealed that MSG responses occur preferentially in single afferent fibers that respond best to NaCl. It is not known whether the transduction pathways for $Na^+$ and glutamate occur in the same receptor cells or if they converge from different cells. We used giga-seal whole cell recording to determine if responses to glutamate and $Na^+$ occur in the same taste cells. Approximately 68% of the cells tested responded to amiloride, indicating that they express functional rENaCs. Responses to glutamate occurred in about 58% of the cells tested. Interestingly, responses to glutamate occurred in the subset of cells that also responded to amiloride, indicating that glutamate receptors

are located preferentially in the same taste cells that also express rENaCs. Further experiments showed that amiloride did not suppress responses to glutamate under voltage-clamp conditions. Taken together, the data suggest that although rENaCs are not involved directly in glutamate transduction, their co-localization with glutamate receptors provides a substrate for the cellular integration of these independent pathways.

## Introduction

Studies in chapter 2 and 3 demonstrated that different receptors and channels mediate the transduction of $Na^+$ and glutamate. Amiloride-sensitive epithelial $Na^+$ channels, rENaCs, are expressed in taste receptor cells of fungiform papillae. $Na^+$ influx through rENaCs depolarizes taste receptor cells, initiating a salty taste (see Chapter 2). The transduction of glutamate is mediated by glutamate receptors. Both NMDA receptor channels and mGluR4 are present in fungiform taste cells and may be involved in transducing glutamate taste (see Chapter 3).

Although the transduction of $Na^+$ and glutamate involve different mechanisms, a number of studies indicate a correlation between NaCl and umami responses. Responses to MSG occur in the subset of chorda tympani nerve fibers that are most sensitive to NaCl; amiloride suppresses the response to both MSG and NaCl (Ninomiya and Funakoshi, 1989; Kumazawa et al., 1991; Nakamura and Norgren, 1991; Hellekant and Ninomiya, 1991; Hellekant et al., 1997). In a behavioral study, hamsters could not distinguish between high concentrations of MSG and NaCl (Yamamoto et al., 1988). These studies raise the question of whether the pathways for glutamate and $Na^+$ are co-

localized and interact in single taste receptor cells or whether these pathways converge onto single nerve fibers.

We have used giga-seal whole-cell recording to determine if functional rENaCs and glutamate receptors occur in the same taste receptor cells and if their transduction pathways are independent. An abstract form of this study has been published (Lin et al., 1996).

## Methods

### Isolation of taste buds

Male Sprague-Dawley rats 4-12 weeks old were used. Taste buds of fungiform papillae were isolated with a method adapted from Béhé et al. (1990). Briefly, rats were sacrificed with $CO_2$ and the tongue was removed. Approximately 0.5 to 0.8 ml of an enzyme mixture containing 3 mg dispase, 0.7 mg collagenase B (Boehringer Mannheim, Indianapolis, IN) and 1 mg trypsin inhibitor (type I-S; Sigma Chemical Corp., St. Louis, MO) in 1.0 ml of Tyrode's was injected between the lingual epithelium and the muscle layers of the tongue. The tongue was then incubated in $Ca^{2+}$ and $Mg^{2+}$-free oxygenated Tyrode's solution for 30 min. After incubation, the lingual epithelium was peeled and pinned serosal side up in a Sylgard-lined petri dish. The taste buds were removed from fungiform papillae by gentle suction with a glass pipette and plated onto Cell-Tak (Collaborative Research, Bedford, MA) coated glass slides. Recording chambers were made by attaching a silicon ring (2 mm thick, 1.5 cm in diameter) to the Cell-Tak coated slides.

## Solutions

Standard bath solution consisted of 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (HEPES), 10 mM glucose and 10 mM sodium pyruvate (pH 7.4 with NaOH). The $Ca^{2+}$- and $Mg^{2+}$-free Tyrode's solution used to isolate taste buds contained 2 mM BAPTA (Molecular Probes, Eugene, OR). Amiloride (Sigma Chemical Corp., St. Louis, MO) was prepared from a stock solution (10 mM) diluted with Tyrode's to a final concentration of 30 μM. This concentration was chosen to maximize the blockage of amiloride -sensitive rENaCs without inhibition of $Na^+/Ca^{2+}$ and $Na^+/H^+$ exchangers (Doolin and Gilbertson, 1996; Kossel et al., 1997). Monosodium glutamate (1 mM; Sigma) also was added to the bath solution. Bath solutions were gravity-fed into the 0.5 ml recording chamber. Flow rates were typically 4-5 ml/min, permitting complete solution exchange in less than 10 sec. The intracellular pipette solution contained 140 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES (pH 7.2 with KOH), 11 mM EGTA, 1 mM ATP, and 0.4 mM GTP (pH 7.2 with KOH).

## Electrophysiological recordings

The whole-cell voltage-clamp configuration was used to record steady-state currents (Hamill et al., 1981). The glass pipettes for patch recording were pulled from microhematocrit capillary tubes (Scientific Products, McGaw Park, IL) with a two-stage vertical puller (model PB-7; Narishige, Tokyo, Japan). Pipette resistance was 3 to 6 MΩ. Membrane currents were filtered at 2 KHz and recorded with an Axopatch patch-clamp amplifier (model 200B; Axon Instruments, Foster City, CA). Voltage-gated $Na^+$ and $K^+$

currents were induced by applying voltage steps from an Indec laboratory computer system (Sunnyvale, CA) and were used to distinguish taste cells from non-sensory epithelial cells. For measurement of the steady-state current, holding current was recorded at -80 mV and 20 mV hyperpolarizing voltage pulses were used to monitor membrane conductance.

## Results

Numerous studies in rodents have implicated the involvement of amiloride-sensitive $Na^+$ channels in the transduction of $Na^+$ salts in taste receptor cells of fungiform papillae. Amiloride, a specific channel blocker of rENaCs, reversibly decreases the holding current and membrane conductance in taste cells of rat fungiform papillae (Doolin and Gilbertson, 1996; Kossel et al., 1997). Therefore, we used the response to amiloride to indicate the presence of functional amloride-blockable rENaCs. In order to investigate possible co-localization of rENaCs and glutamate receptors in taste cells of fungiform papillae, we first examined the response to amiloride. Bath application of 30 μM amiloride reversibly decreased the holding current and membrane conductance in about 68% of fungiform taste cells (Fig 5.1A). These changes were reversible upon washing with Tyrode's. The amplitude of amiloride-sensitive current varied from 5 pA to 500 pA, accounting for 15 % to 98% of total holding current. The mean current amplitude from 40 amiloride-sensitive cells was 33.2 ± 5.6 pA. These data indicate that some taste cells in fungiform papillae are highly sensitive to amiloride. The inhibition constant (Ki) for amiloride-block was between 0.1 to 0.2 μM (n=6 cells; data not shown).

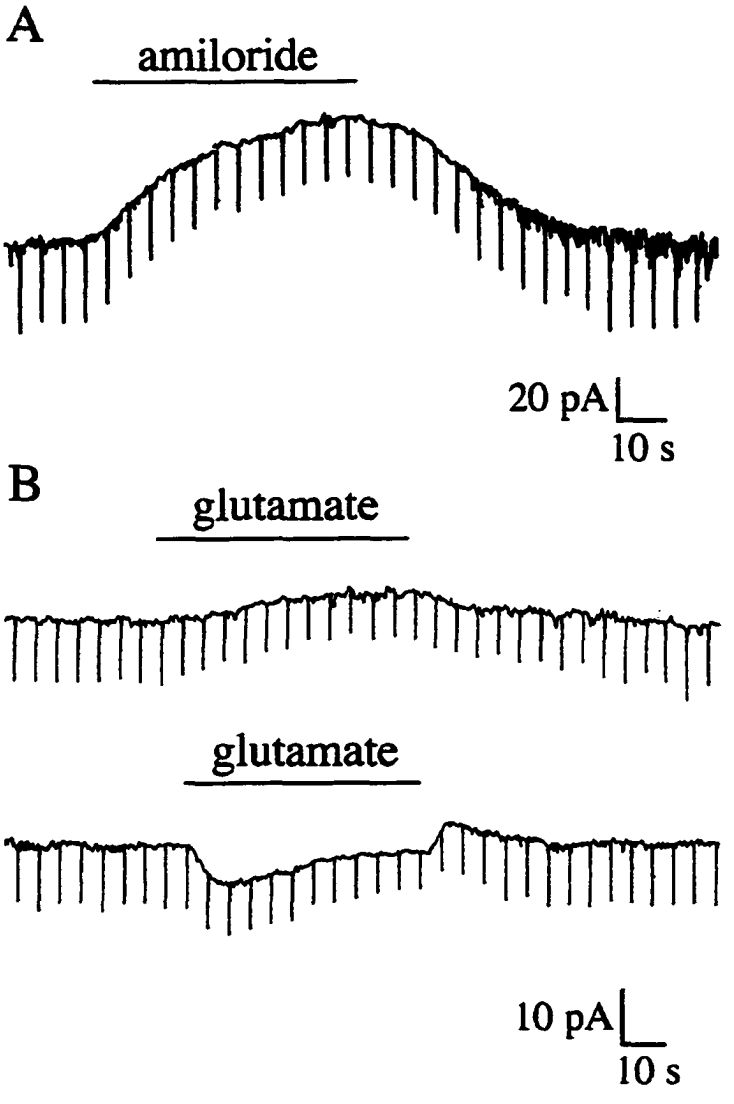



Fig. 5.1. Responses to amiloride and glutamate in taste receptor cells of fungiform papillae. A. Amiloride (30 μM) decreases holding current and membrane conductance. B. Bath application of glutamate (1 mM) induces either a decrease in holding current (top trace) or an increase in holding current (bottom trace) in different cells. The holding potential was -80 mV.

Replacing extracellular $Na^+$ with the non-permeable cation NMDG resulted in a larger decrease in holding current and membrane conductance, suggesting that not all $Na^+$ conductance is amiloride-sensitive (data not shown). These results are consistent with previous studies in rats (Doolin and Gilbertson, 1996; Kossel et al., 1997).

To examine responses to MSG we used a concentration of 1 mM which is sufficient to activate glutamate receptors, but has a negligible effect on the bath $Na^+$ concentration. Three types of glutamate responses were recorded: a decrease in holding current and membrane conductance (Type I), an increase in holding current (Type II), and a combination of the two, e.g. a biphasic response with an increase, followed by a decrease in holding current (Fig. 5.1B). Type I responses correspond to activation of mGluR4, while Type II responses correspond to activation of NMDA receptors (see Chapter 3; Lin et al., 1996; Lin and Kinnamon, 1998). For the purposes of this study, we pooled the different types of glutamate responses.

We tested a total of 136 cells for responses to both amiloride and glutamate. Of these, 73 cells responded to both amiloride and glutamate, 37 cells did not respond to either compound, 20 cells responded to amiloride only and six cells responded to glutamate only (Fig. 5.2). To determine if the glutamate and amiloride responses were independent, we conducted the Chi-Square test. The results showed that the responses were not independent; i.e., responses to glutamate occurred preferentially in the subset of taste cells that also responded to amiloride ($p<0.001$). Therefore, glutamate receptors are preferentially expressed in taste cells that exhibit functional rENaCs. The co-localization

of both rENaCs and glutamate receptors in single taste cells provides the possibility for interaction and integration of $Na^+$ and glutamate pathways in single taste cells.

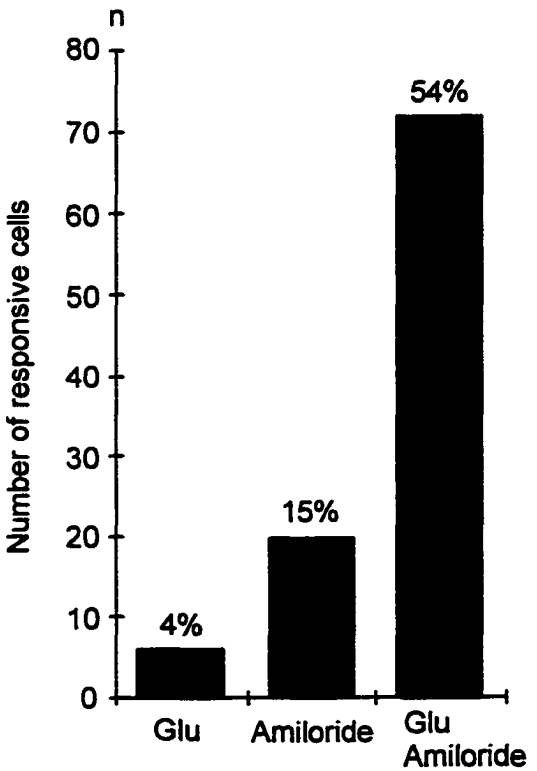



Fig. 5.2: Responses to glutamate occur preferentially in the subset of taste cells that respond to amiloride. All glutamate (Glu)-responsive cells are pooled independent of the type of glutamate response elicited.

To examine whether the transduction pathways for $Na^+$ and glutamate are independent, we examined responses to glutamate in the presence of 30 μM amiloride. The results are shown in figure 5.3. The average amplitude of Type II glutamate responses is 11.4 ± 3.9 pA in the presence of amiloride, compared to 12.5 ± 3.7 pA under

control condition (n=7). Thus, the presence of amiloride in the bath did not suppress the amplitude of glutamate responses (Fig. 5.3). Similar results were obtained from Type I responses to glutamate; peak currents in the presence and absence of amiloride are 16.0 ± 4.0 pA and 15.4 ±5.2 pA respectively (n=5). The data suggest that amiloride does not inhibit glutamate transduction and the transduction pathways for $Na^+$ and glutamate are independent in taste cells. Our results are consistent with a previous study in which plasma membranes from mouse vallate papillae were incorporated into lipid bilayers. Amiloride did not block the conductance induced by glutamate (Brand et al., 1991).

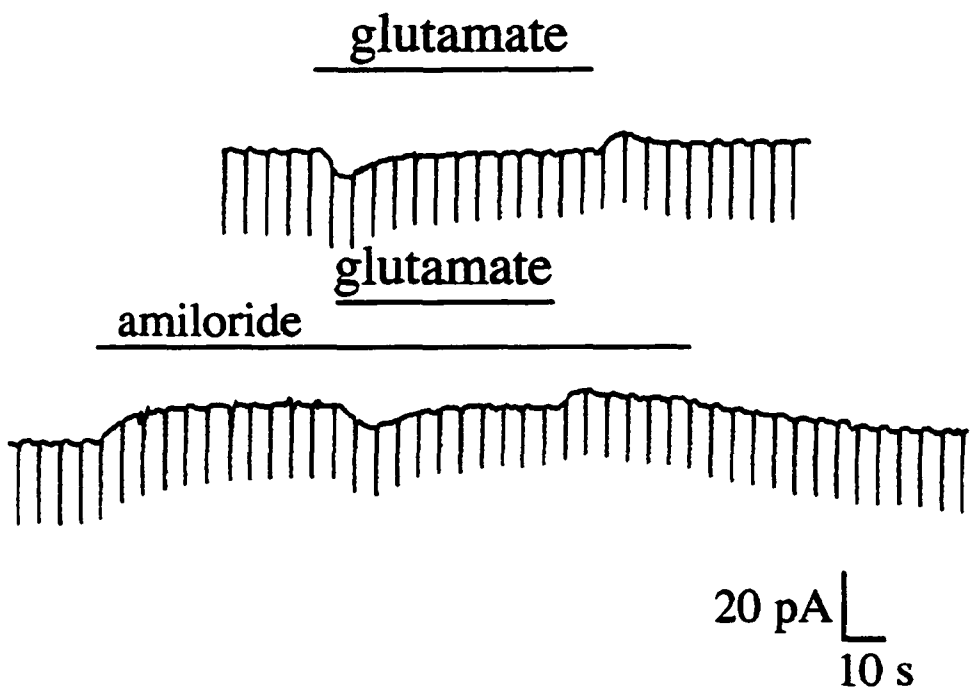



Fig. 5.3. Amiloride does not inhibit responses to glutamate. A glutamate response in the absence (top trace) and presence of 30μM amiloride.

## Discussion

In this study, we showed that the pathways for $Na^+$ and glutamate transduction are independent in taste receptor cells. Amiloride inhibits $Na^+$ transduction, but has little effect on glutamate transduction. However, the transduction pathways for $Na^+$ and glutamate tend to occur together in the same taste receptor cells. Therefore, the transduction of MSG is mediated by both $Na^+$ and glutamate pathways which are integrated in single taste cells of fungiform papillae. This may explain results from previous studies, in which a subset of chorda tympani fibers that respond best to NaCl also respond to MSG (Ninomiya and Funakoshi, 1989; Hellekant and Ninomiya, 1991, Hellekant et al., 1997; Kumazawa et al., 1991; Nakamura and Norgren, 1991). Since MSG responses recorded from the chorda tympani represent responses to both $Na^+$ and glutamate which have already been integrated at the receptor cell level, amiloride should and does suppress the integrated responses to MSG (Nakamura and Kurihara, 1991; Ninomiya et al., 1992).

In addition, the presence of both rENaCs and glutamate receptors provides a possibility for the interaction between these pathways. Although we did not observe an inhibition of glutamate responses by amiloride under voltage-clamp condition, sodium influx through rENaCs may affect taste cells *in vivo* by direct depolarization and by accumulation of intracellular $Na^+$. Since Type I glutamate responses tend to be hyperpolarizing (see chapter 3), the presence of rENaCs may provide a counter-balancing depolarizing effect, allowing transmitter to be released in response to MSG stimulation. Clearly, NaCl can be distinguished from MSG. This may occur because more taste cells

express rENaCs than glutamate receptors, and also because the response to glutamate may have slower kinetics due to activation of second messengers. In support of this idea, a human psychophysical study demonstrated that MSG has a strong aftertaste and shows slow adaptation compared to NaCl (Yamaguchi and Kobori, 1993).

# Chapter 6:

# SUMMARY

In summary, this study provides new information about the transduction of monosodium glutamate in taste cells of fungiform papillae. Since MSG contains two potent taste stimuli, $Na^+$ and glutamate, their transduction mechanisms were studied independently. Data obtained from immunocytochemical and patch-clamping experiments show that the transduction pathways of $Na^+$ and glutamate are independent. However, these transduction pathways occur in single taste cells of fungiform papillae. Therefore, the sensory output for MSG from these taste receptor cells represents integrated responses from both $Na^+$ and glutamate pathways. Based on these studies, a model for the transduction of MSG is shown in Fig. 6.1.

The transduction for $Na^+$ in rodents is primarily mediated by amiloride-sensitive epithelial $Na^+$ channels, rENaCs. As shown in Chapter 2, all three subunits of rENaCs are expressed in fungiform taste cells and constitute functional channels that are sensitive to amiloride. The influx of $Na^+$ through rENaCs depolarizes taste cells and activates voltage-gated $Na^+$, $Ca^{2+}$ and $K^+$ channels, thus resulting in increases in intracellular $Ca^{2+}$ levels and the release of transmitter to afferent nerve fibers. Activation of this pathway by $Na^+$ either from MSG or NaCl results in a salty taste sensation (for review, see Gilbertson and Kinnamon, 1996; Lindemann et al., 1999).

Metabotropic glutamate receptors (mGluRs) may play a central role in the taste transduction of glutamate, since mGluR4 is expressed in taste cells but not in surrounding epithelial cells; and L-AP4 mimics the behavioral response to glutamate (Chaudhari et al., 1996). In the study reported in Chapter 3, activation of mGluR4 decreased the membrane conductance and the response was antagonized by cAMP. Also, synergism between glutamate and 5'-GMP involved a conductance that was antagonized by cAMP. The result implied a role of mGluR4 in the synergy. However, L-AP4 primarily induces a decrease in holding current, or membrane hyperpolarization ( see chapter 3; Bigiani et al., 1997); and decreases in the intracellular $Ca^{2+}$ concentration (Hayashi et al., 1996). This is inconsistent with the observation that glutamate primarily depolarizes taste cells (Hayashi et al., 1996) and increases the frequency of action potentials in afferent nerves (Sato et al., 1970; Ninomiya and Funakoshi, 1989). Thus, mGluR4 is not likely to be the only receptor for the taste transduction of glutamate.

Results in Chapter 3 indicated that NMDA receptor channels are also present in fungiform taste cells. Activation of NMDA receptors depolarizes taste cells and increases the intracellular $Ca^{2+}$ concentration by allowing $Na^{+}$ and $Ca^{2+}$ influx. Although NMDA receptors may be responsible for the depolarization and increase in intracellular $Ca^{2+}$ levels induced by glutamate (Hayashi et al., 1996; Lin et al., 1996), NMDA did not mimic the response to glutamate in a behavioral study (Chaudhari et al., 1996). Thus, the possibility that NMDA receptors serve a different function in taste cells, such as a receptor for synaptic transmission, can not be ruled out. As discussed in chapter 3, further experiments are required to determine if both NMDA and mGluR4 are located on apical membrane, so that they could be co-activated during glutamate taste stimulation.

Another important umami taste stimulus is 5'-GMP. Data in Chapter 4 suggest that 5'-GMP may activate a different set of receptors, since responses to 5'-GMP and glutamate do not always occur in the same cells. The hypothesis of different receptors for 5'-GMP and glutamate is consistent with recordings from afferent nerves and central neurons (Ninomiya and Funakoshi, 1989; Adachi and Aoyama, 1991; Hellekant and Ninomiya, 1991; Scott et al., 1993). Also, as shown in Chapter 4, a small set of taste cells respond to 5'-GMP and glutamate synergistically. Most of these synergistic responses were antagonized by cAMP, which suggests that the synergy may occur through the amplification of intracellular signaling pathways. However, this preliminary study did not reveal any potential mechanisms for intracellular amplification. Further studies are required to determine the steps in the pathway and to determine which glutamate receptors may be involved. Alternatively, 5'-GMP could also bind to glutamate receptors in addition to activating its own receptor. Examination of 5'-GMP responses in the presence and absence of glutamate antagonists may provide useful information about the identity of the receptors involved. A psychophysical study suggested purinergic receptors may be involved in the transduction of 5'-GMP (Schiffman and Gill, 1987). Alternatively 5'-GMP may bind to sweet receptors, since some of the synergistic responses occur in sweet-sensitive fibers and neurons (Ninomiya and Funakoshi, 1989; Adachi and Aoyama, 1991). In addition, It will be interesting to know if mGluR4 and 5'-GMP decrease intracellular cAMP levels by activation of phosphodiesterase (PDE) through gustducin, a taste-specific G protein that has been reported to be involved in sweet and bitter tastes (Wong et al., 1996).

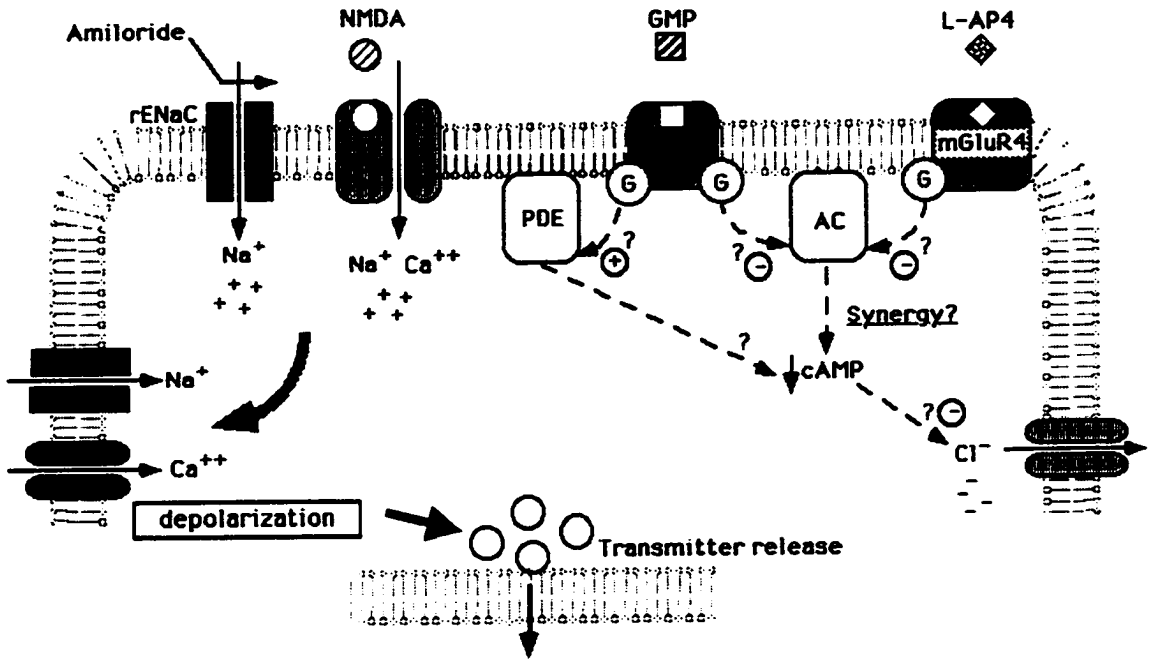



Fig. 6.1. Putative transduction mechanisms of monosodium glutamate.

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# Appendix

My dissertation focuses on the transduction mechanisms of monosodium glutamate in taste. In addition, I participated in two collaborative studies that provided some background information for the dissertation research. Since these projects were not closely related to the main theme of the dissertation, I did not include them in the dissertation. Results of both studies have been published. The first paper, entitled "Development of membrane properties in taste cells of fungiform papillae: functional evidence for early presence of amiloride-sensitive sodium channels" by A.H. Kossel, M. McPheeters, W. Lin and S.C. Kinnamon was published in the Journal of Neuroscience, 17:9634-9641, 1997. In this paper, I provided the dose-dependence of amiloride block for amiloride-sensitive $Na^+$ channels in neonatal and adult rats. The second paper entitled "A basolateral chloride conductance in rat lingual epithelium" by S.L. Wldkowski, W. Lin, M. McPheeters, S.C. Kinnamon and S. Mierson was published in the Journal of Membrane Biology, 164:91-101,1998. I recorded and characterized chloride currents in taste cells and wrote a draft of the results for the manuscript.

I appreciated the opportunity to study and collaborate with other scientists in these projects.