Acetylcholine Increases Intracellular Ca$^{2+}$ in Taste Cells Via Activation of Muscarinic Receptors

TATSUYA OGURA
Department of Anatomy and Neurobiology, Colorado State University, Fort Collins 80523; and Rocky Mountain Taste and Smell Center, University of Colorado Health Sciences Center, Denver, Colorado 80262

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Acetylcholine increases intracellular Ca$^{2+}$ in taste cells via activation of muscarinic receptors. J Neurophysiol 87: 2643–2649, 2002; 10.1152/jn.00610.2001. Previous studies suggest that acetylcholine (ACh) is a transmitter released from taste cells as well as a transmitter in cholinergic efferent neurons innervating taste buds. However, the physiological effects on taste cells have not been established. I examined effects of ACh on taste-receptor cells by monitoring [Ca$^{2+}$]i. ACh increased [Ca$^{2+}$]i in both rat and mudpuppy taste cells. Atropine blocked the ACh response, but D-tubocurarine did not. U73122, a phospholipase C inhibitor, and thapsigargin, a Ca$^{2+}$-ATPase inhibitor that depletes intracellular Ca$^{2+}$ stores, blocked the ACh response. These results suggest that ACh binds to M1/M3/M5-like subtypes of muscarinic ACh receptors, causing an increase in inositol 1,4,5-trisphosphate and subsequent release of Ca$^{2+}$ from the intracellular stores. A long incubation with ACh induced a transient response followed by a sustained phase of [Ca$^{2+}$]i increase. In Ca$^{2+}$-free solution, the sustained phases disappeared, suggesting that Ca$^{2+}$ influx is involved in the sustained phase. Depletion of Ca$^{2+}$ stores by thapsigargin alone induced Ca$^{2+}$ influx. These findings suggest that Ca$^{2+}$ store-operated channels may be present in taste cells and that they may participate in the sustained phase of [Ca$^{2+}$]i increase. Immunocytochemical experiments indicated that the M1 subtype of muscarinic receptors is present in both rat and mudpuppy taste cells.

INTRODUCTION

The synaptic neurotransmitters or neuromodulators released at chemical synapses in taste buds have not been identified. However, several neurotransmitter and neuromodulators are present in taste buds and in nerve fibers innervating taste buds (see Nagai et al. 1996). Previous anatomical studies showed that three types of chemical synapses are present in taste buds: synapses between taste-receptor cells and afferent sensory nerve fibers, synapses between efferent nerve fibers and taste-receptor cells, and, in amphibia and fish, synapses between taste-receptor cells and Merkel-like basal cells (Roper 1992). Afferent synapses relay information about taste quality and intensity to the brain, while efferent synapses in taste buds and basal cells likely regulate or modulate signal transduction in taste-receptor cells. In addition to the synapses that release neurotransmitters, ACh may play a role in neuronal activity by acting on postsynaptic receptors located on afferent nerve terminals or taste cells. In the present study, Ca$^{2+}$ imaging and immunocytochemistry were used to examine whether ACh receptors are present in taste cells of mudpuppy and rat. The data suggest that muscarinic receptors are present in taste cells and that ACh may play a physiological role in taste receptor function.
induces both Ca\(^{2+}\) release from the internal stores and Ca\(^{2+}\) influx.

**METHODS**

**Isolation of taste-receptor cells of mudpuppy**

Mudpuppies (*Necturus maculosus*) were obtained from commercial sources and housed in fish tanks at 10°C and regularly fed with minnows. Taste-receptor cells were isolated as described previously (Kinnamon et al. 1988a; Ogura et al. 1997). Briefly, mudpuppies were decapitated after anesthesia in ice-cold water, and the lingual epithelium was dissected from the underlying connective tissue. The apical surface of the stripped epithelium was then incubated for 15 min in fluorescein-conjugated wheat germ agglutinin (Molecular Probes: 0.5 mg/ml in amphibian physiological saline, APS), so that mature taste cells could be distinguished from other cell types after isolation (Kinnamon et al. 1988b). The epithelium was then incubated in APS containing collagenase (0.5 mg/ml; Sigma, Type I), bovine albumin (1 mg/ml), and glucose (5 mM), for about 20 min. The epithelium was then gently separated from the underlying connective tissue, leaving the taste buds atop their connective tissue papillae. Incubation with Ca\(^{2+}\)-free APS breaks connection between adjacent cells. Isolated taste cells were collected by gentle suction with a glass pipette and plated onto recording chambers made with cover slips coated with Cell-Tak (Collaborative Research).

**Isolation of taste buds of rat**

Taste buds were isolated from rat circumvallate, foliate, and fungiform papillae according to the method of Béhé et al. (1990). Briefly, adult Sprague-Dawley rats were killed with CO\(_2\), and their tongues, tissues were labeled with the fluorescent dye fura-2 AM for 10–20 min, then washed with normal bath solution for 20 min. Images were acquired with an intensified CCD camera (IC100-ICCD, Pauletek Imaging) through an oil-immersion objective lens (Fluor 1.3 NA, Nikon) of an inverted microscope (Diaphot TMD, Nikon). The video signal from the camera was captured using Axon Imaging Workbench software with Axon Image Lightning 2000 video capture board on a PC computer. For dual-wavelength ratiometric measurement, fura-2 images were obtained at EX350 and EX380 nm. A set of F350- and F380-nm images was captured every 2 s to record fast responses or at 5- or 10-s intervals during slow response or under control conditions to prevent bleaching of the fura-2 fluorescence. Averaged Ca\(^{2+}\) levels over the entire cell area were plotted as F350/F380 over time.

**Intracellular calcium measurement**

[Ca\(^{2+}\),] in isolated taste-receptor cells from mudpuppy was measured using the membrane-permeable Ca\(^{2+}\)-sensitive dye fura-2 AM by a method adapted from our previous study (Ogura et al. 1997). Briefly, cells were loaded with fura-2 AM (2 \(\mu M\), Molecular Probes) for 10–20 min, then washed with normal bath solution for 20 min. Images were acquired with an intensified CCD camera (IC100-ICCD, Pauletek Imaging) through an oil-immersion objective lens (Fluor X40, 1.3 NA, Nikon) of an inverted microscope (Diaphot TMD, Nikon). The video signal from the camera was captured using Axon Imaging Workbench software with Axon Image Lightning 2000 video capture board on a PC computer. For dual-wavelength ratiometric measurement, fura-2 images were obtained at EX350 and EX380 nm. A set of F350- and F380-nm images was captured every 2 s to record fast responses or at 5- or 10-s intervals during slow response or under control conditions to prevent bleaching of the fura-2 fluorescence. Averaged Ca\(^{2+}\) levels over the entire cell area were plotted as F350/F380 over time.

**Immunohistochemistry**

Rats were anesthetized and perfused with 4% paraformaldehyde in 0.1 M PBS. Tongues were removed and post fixed for 2 h. For mudpuppies, tongues were removed and fixed overnight. The tissue was frozen and cut into 30-μm-thick sections. Sections were incubated with an affinity-purified polyclonal antibody against the human M1 subtype of muscarinic ACh receptor (1:100–200, Alomone Labs). The antibody is raised in rabbit against purified glutathione-S-transferase (GST)-fusion proteins containing a part of the i3 intracellular loop of the human m1 muscarinic acetylcholine receptor (amino acids 227–353) (Peralta et al. 1987). Immunoreactivity was visualized with rhodamine-conjugated secondary antibody (Jackson Immuno Research Laboratories, Lissamine rhodamine-conjugated Affinytide Fluor Fragment goat-anti rabbit IgG, No. 111-087-003). Fluorescence images were obtained using a confocal microscope system (Olympus).

To estimate the percentage of immunoreactive taste-receptor cells of rat foliate and circumvallate papillae, nuclei were counterstained with propidium iodide, and both immunoreactive and nonimmunoreactive cells were counted. Briefly, tissues were labeled with the antibody against the M1 subtype receptor as described in the preceding text except the secondary antibody was conjugated with Alexa Fluor 488 (Molecular Probes). Sections were pretreated with 0.5 mg/ml RNase A (Boehringer) at 37°C for 30 min. The RNase was preboiled 5 min to inactivate residual DNase. Finally, sections were treated with propidium iodide (1 μg/ml in PBS) for 1 min.

**Solutions**

Normal APS contained (in mM) 112 NaCl, 2 KCl, 8 CaCl\(_2\), and 3 HEPES, buffered to pH 7.2 with NaOH. Ca\(^{2+}\)-free APS contained either 1 mM bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid (BAPTA, for cell isolation) or 1 mM EGTA (for Ca\(^{2+}\) imaging) without CaCl\(_2\) in normal APS. Normal Tyrode’s solution contained (in mM) 140 NaCl, 5 KCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 10 Na pyruvate, 10 glucose, and 10 HEPES, buffered to pH 7.2 with NaOH. Ca\(^{2+}\)-free Tyrode’s solution contained either 2 mM BAPTA (for cell isolation) or 1 mM EGTA (for Ca\(^{2+}\) imaging) without CaCl\(_2\) and MgCl\(_2\) in normal Tyrode’s.

**RESULTS**

**ACH increases [Ca\(^{2+}\)]\(_i\) in taste-receptor cells**

Intracellular Ca\(^{2+}\) levels were measured in isolated taste cells of mudpuppy and rat circumvallate papillae using calcium imaging with the Ca\(^{2+}\)-sensitive fluorescent dye fura-2. Responses to ACh were quite similar in both mudpuppy and rat of some of the cells in the focal plane is distinguishable (see Bernhardt et al. 1996). Averaged Ca\(^{2+}\) levels over cell areas were plotted as F350/F380 over time. One focal plane per bud was captured; this eliminated repeat measurements from the same cells.

Cells were bathed in normal saline until the resting intracellular calcium level was stable. The bath was then perfused with acetylcholine chloride (ACh) solution (10 nM to 1 mM, Sigma). Washing with normal saline followed until the intracellular calcium again reached prestimulus levels. Other treatments included: t-tubocurarine (250 μM, Sigma), atropine (0.5 μM, Sigma), thapsigargin (1 μM for 10–15 min, Sigma), U73122 (5 μM for 5–10 min, Calbiochem), and Ca\(^{2+}\)-free solution.

Cells were considered to respond to ACh if the increase in [Ca\(^{2+}\)]\(_i\), was more than 2 SDs above the mean resting level obtained by averaging 5 data points before applying ACh in each cell tested. The effects of drug treatments on the ACh response were assessed using paired Student’s t-tests. Statistical values are presented as mean [Ca\(^{2+}\)]\(_i\), ± SE.
taste cells. ACh (10 μM) induced increases in [Ca^{2+}]_i in many cells tested (72 of 86 cells in mudpuppy and 79 of 120 cells in rat). The peak response occurred within 10 s from the beginning of ACh application (Fig. 1, A and C). A muscarinic ACh receptor antagonist atropine (0.5 μM) blocked the ACh responses, but a nicotinic ACh receptor antagonist d-tubocurarine (250 μM) did not (Figs. 1, A and C, and 3). The effect of atropine was statistically significant (P < 0.001, Fig. 3). The effect of d-tubocurarine was not significant (P > 0.05, Fig. 3). The data suggest that ACh induces increases in [Ca^{2+}]_i via muscarinic ACh receptors in both mudpuppy and rat circumvallate taste cells. The magnitude of the peak response was dose dependent between 10 nM and 1 mM (Fig. 1, B and D).

In other cell types, ACh activates G-protein-coupled muscarinic receptors, causing an increase in inositol 1,4,5-trisphosphate (IP3) and subsequent release of Ca^{2+} from intracellular Ca^{2+} stores. To determine whether ACh induces Ca^{2+} release from Ca^{2+} stores, I used a Ca^{2+}-ATPase inhibitor thapsigargin that causes depletion of Ca^{2+} stores. Thapsigargin (1 μM) increased [Ca^{2+}]_i to a variable extent in taste cells as reported previously (Ogura et al. 1997; discussed later). After incubation with thapsigargin, ACh failed to increase [Ca^{2+}]_i (Figs. 2, A1 and B1, and 3). This effect of thapsigargin on the ACh response was statistically significant (P < 0.001, Fig. 3). The data strongly suggest that ACh releases Ca^{2+} from the stores. To determine whether ACh activates phospholipase C (PLC)
and consequently produces IP₃, taste cells were treated with the PLC inhibitor U73122 (5 μM). After incubation with U73122, the ACh-induced Ca²⁺ responses were inhibited (Figs. 2, A2 and B2, and 3). This effect of U73122 on the ACh responses was significant (P < 0.001, Fig. 3). The data strongly suggest that the IP₃ pathway is involved in the ACh response in both mudpuppy and rat circumvallate taste cells.

Increases in [Ca²⁺], in response to ACh were also observed in taste cells of rat foliate and fungiform papillae (data not shown). These data strongly suggest that taste cells in rat as well as mudpuppy respond to ACh via muscarinic receptors.

Currently five subtypes of muscarinic ACh receptors have been cloned in muscarinic cells. Three types of them, M1, M3, and M5 are considered to activate PI metabolism (Caulfield 1993; Felder 1995). Thus M1, M3-, or M5-like receptors are involved in the Ca²⁺ response to ACh in taste-receptor cells.

**Ca²⁺ entry during sustained ACh responses**

In the presence of extracellular Ca²⁺, a long incubation with ACh induced a transient response followed by a sustained phase in mudpuppy taste cells (n = 32; Fig. 4). In Ca²⁺-free saline, only transient responses persisted and sustained phases disappeared (n = 16), suggesting that Ca²⁺ influx is involved in the sustained phase. Subsequently, adding external Ca²⁺-induced increases in [Ca²⁺], suggesting Ca²⁺ entry through Ca²⁺-store-operated channels (SOC) (Parekh and Penner 1997). In control experiments, where Ca²⁺-free solution was added in the absence of ACh, there were no large increases in [Ca²⁺], on return to a Ca²⁺-containing solution (n = 4; data not shown). SOCs are activated solely by store depletion without requirement of a receptor-mediated mechanism, a mechanism also known as “capacitative calcium entry” (CCE) (see Putney and McKay 1999). Therefore it was examined whether SOCs are present in taste cells using thapsigargin to deplete Ca²⁺ stores. After incubation with thapsigargin in the absence of external Ca²⁺, addition of external Ca²⁺ induced a large increase in [Ca²⁺], (n = 13); this is typical following SOC activation (see Putney and McKay 1999). Interestingly, during incubation with thapsigargin, increases in [Ca²⁺], in the absence of external Ca²⁺ appeared to be smaller than those in the presence of external Ca²⁺ (compare Figs. 2A1 and 4). This suggests that Ca²⁺ influx contributes part of the increase in [Ca²⁺], during incubation with thapsigargin in the presence of external Ca²⁺. These data strongly suggest that SOCs are present in ACh-responsive taste cells. It is possible that the sustained part of the ACh-induced calcium response is mediated in part by Ca²⁺ influx through SOCs.

**Immunoreactivity for muscarinic ACh receptor protein**

Physiological data suggest that ACh activates the IP₃ pathway via M1/M3/M5-like muscarinic ACh receptors. To determine if muscarinic receptor proteins are present in taste-receptor cells, I examined immunoreactivity for the human M1 subtype of muscarinic ACh receptors. In sections of rat circumvallate and foliate papillae, immunoreactivity for the M1 subtype of muscarinic ACh receptors was present in many taste cells of each taste bud (Fig. 5, A and B). Estimated percentages

![FIG. 3. Summarized data of ACh-induced changes in intracellular Ca²⁺ levels in mudpuppy (A) and rat (B). Maximum ACh-induced changes are expressed as a percentage of resting level before applying ACh. Cells were tested twice, before (○) and during or after (●) the treatments indicated, as illustrated in Figs. 1 and 2 (atropine; mudpuppy: n = 10, paired Student’s t-test = 5.73; rat: n = 56, paired Student’s t-test = 5.28), d-tubocurarine (dTC; n = 9, paired Student’s t-test = 0.74; rat: n = 28, paired Student’s t-test = 0.98), thapsigargin (mudpuppy; n = 8, paired Student’s t-test = 9.97; rat: n = 13, paired Student’s t-test = 4.92), and U73122 (mudpuppy; n = 8, paired Student’s t-test = 5.21; rat: n = 49, paired Student’s t-test = 11.7)]. *; significant effect (P < 0.001).](http://jn.physiology.org/doi/10.1152/jn.00952.2002)
of immunoreactive taste cells were 65% in circumvallate papillae (n = 230 cells) and 59% in foliate papillae (n = 263 cells). Interestingly, heavy label was observed in the apical regions of taste buds (see Fig. 5C). No selective labeling was observed in control sections, in which primary antibody was omitted (D1; fluorescent image, D2; light image of the same section). Preabsorption with antigen signiﬁcantly reduced the labeling. The results indicated that M1 subtype of muscarinic ACh receptor is present in rat and mudpuppy taste cells.

**DISCUSSION**

**Muscarinic ACh receptors in taste cells**

The data presented here demonstrate that muscarinic ACh receptors are present in many taste cells of mudpuppy and rat. ACh induced increases in [Ca\(^{2+}\)]\(_i\) were inhibited by the muscarinic receptor antagonist atropine. In addition, responses were inhibited by both the PLC inhibitor U73122, and thapsigargin, an inhibitor of Ca\(^{2+}\)-ATPase at membrane of Ca\(^{2+}\) stores. These data suggest that ACh produces IP\(_3\) via PLC activation resulting in release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores (see Fig. 7). These data are consistent with a previous study showing that carbachol, a cholinergic agonist, increases IP\(_3\) levels of rat lingual epithelium containing rat taste buds (Hwang et al. 1990). Currently, five subtypes of muscarinic ACh receptors have been cloned from other types of tissues. M1, M3, and M5 subtypes of muscarinic receptors are considered to activate the G\(_{q/11}\) class of G proteins to produce IP\(_3\) via PLC (see Felder 1995). Accordingly, immunoreactivity to the human M1 receptors was observed in both rat and mudpuppy taste buds, suggesting that this receptor mediates the response. Previous reports showed that antibodies against human M2 and human M4 receptors could detect the M2 and M4 receptors in newt retina (Cheon et al. 2001) and that the receptor subtypes are well conserved in mammalian species (89–98%) (Bonner 1989), so it is not surprising that an antibody to the human M1 receptor recognized the amino acid sequence in both mudpuppy and rat taste cells. The epitope for the antibody tested is unique for M1 receptors and is not present in other related proteins, including other muscarinic receptor subtypes (Peralta et al. 1987). The percentage of ACh responsive taste cells and the percentage of immunoreactive taste cells to the M1 subtype receptor in rat circumvallate papillae were similar. However, this does not rule out of the possibility of the presence of M3 and/or M5 receptor subtypes in taste cells. Further studies would reveal whether other subtypes of muscarinic receptors are present in taste cells.

**Possible location of muscarinic ACh receptors**

A previous study showed that choline acetyltransferase, a key biosynthetic enzyme for ACh, has been found in taste-bud cells and in axons innervating taste buds in rats and mice (Kim and Roper 1994). These data suggest that ACh could be released from taste-receptor cells and/or from nerve endings of efferent nerve fibers innervating taste buds. Interestingly, previous anatomical reports indicated that only subsets of taste-receptor cells have synaptic connections with afferent nerve fibers in mammalian taste buds (Kinnamon et al. 1988c; Royer and Kinnamon 1988, 1994). In the present study, however, many taste cells responded to ACh and many taste cells were immunoreactive for the M1 receptor. These findings suggest that ACh released from taste cells may have a role other than...
synaptic transmission between taste cells and nerve endings. ACh released from taste cells may regulate adjacent taste cells through autoregulatory mechanisms. Synapses have not been observed between taste-receptor cells, although synapses between taste-receptor cells and basal cells are common in mud-puppy taste buds (Delay and Roper 1988).

Several studies have provided some evidence for efferent synapses or bidirectional synapses between taste cells and nerve fibers in both mudpuppies and rat (Delay and Roper 1988; Ewald and Roper 1994a; Yang et al. 2000). Clear vesicles are found at synaptic sites in taste buds (Delay and Roper 1988; Yang et al. 2000), which, in other systems, are known to contain ACh (Betz and Henkel 1994; Wiley et al. 1987).

ACh-induced Ca\(^{2+}\) influx

A long incubation with ACh induced a sustained phase of [Ca\(^{2+}\)]\(_i\) in taste cells due to [Ca\(^{2+}\)]\(_e\) entry from extracellular sources. Stimulation of muscarinic ACh receptors activates SOCs in neuroblastoma cells (Mathes and Thompson 1995), smooth muscle (Wayman et al. 1996), parotid acinar cells (Takeamura et al. 1989), and lacrimal acinar cells (Kwan et al. 1990). The present data suggest that SOCs may contribute to the sustained phase of responses to ACh in taste cells (see Fig. 7). However, I cannot rule out the participation of other receptor-operated Ca\(^{2+}\) channels and nonselective cation channels in mediating Ca\(^{2+}\) influx. Currently, there are no specific inhibitors to distinguish SOCs from other Ca\(^{2+}\)-permeable channels. Because several bitter stimuli cause release of Ca\(^{2+}\) from intracellular stores, SOCs may contribute to these responses as well (Ogura et al. 2002). Because sustained elevation of [Ca\(^{2+}\)]\(_i\) is a key factor for intracellular signaling, ACh-induced Ca\(^{2+}\) influx may play an important role in taste responses.

One of the physiological functions of ACh might be to modulate taste responses because preliminary results suggest that preincubation with ACh could attenuate responses to the bitter stimulus denatonium (Ogura 2001). Further study will be required to determine the mechanisms by which ACh modulates taste responses.

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FIG. 7. Proposed model for intracellular signaling mechanisms in response to ACh in taste-receptor cells. ACh binds to M1 like muscarinic receptors; this then induces Ca\(^{2+}\) release from Ca\(^{2+}\) stores via the IP3 pathway. Depletion of the Ca\(^{2+}\) stores induces Ca\(^{2+}\) entry, possibly through store-operated channels.


