理學博士學位論文

The role of G-protein-coupled receptor induced Ca^{2+} signaling in salivary gland cells

타액선 G-protein-coupled receptor에 의한 세포내 칼슘신호의 역할

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이 계 민

ABSTRACT

The role of G-protein-coupled receptor induced-Ca^{2+} signaling in salivary gland cells

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Salivation is an important function of the salivary gland. The cells of salivary gland are non-excitable cells which lack the expression of voltage-sensitive channel but express G-protein coupled receptor (GPCR) which receives external signals and conducts physiological functions. In salivary gland, the function of GPCR is crucial for salivation. Therefore, a change in the signal transduction conducted by GPCR is thought to cause changes in salivation. The excretion of saliva from salivary gland is well known to be controlled by different external factors including neural input; however, not much research has been done on identifying the different types of GPCR that are involved in transferring these external signals. Also, the effect of different pharmaceutical drugs that cause xerostomia on the function of GPCR is not well established. Therefore, doing a research on identifying the role of GPCR signal transduction in salivary gland cell withholds a great

significance and it has the potential to improve the field of exocrine physiology and clinical dentistry. By observing the effect of lipid raft disruption in three form of membrane events involved in muscarinic transcellular water movement, I attempted to understand the contribution of lipid raft in each steps of water movement. Thus, it was focused on identifying the correlation between the underlying mechanism of salivation and antidepressant. Especially, the results were revealed the involvement of the signal transduction conducted by GPCR with salivation. Furthermore, I revealed many different receptors, ion channels and membrane transporters that are yet undiscovered and understand the external signal transmission mechanism to identify the connection between the salivation and GPCR signal transduction pathway. First, I examined for changes in physiology functions of the GPCR in salivary gland cells by disruption of the lipid raft which is known to play an important role in membrane physiology. In this study, through the depletion of cholesterol, I can explain that MβCD effects on the salivary GPCR. Next, I treated desipramine in the salivary gland cells and confirmed mechanisms accordingly. Desipramine is known to cause dry mouth when taken long term. However, because not yet clarified for its mechanism, my results can be explained due to dry mouth caused

desipramine. Finally, I found new GPCR in the human salivary gland cells. I was confirmed the expression of the bradykinin receptor, and my results suggested the role of bradykinin in human salivary gland cells.

Taken together, through this paper, the relationship between the lipid raft depletion and GPCR signaling were identified. And I described the mechanism of dry mouth caused by desipramine in salivary gland cells. Also I demonstrated the role of bradykinin in human salivary gland cells. So, I suggested that it is important to study the function of the salivary GPCR-induced calcium signals.

Key Words: Salivary glands, GPCR, Calcium, Aquaporin5, Lipid raft, Xerostomia, Desipramine, Bradykinin

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General Introduction

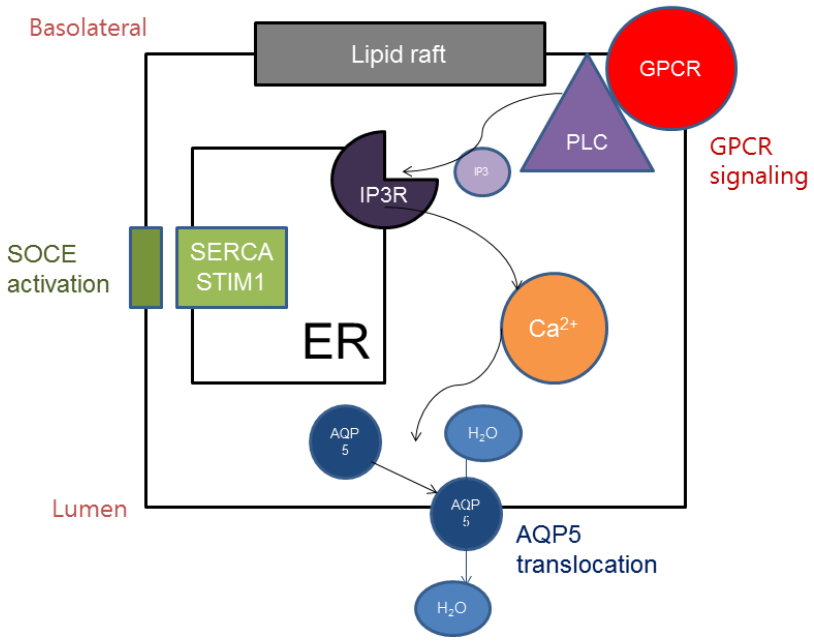
The salivary glands are exocrine organs formed saliva went to the inside of the epithelial surface is to be released into the oral cavity. The salivary glands are composed of acinar cell and ductal cell. The position can be divided into the parotid gland, submandibular gland, sublingual gland.

Saliva is known to play an important role in preserving homeostasis in the oral cavity. Homeostasis, including osmolarity, pH and hydration level, is controlled by the excretion of saliva. Salivation is an important function of the salivary gland. The cells of salivary gland are a non-excitable cells which lack the expression of voltage-sensitive channel but express G-protein coupled receptor (GPCR) which receive external signals and conduct physiological functions. The activation of GPCR in the salivary gland cells is already known to increase the intracellular Ca^{2+} level. As an example, acetylcholine released from parasympathetic nerve terminal activates muscarinic M3 receptors, Gq/11 protein and phospholipase C and cause an increase in intracellular Ca^{2+} level. This change in intracellular Ca^{2+} level causes movement of water in the salivary gland cell and eventually leads to excretion of saliva into the environment. There are two main types of water transportation in the salivary gland, transcellular pathway and paracellular

pathway. Transcellular pathway is involved with the movement of a water channel called aquaporin under external stimulus. Aquaporin is water-permeable transmembrane protein involved in the physiology of these secretory gland functions. The expression and roles of AQPs have been studied in secretory glands where their main function is in fluid secretion. When acetylcholine is present, AQP5 traffics in vitro from intracellular vesicles to the acinar plasma membrane. Paracellular pathway is involved with the release of Na^+ and water through intercellular space under particular condition produced by transporter mediated ion movement and movement of Cl^- to the luminal surface. These two pathways are known to be activated by the change in intracellular Ca^{2+} level. In salivary gland, the function of GPCR is crucial for salivation. Therefore, a change in the signal transduction conducted by GPCR is thought to cause changes in salivation. Xerostomia, also known as “dry mouth”, is a condition in which the level of salivation decrease consistently. The patients with xerostomia usually experience difficulty in maintaining homeostasis in their oral cavity and usually these patients end up with a secondary disease called the rampant caries. The excretion of saliva from salivary gland is well known to be controlled by different external factors including neural input; however, not much research has been done on identifying the different types of GPCR that are involved

in transferring these external signals. Also, the effect of different pharmaceutical drugs that cause xerostomia on the function of GPCR is not well established. Therefore, doing a research on identifying the role of GPCR signal transduction in salivary gland cell withholds a great significance and it has the potential to improve the field of exocrine physiology and clinical dentistry.

In the field of oral biology, many researchers have been focusing their experiments on the salivation of the patients with Sjogren's syndrome. In addition, more research has been conducted to explore the side effect of radiation therapy for Sjogren's syndrome which is also known to decrease salivation. With the aids of these researches, the clinical therapy for Sjogren's syndrome has improved with greater efficiency. Unlike the effect of Sjogren's syndrome and radiation therapy, not much experiment are being done on the effect of pharmaceutical drug on the salivary gland. In fact, drugs ingestion is a form of therapy which is more frequently practiced in my daily life and therefore, greater understanding of its side effect is urgent. In this thesis, the effect of different pharmaceutical drugs on the physiology of salivary gland and salivation is observed and greater emphasis is made on its linkage to the GPCR signaling transduction.



Ca^{2+} signaling and regulation of fluid secretion in salivary gland acinar cells.

CHAPTER 1:

Salivary G-protein coupled receptor mediated Ca^{2+} signaling and Lipid Rafts

Abstract

Lipid raft is located within the cell membrane and it is known to have an important physiological function; however, it is unclear whether the disruption of lipid raft has an impact on the function of the whole cell membrane or have only a partial impact. The functions that are influenced by the disruption of the lipid raft can have a constituent element that is either placed on top of the lipid raft or be regulated by the structure of the lipid raft. By observing the effect of lipid raft disruption in three form of membrane events involved in muscarinic transcellular water movement, I attempted to understand the contribution of lipid raft in each steps of water movement. In my lab, I demonstrated how MβCD incubation, which disrupts the lipid raft by depleting cholesterol**,** inhibits muscarinic and histamine receptor mediated Ca^{2+} signaling but do not effect thapsigargin-induced Ca^{2+} signaling. In addition, I also demonstrated how MβCD incubation inhibits all muscarinic histamine and thapsigargin mediated translocation of aquaporin.

Introduction

Lipid raft is known that many proteins together to send and receive signals. In particular, lipid raft in polarity cells play a critical role in the physiological function (Kai Simons and Derek Toomre, 2000). There are also a variety of lipid rafts in the receptor, and a platform that transfers the signal into the cell outside the cell. Lipid rafts have been mainly studied in epithelial and immune cell, it has recently been revealed about the functions of the exocrine. The function of the lipid raft has been reported in many types of cells, but little was known about its role in the salivary glands. Salivation is an important function of the salivary gland. The cells of salivary gland are a non-excitable cells which lack the expression of voltage-sensitive channel but express G-protein coupled receptor (GPCR) which receive external signals and conduct physiological functions. The activation of GPCR in the salivary gland cells is already known to increase the intracellular Ca^{2+} level. GPCR activation, store operated Ca^{2+} entry (SOCE) activation and AQP5 translocation are activated by membrane protein. However, it was unclear how lipid raft works with them. A recent study shows that activation of M3 mAChRs increases flotillin (lipid raft marker) and AQP5 co-localization (Ishikawa et al., 2005). However, there is not study about comparison GPCR

signaling with SOCE activation in lipid raft disruption. Therefore, I are going to study how lipid raft structure change after M3 receptor activation and AQP5 translocation in membrane. I demonstrate GPCR- Ca^{2+} signaling and AQP5 translocation relationship. In order to understand molecular mechanism of salivary lipid raft, I explain effect of MβCD incubation to GPCR signaling. In this study, I monitored the effect of MβCD on salivary Ca^{2+} signaling. Ca^{2+} signaling and aquaporin translocation in human salivary gland HSG cells have been used to study the pathological mechanisms of salivary impairment such as in Sjogren's syndrome (Ishikawa et al., 2005; Jin et al., 2012a; Lee et al., 2013). I tested the effect of MβCD on the salivary receptors expressed in salivary glands and HSG cells and known to induce Ca^{2+} signaling in an attempt to understand the correlation GPCR and lipid raft.

MATERIALS AND METHODS

Cell Preparation

Human submandibular salivary gland cells were prepared as previously described (Kim et al., 2009). HSG cells were grown in Modified Eagle's Medium (GIBCO, Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (GIBCO) and 1% (v/v) penicillin (5000 U/ml) + streptomycin (5000 μg/ml) solution (GIBCO). The cells were cultured in a humidified atmosphere of 95% air + 5% CO_2. The culture medium was changed every 2 days, and the cells were subcultured weekly.

$[Ca^{2+}]_i$ Measurement

Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was analyzed with fura-2/AM (Seo et al., 2010). Briefly, the cell suspension was incubated with 3 μM fura-2/AM at 37^{o}C for 60 minutes with continuous stirring. Sulfinpyrazone (250 μM) was added to prevent dye leakage. The cells were then washed with Locke's solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 2.2 mM $CaCl_2$, 5.0 mM HEPES, 10 mM glucose, pH 7.4 with NaOH). Changes in the fluorescence ratio were measured at dual excitation wavelengths of 340 and

380 nm with an emission wavelength of 500 nm.

Construction of Aquaporin-5 and Western blotting

Total RNA was isolated from HSG cells, and the full-length cDNA extracted from it was amplified with reverse-transcriptase PCR (RT-PCR). PCR utilized 5'-GAATTCTATGAAGAAGGAGGTGTGCTCC as the upstream primer and 5'-GGTACCGCGGGTGGTCAGCTCCATGGT as the downstream primer (the introduced EcoRI and KpnI restriction sites are underlined). The 798-bp human aquaporin-5 gene was then cloned into pEGFP-C1. HSG cells were transiently transfected with the aquaporin-5 gene using LipofectAMINE 2000 (Invitrogen). Aquaporin-5-transfected HSG cells were grown in a 60-mm dish, pre-incubated with MβCD for 30 min, and incubated with carbachol or histamine for 20 min at 37°C. The apical membrane fraction was sonicated in ice-cold 20 mM HEPES solution containing 1 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, and 0.3 mM phenylmethylsulfonyl fluoride (3×30 s, Branson Sonifer) at pH 7.4. The samples were centrifuged at 600×g at 4 °C, and the supernatants were further centrifuged at 20,000×g at 4 °C. The pellets (P2 membrane fractions) were subjected to SDS/PAGEβ immunoblotting. After electrophoresis in SDS-

PAGE (10 %) gel, the protein samples were transferred to polyvinylidene difluoride membranes (0.45 μm, Millipore). After blocking, the membrane was incubated with anti-aquaporin-5 as the primary antibody (1:200, Santa Cruz, sc-9891). The membrane was then washed and incubated with donkey anti-goat IgG-HRP. The membrane was finally subjected to an electrochemiluminescence assay. For normalization of the antibody signal, the membranes were stripped and re-probed with antibody for anti-α1 Na^+/K^+-ATPase (1:1000, Abcam, ab7671).

Fluorescence imaging

GFP-tagged cells transfected with aquaporin-5 were plated on glass coverslips coated with poly-L-ornithine. MβCD for 30 min, and incubated with carbachol or histamine or thapsigargin for 20 min at 37°C. The cells were fixed with 4% paraformaldehyde in PBS for 15 min. Slides were mounted and visualized using a laser scanning confocal microscopy (Zeiss LSM 700).

RESULT

MβCD preincubation inhibits muscarinic and histaminergic $[Ca^{2+}]_i$ increase without effect on thapsigargin-mediated $[Ca^{2+}]_i$

I monitored the effect of lipid raft depletion on salivary G-protein coupled receptor signaling in human salivary gland HSG cells, which have been broadly used for salivary Ca^{2+} studies. 10 μM MβCD inhibited the cytosolic increases in Ca^{2+} induced by carbachole (Figure 1-1A). MβCD also inhibited the histamine-induced increases in $[Ca^{2+}]_i$ (Figures 1-1B). However, MβCD did not affect the increase in $[Ca^{2+}]_i$ induced by thapsigargin, which mimics the downstream signaling of phospholipase C activation such as the depletion of the cytosolic Ca^{2+} pool and the induction of store-operated Ca^{2+} entry (Figure 1-1C). These results suggest that MβCD selectively inhibits GPCR Ca^{2+} signaling.

MβCD depletes cholesterol in HSG cells.

It is already well known that MβCD deplets cholesterol and eliminates lipid raft. But I do not have any information about the relevant MβCD incubating condition. Therefore, I need to check for a condition which can deplete cholesterol. After MβCD incubation, I can find cholesterol contents changes

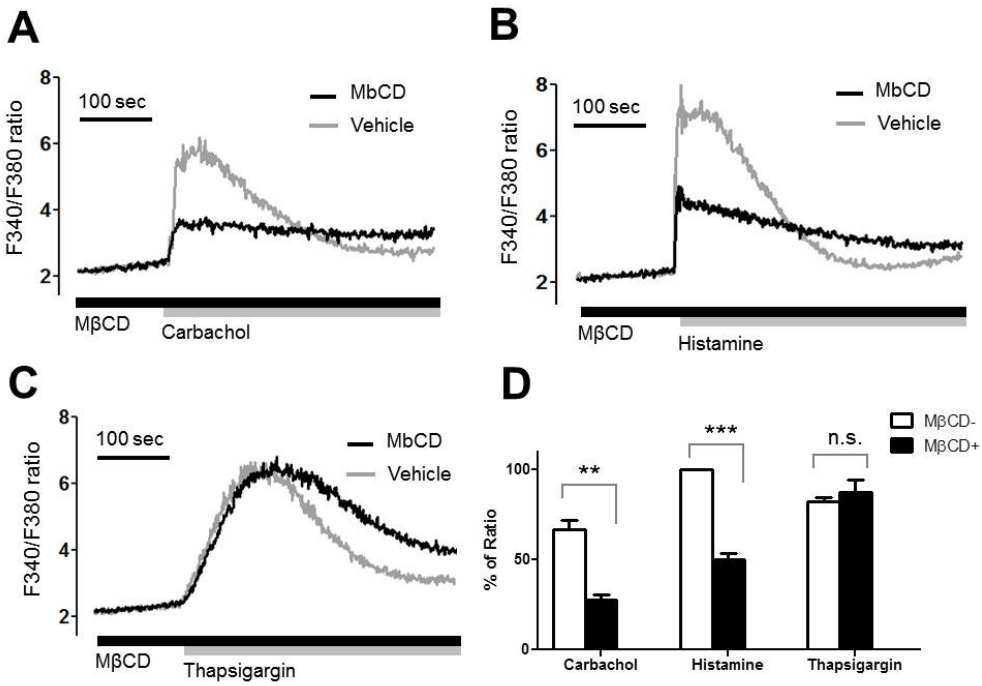
and MβCD treat condition. In 10mM MβCD for 30min, cholesterol of HSG cell membrane decreased (Figure 1-2).

MβCD that inhibits trafficking of AQP5 is controlled by carbachol and histamine.

Trafficking of AQP5 in salivary gland cells is the result of increasing $[Ca^{2+}]_i$ activated by carbachol and histamine. My research is based on a method that has been used for the previous studies (Lee et al., 2015), I studied about the impact of MβCD the trafficking of AQP5. When using a Membrane fraction, the groups treated with histamine, carbachol showed that AQP5 translocation is reduced, the thapsigargin was no significant effect (Figure 1-3) When I use the AQP5-GFP were treated with the Carbachol Histamine, AQP5 was shifted to the plasma membrane, the MβCD was treated, it was confirmed that the tendency is reduced. However, the experimental group treated with thapsigargin was shown to be unaffected by MβCD (Figure 1-4). Through this result, MβCD blocks the calcium signal is controlled by the muscarinic and histamine and suggests that accordingly involved in the regulation AQP5 translocation.

Figure 1-1. The preincubation of MβCD inhibits carbachol- and histamine-induced Ca^{2+} increase.

(A-C) Fura-2-loaded HSG cells were treated by 100 μM carbachol (A), 100 μM histamine (B), or 1 μM thapsigargin (C) with (black trace) or without (gray trace) of preincubation of 10 mM MβCD for 30 min. Typical Ca^{2+} transients from more than five separate experiments are presented. (D) Cells were preincubated with the indicated MβCD concentrations, and then challenged with 100 μM carbachol, 100 μM histamine, or 1 μM thapsigargin. The peak height of Ca^{2+} elevation was monitored. Each point represents mean +/- SEM. All results were reproducible.


A
F340/F380 ratio
8
6
4
2
100 sec
MbCD
Vehicle
MβCD
Carbachol
B
F340/F380 ratio
100 sec
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Vehicle
MβCD
Histamine
C
F340/F380 ratio
100 sec
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Thapsigargin
D
% of Ratio
100
50
0
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***
n.s.
MβCD-
MβCD+
Carbachol
Histamine
Thapsigargin

Figure 1-2. MβCD depletes cholesterol in HSG cells.

(A) HSG cells were incubated with 10 mM MβCD for indicated preincubation time. (B) HSG cells were incubated with the indicated concentration of MβCD for 30 min. An Amplex Red Cholesterol Assay Kit was used to measure cholesterol contents as described in the Materials and Methods. The cholesterol contents of cell lysates are depicted as % of vehicle-treated control. The values represent the average ± SEM. **$p < 0.01$; one way repeated ANOVA test. ($n = 3 - 6$).

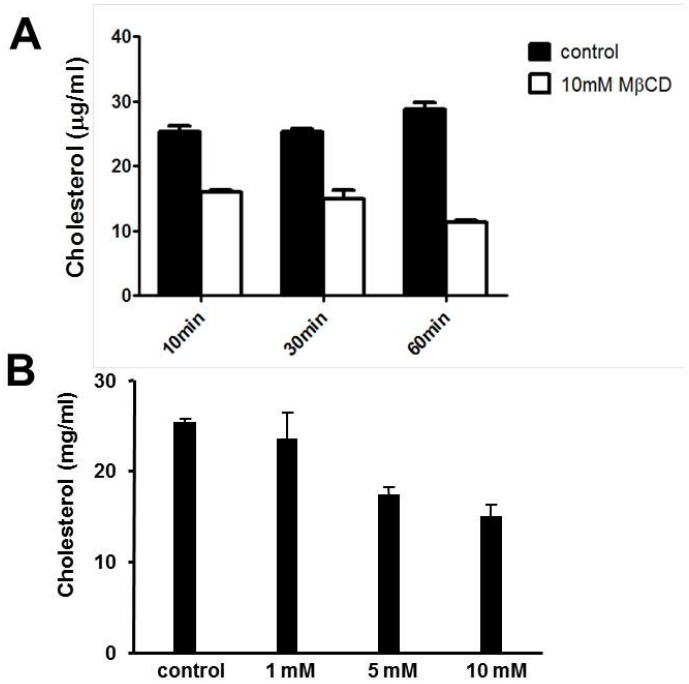
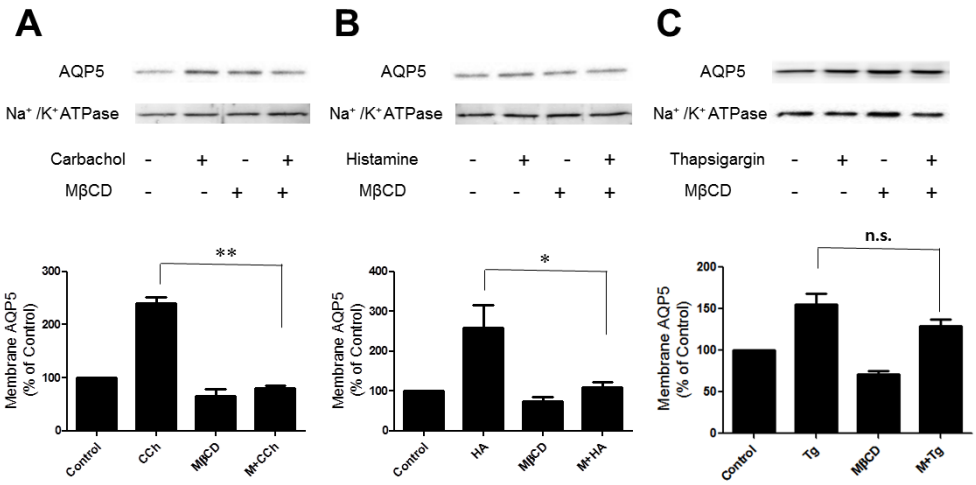

A
Cholesterol (µg/ml)
40
30
20
10
0
control
10mM MβCD
10min
30min
60min
B
Cholesterol (mg/ml)
30
20
10
0
control
1 mM
5 mM
10 mM

Figure 1-3. MβCD inhibits the carbachol- and histamine- mediated increase of AQP5 in the plasma membrane fraction, it does not affect the increasing in AQP5 by thapsigargin.

HSG cells transfected AQP5-GFP were pre-treated in the absence or presence of 10mM MβCD for 30min and then treated with (A) 100 μM carbachol, (B) 100 μM histamine and (C) 1 μM thapsigargin for 20 min. The western blots with membrane fractionation method is described in Materials and Methods. Each data quantification of the membrane AQP5 levels were normalized to the levels of the Na^+-K^+-ATPase subunit. Each values (% of control) was depicted as mean ± SEM. n.s., not significant; *$P < 0.05$.


A
AQP5
Na+ /K+ ATPase
Carbachol - + - +
MβCD - - + +
Membrane AQP5 (% of Control)
300
200
100
0
**
Control
CCh
MβCD
M+CCh
B
AQP5
Na+ /K+ ATPase
Histamine - + - +
MβCD - - + +
Membrane AQP5 (% of Control)
400
300
200
100
0
*
Control
HA
MβCD
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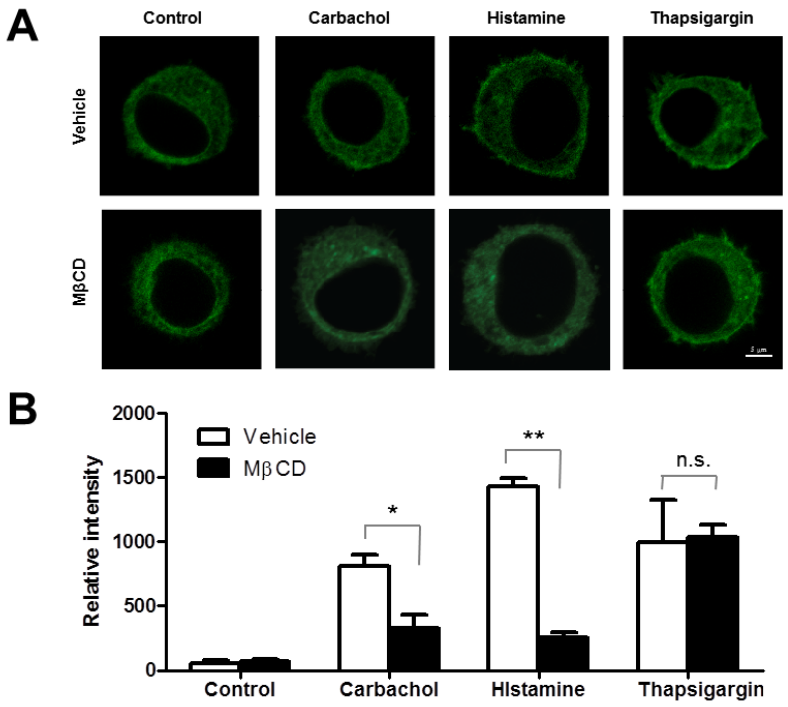
AQP5
Na+ /K+ ATPase
Thapsigargin - + - +
MβCD - - + +
Membrane AQP5 (% of Control)
200
150
100
50
0
n.s.
Control
Tg
MβCD
M+Tg

Figure 1-4. MβCD blocks the carbachol- and histamine- induced AQP5 translocation, but it does not affect thapsigargin-mediated AQP5 translocation.

HSG Cells transfected AQP5-GFP were pre-incubated in the absence of presence of 10mM MβCD for 30min and then challenged with 100 μM carbachol, 100 μM histamine and 1 μM thapsigargin for 20 min. (A) the figure shows trend of trafficking AQP5-GFP. The images were made by a confocal microscope. (B) I quantified the AQP5 to location on the membrane statistically by measuring the fluorescence intensity.


A
Control
Carbachol
Histamine
Thapsigargin
Vehicle
MβCD
B
Relative intensity
2000
1500
1000
500
0
Vehicle
MβCD
*
**
n.s.
Control
Carbachol
HIstamine
Thapsigargin

DISCUSSION

I described to influence the AQP5 translocation as calcium signal is controlled by the muscarinic and histamine receptor lipid raft depletion by MβCD in salivary gland cells. The depletion of lipid raft not been reported to influence the function of saliva from the salivary gland cells. In salivary gland muscarinic receptor is known to influence in controlling the increase in intracellular calcium and salivation. In the salivary control, the movement of water can be divided into two main methods. One of the water movement in paracellular pathway due to ion exchange by cell membrane transporter, and the other is moved to the channel of AQP5 the plasma membrane to pass through the water there is a transcellular pathway by increasing the activity. My results suggest the lipid raft depletion inhibits the calcium signal of histamine and muscarinic receptor (Fig.1-1, 1-2) and shows that reduced translocation of AQP5 (Fig1-3, 1-4). In addition, lipid raft depletion does not affect thapsigargin induced $[Ca^{2+}]_i$ changes. And MβCD does not inhibit thapsigargin induced AQP5 translocation. Through these results, I suggest that lipid raft depletion does not affect SOCE.

I found that MβCD deplete lipid raft, and inhibit not only the muscarinic receptor, but also the histamine receptor. MβCD did not inhibit the $[Ca^{2+}]_i$ –

mediated increase in thapsigargin. Thapsigargin is a sarcoplasmic reticular Ca^{2+}-ATPase inhibitor that triggers Ca^{2+} release from the intracellular Ca^{2+} pool and store-operated calcium entry.

My results suggested that MβCD blocks salivary muscarinic and histamine – induced Ca^{2+} signaling and AQP5 translocation. But SOCE does not have any relationship with lipid raft depletion. These data will be helpful for understanding the cellular mechanism of lipid raft depletion in salivary gland and for studying effect of lipid raft depletion in dry mouth.

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CHAPTER 2*:

Desipramine-mediated inhibition of salivary Ca^{2+} signaling and aquaporin translocation

***This chapter has been largely reproduced from an article published Lee K, Choi S, Choi LM, Lee J, Kim JH, Chung G, Lee G, Choi SY, Park K. Oral Dis.2015 May;21(4):530-5**

ABSTRACT

Desipramine is a tricyclic antidepressant with a negative side effect of dry mouth. The Na^+/H^+ exchanger was suggested to be a target of desipramine in salivary gland cells. However, it is unclear whether desipramine has other targets in the salivary secretion pathway. Here I studied the effect of desipramine on salivary Ca^{2+} signaling. Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined with the fluorescent Ca^{2+} indicator fura-2/AM. Aquaporin translocation was analyzed by Western blotting and immunocytochemistry of confocal microscopy. Desipramine inhibited the carbachol- and histamine-mediated increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in a concentration-dependent manner. However, desipramine did not affect increases in $[Ca^{2+}]_i$ mediated by extracellular ATP, sphingosine-1-phosphate, or thapsigargin. The adrenergic receptor blockers prazosin and propranolol did not reverse the desipramine-mediated inhibition of carbachol- and histamine-induced increases in $[Ca^{2+}]_i$. I also found that desipramine inhibits the increase in membrane aquaporin-5 level triggered by carbachol and histamine treatments. These results imply that desipramine blocks muscarinic and histamine receptor-mediated Ca^{2+} signaling and the subsequent translocation of aquaporin-5 in human salivary gland cells, suggesting a novel mechanism for the xerogenic effects of desipramine.

INTRODUCTION

Xerostomia, or dry mouth, is one of the most common oral diseases, manifesting as decrease in salivation (Turner and Ship, 2007). The most common causes of dry mouth are extrinsic medicines, salivary gland damage caused by radiation therapy, and Sjögren's syndrome. To date, approximately 400-600 chemicals are reported to induce dry mouth (Sreebny and Schwartz, 1997; Scully, 2003; Guggenheimer and Moore, 2003). In particular, psychiatric drugs such as antidepressants, antipsychotics, and drugs for Parkinson's disease produce serious dry mouth.

Desipramine is an orally administrable tricyclic antidepressant that is widely prescribed for trigeminal neuropathic pain and major mood disorders (Potter et al., 1991). However, desipramine is reported to induce xerostomia (Scarpace et al., 1993; Koller et al., 2000; Galanter et al., 2002). With its inhibition of catecholamine reuptake in the nerve terminal, desipramine increases synaptic catecholamines and prolongs adrenergic signaling (Nelson et al., 1984). However, the relationship between adrenergic signaling and reduced salivation has not yet been proven in salivary glands. In a previous study, I reported that desipramine inhibits Na^+/H^+ exchanger activity (Choi et al., 2006), which serves an important role in salivary secretion (Turner and

Sugiya, 2002). However, it is unclear whether the Na^+/H^+ exchanger is the sole target of desipramine, or whether desipramine may have other critical targets such as members of the salivary Ca^{2+} signaling and aquaporin translocation pathways.

In this study, I monitored the effect of desipramine on salivary Ca^{2+} signaling. Ca^{2+} signaling and aquaporin translocation in human salivary gland HSG cells have been used to study the pathological mechanisms of salivary impairment such as in Sjögren's syndrome (Ishikawa et al., 2005; Jin et al., 2012a; Lee et al., 2013). I tested the effect of desipramine on the salivary receptors expressed in salivary glands and HSG cells and known to induce Ca^{2+} signaling (i.e., muscarinic M3 receptor, histamine H1 receptor, P2X7 purinoceptor, and sphingosine-1-phosphate S1P1 receptor) in an attempt to understand the xerogenic mechanism of desipramine.

MATERIALS AND METHODS

Chemicals

Desipramine, histamine, carbachol, desipramine, ATP, thapsigargin, propranolol, and prazosin were purchased from Sigma (St. Louis, MO, USA). Fura-2-acetoxymethyl ester (Fura-2-AM) was obtained from Molecular Probes (Eugene, OR, USA). Modified Eagle's Medium, bovine calf serum, and penicillin/streptomycin were purchased from GIBCO (Grand Island, NY, USA).

Cell Preparation

Human submandibular gland HSG cells were grown in Modified Eagle's Medium supplemented with 10% (v/v) heat-inactivated bovine calf serum and 1% (v/v) penicillin (5,000 U/ml) + streptomycin (5,000 μg/ml) solution. The cells were cultured in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was changed every two days, and the cells were sub-cultured weekly.

$[Ca^{2+}]_i$ *Measurement*

Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined with the

fluorescent Ca^{2+} indicator fura-2/AM. Briefly, a cell suspension was incubated with fresh Modified Eagle's Medium containing fura-2/AM (4 μM) for 40 min at 37°C with continuous stirring. The cells were then washed with a HEPES-buffered solution (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH adjusted to 7.4 with NaOH) or Locke's solution (154 mM NaCl; 5.6 mM KCl; 1.2 mM $MgCl_2$; 2.2 mM $CaCl_2$; 5.0 mM HEPES; 10 mM glucose, pH 7.4 with NaOH) and left at room temperature until use. Sulfinpyrazone (250 μM) was added to all solutions to prevent dye leakage. Cells were transferred to poly-L-lysine-coated 25-mm coverslips for cell attachment and held for 40 min in an incubator, and then the coverslips were mounted onto the chamber. Changes in the fluorescence ratio were monitored by a Ca^{2+} imaging machine with MetaFlour software (Molecular Devices, Sunnyvale, CA, USA) with dual excitation at 340 and 380 nm and emission at 500 nm. Experiments with suspended HSG cells were performed with a spectrofluorophotometer using the same principle.

Construction of Aquaporin-5 and Western blotting

Total RNA was isolated from HSG cells, and the full-length cDNA extracted from it was amplified with reverse-transcriptase PCR (RT-PCR).

PCR utilized 5’-GAATTCTATGAAGAAGGAGGTGTGCTCC as the upstream primer and 5’-GGTACCGCGGGTGGTCAGCTCCATGGT as the downstream primer (the introduced EcoRI and KpnI restriction sites are underlined). The 798-bp human aquaporin-5 gene was then cloned into pEGFP-C1. HSG cells were transiently transfected with the aquaporin-5 gene using LipofectAMINE 2000 (Invitrogen). Aquaporin-5-transfected HSG cells were grown in a 60-mm dish, pre-incubated with desipramine for 3 min, and incubated with carbachol or histamine for 20 min at 37°C. The apical membrane fraction was sonicated in ice-cold 20 mM HEPES solution containing 1 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, and 0.3 mM phenylmethylsulfonyl fluoride (3×30 s, Branson Sonifer) at pH 7.4. The samples were centrifuged at 600×g at 4 °C, and the supernatants were further centrifuged at 20,000×g at 4 °C. The pellets (P2 membrane fractions) were subjected to SDS/PAGE immunoblotting. After electrophoresis in SDS-PAGE (10 %) gel, the protein samples were transferred to polyvinylidene difluoride membranes (0.45 μm, Millipore). After blocking, the membrane was incubated with anti-aquaporin-5 as the primary antibody (1:200, Santa Cruz, sc-9891). The membrane was then washed and incubated with donkey anti-goat IgG-HRP. The membrane was finally subjected to an electrochemiluminescence assay. For normalization of the antibody signal,

the membranes were stripped and re-probed with antibody for Gβ (1:500, Santa Cruz, sc-25413) or anti-α1 Na^+/K^+-ATPase (1:1000, Abcam, ab7671).

Immunocytochemistry

GFP-tagged cells transfected with aquaporin-5 were plated on glass coverslips coated with poly-L-ornithine. Cells were pre-treated with desipramine for 3 min, followed by treatment with carbachol and histamine for 20 min. The cells were fixed with 4% paraformaldehyde in PBS for 15 min and permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature (20-25 ^oC). They were then incubated for 1 h at room temperature in blocking solution, 1% BSA in PBS. The samples were incubated with primary antibodies for 2 h at room temperature or overnight at 4°C and incubated with fluorescence-conjugated secondary antibodies for 1 h at room temperature The following antibodies were used in this study: anti-alpha1 Na^+-K^+-ATPase (1:100, Abcam, ab7671) and Cy3-conjugated secondary antibodies (1:200, Jackson Laboratories). Slides were mounted and visualized using a laser scanning confocal microscopy (Zeiss LSM 700).

Data Analysis

All quantitative data are expressed as mean ± SEM. We calculated the half-maximal inhibitory concentration (IC_{50}) with the Microcal Origin program. Differences were determined by one-way analysis of variance with post hoc tests and were considered to be significant when $P < 0.05$.

RESULT

Desipramine inhibits muscarinic and histamine Ca^{2+} signaling without an effect on extracellular ATP-, spingosine-1-phosphate-, or thapsigargin-mediated $[Ca^{2+}]_i$ increases

I monitored the effect of desipramine on salivary Ca^{2+} signaling in human salivary gland HSG cells, which have been broadly used for salivary Ca^{2+} studies. Desipramine inhibited the cytosolic increases in Ca^{2+} induced by carbachol, a strong salivary secretagogue (Figure 2-1A). The inhibitory effect of desipramine on carbachol-mediated increases in $[Ca^{2+}]_i$ showed concentration dependence with a 369 ± 95 nM IC_{50} (Figure 2-1B). Desipramine also inhibited the histamine-induced increases in $[Ca^{2+}]_i$ in a concentration-dependent manner (IC_{50}: 184 ± 35 nM) (Figures 2-1C, 2-1D). However, desipramine did not affect the ionotrophic P2X7 receptor-mediated increase in $[Ca^{2+}]_i$ (Hwang et al., 2009; Figure 2-2A). Desipramine also showed no inhibitory effect on the increase in $[Ca^{2+}]_i$ mediated by sphingosine-1-phosphate, another phospholipase C-linked G-protein coupled receptor in HSG cells (Seo et al., 2010; Figure 2-2B). In addition, desipramine did not affect the increase in $[Ca^{2+}]_i$ induced by thapsigargin, which mimics the downstream signaling of phospholipase C activation such

as the depletion of the cytosolic Ca^{2+} pool and the induction of store-operated Ca^{2+} entry (Figure 2-2C). These results suggest that desipramine selectively blocks muscarinic and histamine Ca^{2+} signaling.

The adrenergic receptor blockers propranolol and prazosin do not reverse desipramine-mediated inhibition

Desipramine inhibition of catecholamine reuptake is blocked by adrenergic receptor antagonists (Kleven and Koek, 1998). However, both prazosin, an alpha-adrenergic antagonist (Figures 2-3A), and propranolol, a beta-adrenergic antagonist (Figures 2-3C), failed to reverse the desipramine-mediated inhibition of carbachol-induced increases in $[Ca^{2+}]_i$. The same trend was observed with histamine-induced increases in $[Ca^{2+}]_i$ (Figures 2-3B, 2-3D), suggesting that the effect of desipramine is independent from the inhibition of catecholamine reuptake.

Desipramine inhibits carbachol and histamine-mediated aquaporin-5 translocation in HSG cells

Muscarinic and histamine Ca^{2+} signaling trigger the membrane trafficking of aquaporin-5, a ‘water channel’ protein in the salivary gland (Delporte and Steinfeld, 2006; Kim et al., 2009; Lee et al., 2013). To determine the effect of

desipramine on aquaporin, I analyzed the expression level of aquaporin-5 in the membrane fraction. I searched for optimal stimulation conditions for the quantification of AQP5 translocation and found that carbachol and histamine stimulation for 10 min and 20 min showed clear AQP5 translocation. With 20 min stimulation, carbachol and histamine increased membrane aquaporin-5 levels, while desipramine blocked both carbachol- and histamine-induced aquaporin-5 translocations (Figures 2-4). I confirmed this finding with GFP-tagged aquaporin-5, which moved to the plasma membrane with carbachol and histamine treatment. Cells treated with carbachol and histamine showed stronger fluorescence in the plasma membrane than in the cytosol, whereas cells co-treated with desipramine, carbachol, and histamine showed similar fluorescence intensities in both the cytosol and the plasma membrane (Figure 2-5). These results suggest that desipramine blocks muscarinic and histamine receptor-mediated Ca^{2+} signaling and subsequent aquaporin-5 translocation in human salivary gland cells.

Figure 2-1. Desipramine inhibits muscarinic and histamine-induced $[Ca^{2+}]_i$ increases in human salivary gland HSG cells.

(A) Fura-2-loaded HSG cells were pre-incubated with 10 μM desipramine (Desi, black) or vehicle (gray) for 3 min and treated with 300 μM carbachol. Changes in the fluorescence ratio of F340/F380 were monitored as described in Materials and Methods. Typical Ca^{2+} traces from more than five separate experiments are presented. (B) Cells were pre-incubated with desipramine with the indicated concentrations, and the inhibition of 300 μM carbachol-induced increases in Ca^{2+} was monitored. The peak height of Ca^{2+} elevation was analyzed, and each point represents the mean ± SEM. (C) Cells were pre-incubated with 10 μM desipramine (Desi, black) or vehicle (gray) for 3 min and then treated with 100 μM histamine. Changes in the fluorescence ratio of F340/F380 were monitored, and typical Ca^{2+} traces from more than four separate experiments are presented. (D) Cells were pre-incubated with desipramine at the indicated concentrations, and the inhibition of 100 μM histamine-induced increases in Ca^{2+} was monitored. The peak height of Ca^{2+} elevation was analyzed, and each point represents the mean ± SEM. All results were reproducible.

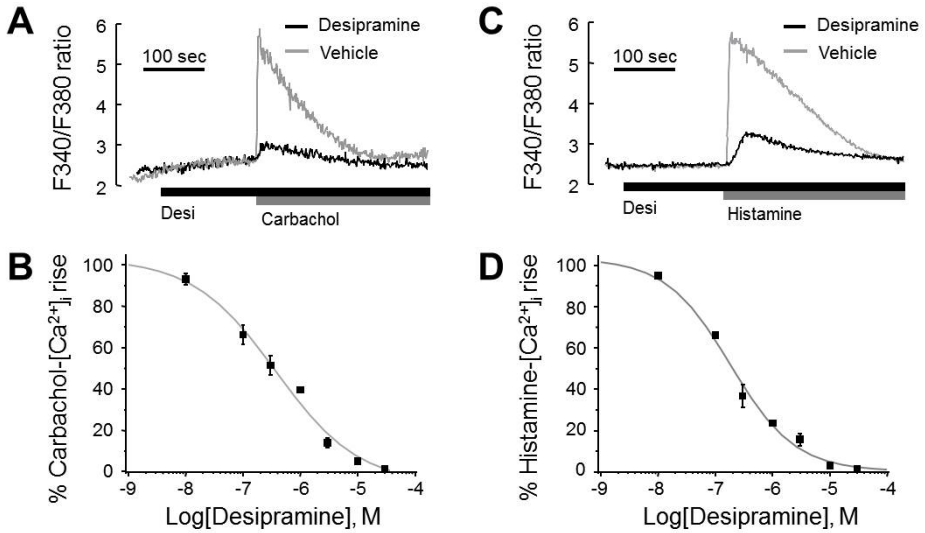

A
F340/F380 ratio
6
5
4
3
2
100 sec
Desipramine
Vehicle
Desi
Carbachol
C
F340/F380 ratio
6
5
4
3
2
100 sec
Desipramine
Vehicle
Desi
Histamine
B
% Carbachol-[Ca2+]i rise
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
Log[Desipramine], M
D
% Histamine-[Ca2+]i rise
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
Log[Desipramine], M

Figure 2-2. Desipramine does not inhibit extracellular ATP-, spingosine-1-phosphate-, or thapsigargin-mediated $[Ca^{2+}]_i$ increase in HSG cells.

(A-C) Fura-2-loaded HSG cells were pre-incubated with 10 μM desipramine (Desi, black) or vehicle (gray) for 3 min and treated with 300 μM ATP (B), 10 μM sphingosine-1-phosphate (S-1-P), or 3 μM thapsigargin (C). Changes in the fluorescence ratio of F340/F380 were monitored, and typical Ca^{2+} traces from more than five separate experiments are presented. All results were reproducible.

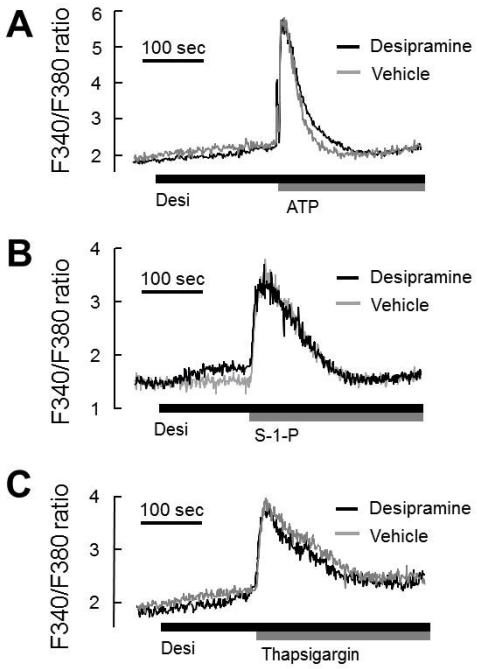
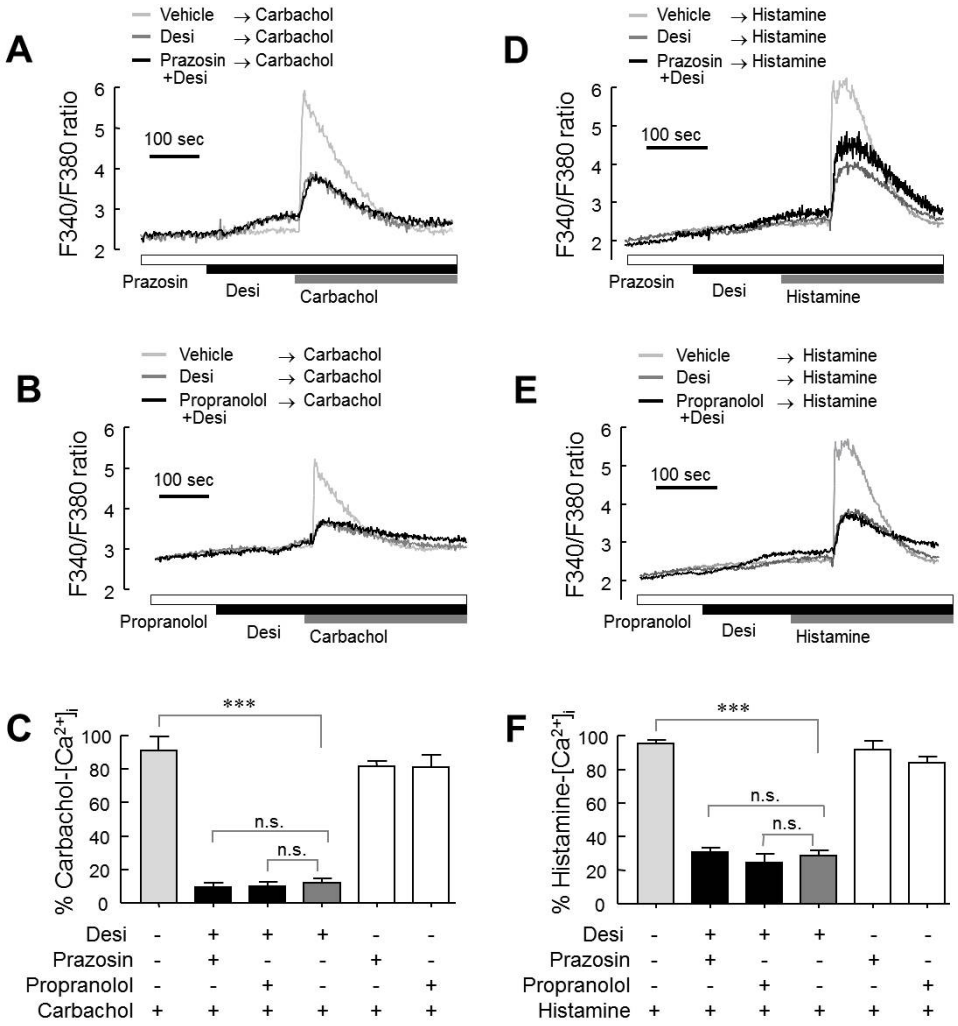

A
F340/F380 ratio
6
5
4
3
2
100 sec
Desipramine
Vehicle
Desi
ATP
B
F340/F380 ratio
4
3
2
1
100 sec
Desipramine
Vehicle
Desi
S-1-P
C
F340/F380 ratio
4
3
2
100 sec
Desipramine
Vehicle
Desi
Thapsigargin

Figure 2-3. The adrenergic receptor blockers propranolol and prazosin do not reverse desipramine-mediated inhibition.

(A-D) Fura-2-loaded HSG cells were pre-incubated with vehicle (light gray), 10 μM desipramine (Desi, dark gray), 10 μM desipramine with 3 μM prazosin (A, B) (Prazosin+Desi, black), or 10 μM desipramine with 3 μM propranolol (C, D) (Propranolol+Desi, black) for 3 min and treated with 300 μM carbachol (A, C) or 100 μM histamine (B, D). Changes in the fluorescence ratio of F340/F380 were monitored, and typical Ca^{2+} traces from more than five separate experiments are presented. (E-F) Cells were pre-incubated with desipramine with desipramine, prazosin, or propranolol, and then 300 μM carbachol (E)- or 100 μM histamine (F)-induced increases in Ca^{2+} were monitored. The peak height of Ca^{2+} elevation was analyzed, and each point represents the mean ± SEM.


A
Vehicle → Carbachol
Desi → Carbachol
Prazosin +Desi → Carbachol
F340/F380 ratio
100 sec
Prazosin
Desi
Carbachol
D
Vehicle → Histamine
Desi → Histamine
Prazosin +Desi → Histamine
F340/F380 ratio
100 sec
Prazosin
Desi
Histamine
B
Vehicle → Carbachol
Desi → Carbachol
Propranolol +Desi → Carbachol
F340/F380 ratio
100 sec
Propranolol
Desi
Carbachol
E
Vehicle → Histamine
Desi → Histamine
Propranolol +Desi → Histamine
F340/F380 ratio
100 sec
Propranolol
Desi
Histamine
C
% Carbachol-[Ca2+]i
***
n.s.
n.s.
Desi - + + + - -
Prazosin - + - - + -
Propranolol - - + - - +
Carbachol + + + + + +
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% Histamine-[Ca2+]i
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n.s.
n.s.
Desi - + + + - -
Prazosin - + - - + -
Propranolol - - + - - +
Histamine + + + + + +

Figure 2-4. Desipramine blocks the carbachol- and histamine-mediated increase of aquaporin-5 in the plasma membrane fraction.

GFP-tagged HSG cells transfected with aquaporin-5 were pre-incubated in the absence or presence of 10 μM desipramine for 3 min and then challenged with 100 μM carbachol and 100 μM histamine for 20 min. (A) Western blots show the membrane aquaporin-5 (AQP5) and G-β subunit (Gβ). The preparation of the membrane fractionation is described in Materials and Methods. (B) Quantification of the membrane aquaporin-5 with G-β subunit control (n = 3 - 6). Membrane aquaporin-5 levels were normalized to the level of the membrane G-β subunit. (C) Western blots show membrane aquaporin-5 (AQP5) and Na^+-K^+-ATPase. (D) Quantification of the membrane aquaporin-5 with Na^+-K^+-ATPase (NKE) control (n = 3 - 6). Membrane aquaporin-5 levels were normalized to the levels of the membrane Na^+-K^+-ATPase subunit. Each values (% of control) was depicted as mean ± SEM. n.s., not significant; *$P < 0.05$.

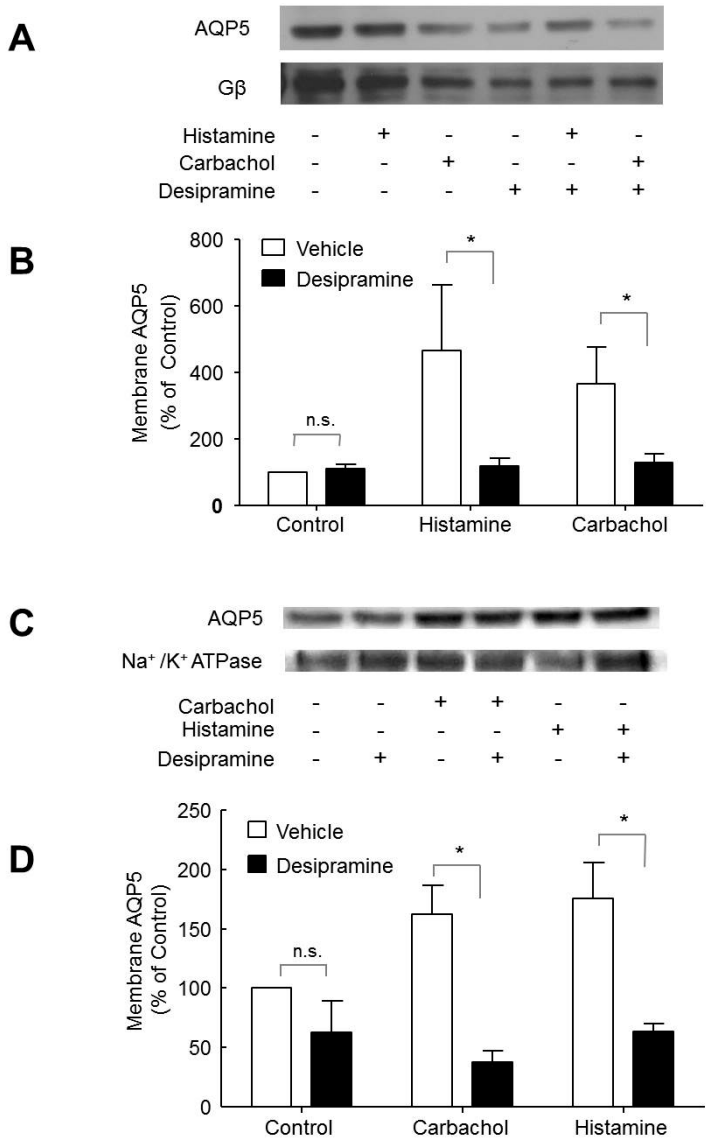
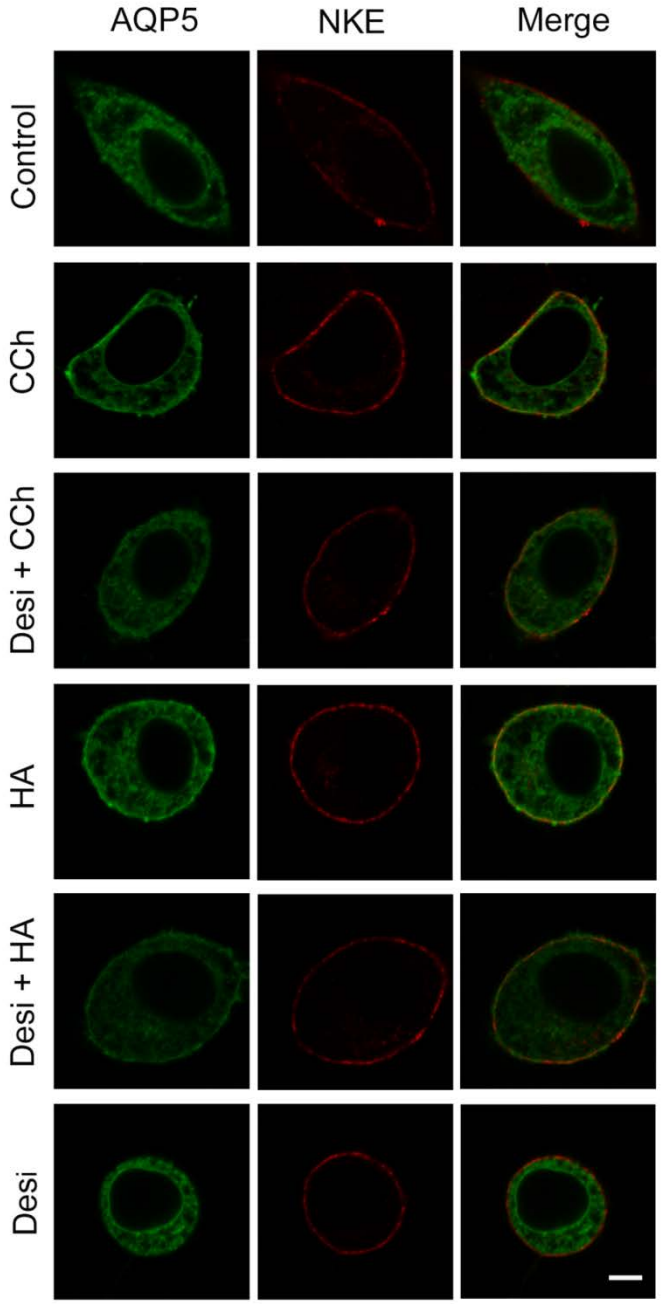

A
AQP5
Gβ
Histamine - + - - + -
Carbachol - - + - - +
Desipramine - - - + + +
B
Vehicle
Desipramine
Membrane AQP5 (% of Control)
800
600
400
200
0
n.s.
*
*
Control
Histamine
Carbachol
C
AQP5
Na+ /K+ ATPase
Carbachol - - + + - -
Histamine - - - - + +
Desipramine - + - + - +
D
Vehicle
Desipramine
Membrane AQP5 (% of Control)
250
200
150
100
50
0
n.s.
*
*
Control
Carbachol
Histamine

Figure 2-5. Desipramine inhibits carbachol- and histamine-mediated aquaporin-5 translocation.

Cells transfected with GFP-tagged aquaporin-5 were pre-incubated in the absence or presence of 10 μM desipramine (Desi) for 3 min and then challenged with 100 μM carbachol (CCh) or 100 μM histamine (HA) for 20 min. Na^+-K^+-ATPase (NKE, red) was used as the membrane marker and is depicted together with GFP-AQP5 (AQP5, green). The images were obtained with a confocal microscope as described in the Materials and Methods.


AQP5
NKE
Merge
Control
CCh
Desi + CCh
HA
Desi + HA
Desi

Discussion

I have shown that desipramine inhibits muscarinic and histamine receptor-mediated Ca^{2+} signaling and aquaporin-5 translocation in human salivary gland HSG cells, which I suggest is one of the mechanisms underlying desipramine-mediated decrease in salivation.

The inhibition of salivary muscarinic receptors is generally accepted as a causal mechanism of hyposalivation commonly seen in Sjögren's syndrome (Jin et al., 2012b; Lee et al., 2013). The activation of salivary muscarinic receptors is the most important mediator of the parasympathetic control of salivation and triggers increases in $[Ca^{2+}]_i$ (Turner and Sugiya, 2002; Li et al., 2006). Ca^{2+} signaling modulates salivary secretion via two pathways (Ambudkar, 2011). One pathway creates an osmotic gradient of anions using Ca^{2+}-activated anion channels, resulting in transepithelial secretion. The other increases the plasma membrane surface level of aquaporin, creating 'water channels' that result in transcellular secretion. Aquaporin-5 is the predominant water channel in salivary gland cells (Ishikawa et al., 2005). My results clearly demonstrate the desipramine-mediated inhibition of muscarinic and histamine Ca^{2+} signaling and subsequent aquaporin-5 translocation. My previous report showed that desipramine inhibits the

Na^+/H^+ exchanger in human primary salivary gland cells and HSG cells (Choi et al., 2006). Thus, desipramine affects both Ca^{2+} signaling-mediated transcellular salivary secretion and Na^+/H^+ exchanger-mediated transepithelial salivary secretion, thus producing a xerogenic effect.

I found that desipramine inhibits not only the muscarinic receptor, but also the histamine receptor. I previously reported that the salivary histamine receptor triggers Ca^{2+} signaling and aquaporin-5 translocation independent of the muscarinic receptor, suggesting an antihistamine-induced xerogenic mechanism (Kim et al., 2009). I believe that desipramine acts on both muscarinic and histamine receptors. The finding that desipramine did not inhibit the $[Ca^{2+}]_i$-mediated increase in sphingosine-1-phosphate receptor (another phospholipase C-linked receptor) and thapsigargin (a sarcoplasmic reticular Ca^{2+}-ATPase inhibitor that triggers Ca^{2+} release from the intracellular Ca^{2+} pool and store-operated calcium entry) strongly suggests that the inhibitory mechanism of desipramine acts at the receptor level before phospholipase C activation. Desipramine's lack of effect on the P2X7 receptor also supports this hypothesis. Finally, the results of the adrenergic antagonist experiment confirm that the effect of desipramine is not mediated by catecholamine reuptake inhibition or adrenergic potentiation, which are unique characteristics of tricyclic antidepressants. In fact, tricyclic

antidepressants including desipramine are reported to show a binding affinity to muscarinic and histamine receptors (Taylor and Richelson, 1980; Batra and Biörklund, 1986; Somogyi and Perel, 1989). Due to the similar characteristics of these receptors, some antihistamine drugs (i.e., diphenhydramine and promethazine) block muscarinic receptors (Liu et al., 2006; Brown and Eckberg, 1997), which possibly explains the inhibitory specificity of desipramine. Based on these findings, I believe there is potential in exploring the alternative prescription of antidepressants with fewer anti-cholinergic and anti-histamine side effects for patients with dry mouth and rampant caries caused by long-term desipramine treatment.

Taken together, my results demonstrate that desipramine inhibits salivary muscarinic and histamine receptor-induced Ca^{2+} signaling and aquaporin-5 translocation. These data will be informative for understanding the cellular and molecular mechanisms of desipramine-induced dry mouth and for assisting in the further understanding of the xerogenic mechanisms of extrinsic medicines.

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CHAPTER 3:

Human salivary gland cells modulation of the proinflammatory cytokine expression by bradykinin

ABSTRACT

Bradykinin is an important peptide modulator that affects the function of neurons, immune cells and exocrine cells. However, there is no evidence of the bradykinin receptors and their functions in human salivary glands. Here I have studied the identification and characterization of bradykinin receptors on human submandibular gland cells. In the human submandibular gland, A253 cells and HSG cells, both B1 and B2 receptors are expressed. Bradykinin increased cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in a concentration-dependent manner, implying that bradykinin receptors are functional in human salivary gland cells. Bradykinin did not affect a carbachol-induced $[Ca^{2+}]_i$ rise. Bradykinin did not modulate aquaporin-5 translocation but promote the expression of proinflammatory cytokines including TNF-α, implying the role of bradykinin in the salivary gland inflammation without any modulation of salivary secretion. These data suggest that bradykinin receptors serve GPCR-mediated functional modulation in human submandibular gland cells.

INTRODUCTION

The neurotransmitters and hormones produced by neurons and immune cells control exocrine functions. Salivary gland is one of exocrine glands communicating vigorously with other cells. Salivary gland cells are classified as non-excitable cells, as they lack voltage-sensitive channels, and they communicate with other cells through G-protein-coupled receptors (GPCRs). For example, salivary secretion, one of the essential functions of salivary glands, is mediated by the activation of GPCRs and the subsequent increase in cytosolic Ca^{2+} levels ($[Ca^{2+}]_i$) (Roussa, 2011; Lee et al., 2012; Ambudka, 2014). Therefore, impairment of GPCR-mediated signaling results in the dysfunction of salivary glands (Jin et al., 2012). In the human salivary gland, a series of GPCRs, including P2Y2, histamine, and S1P have been investigated, elucidating their unique functions (Baker et al., 2008; Kim et al., 2009; Seo et al., 2010).

Bradykinin is one of the neuropeptides of the kinin family, and is involved in many physiological functions such as neuronal function (pain), immune function (inflammation), and cardiovascular function (vasodilation and smooth muscle contraction) (Marceau and Regoli, 2004; Maurer et al., 2011). More specifically, bradykinin is known to play a significant role in the

pathological mechanisms of inflammation, angioedema, and ischemia (Kaplan and Joseph, 2014; Heusch et al., 2015). The effect of bradykinin is mediated by its GPCRs, B1 and B2 receptors, which induce phospholipase C activation and a subsequent $[Ca^{2+}]_i$ increase (Leeb-Lundberg et al., 2005). In oral tissues, bradykinin is reported to induce nitric oxide production in odontoblasts (Korkmaz et al., 2006), and modulate the expression of cyclooxygenase (COX) and toll-like receptors in gingival fibroblasts (Nakao et al., 2001; Gutiérrez-Venegas et al., 2012). Interestingly, heat reflex triggers the secretion of kallikrein from rat submaxillary glands and increases bradykinin levels, thereby inducing thermolytic salivation (Damas and Bourdon, 1994). Bradykinin also potentiates chorda tympani neuron-mediated salivation in cats (Stojic, 1999). Additionally, the bradykinin receptor mediates nitric oxide (NO) and PGE_2 production in the submandibular glands of rats (Genaro et al., 2000).

In human salivary glands, however, bradykinin receptors have not yet been identified nor have their functions and signaling mechanism been characterized. The study of bradykinin in human salivary gland cells is of significance because bradykinin signaling has often been demonstrated to function in a species-dependent manner (Gobeil et al., 1996; Décarie et al.,

1996; Sawada et al., 2004). In this study, my aim was to identify bradykinin receptors in human salivary gland cells and to characterize their functions and signaling mechanism.

MATERIALS AND METHODS

Cell and Tissue Preparation

Human submandibular salivary gland cells were prepared as previously described (Kim et al., 2009). Pieces of human submandibular glands were obtained from six malignant neoplasms from patients (males and females ranging from 26 to 81 years of age) who had provided informed consent. The glands did not contain histologically atypical cells. The experiments were performed according to the Declaration of Helsinki, the World Medical Association, and were approved by the Institutional Review Board (CRI06002) of Seoul National University Dental Hospital. The A253 cells and HSG cells were grown in Modified Eagle's Medium (GIBCO, Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (GIBCO) and 1% (v/v) penicillin (5000 U/ml) + streptomycin (5000 μg/ml) solution (GIBCO). The cells were cultured in a humidified atmosphere of 95% air + 5% CO_2. The culture medium was changed every 2 days, and the cells were subcultured weekly.

$[Ca^{2+}]_i$ Measurement

Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was analyzed with fura-2/AM (Seo et al., 2010). Briefly, the cell suspension was incubated with 3 μM fura-2/AM at 37^{o}C for 60 minutes with continuous stirring. Sulfinpyrazone (250 μM) was added to prevent dye leakage. The cells were then washed with Locke's solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 2.2 mM $CaCl_2$, 5.0 mM HEPES, 10 mM glucose, pH 7.4 with NaOH). Changes in the fluorescence ratio were measured at dual excitation wavelengths of 340 and 380 nm with an emission wavelength of 500 nm.

Immunofluorescent staining

Human submandibular glands were fixed in 10% paraformaldehyde overnight, embedded in paraffin, and sectioned. The 5-μm-thick paraffin sections were deparaffinized and antigen retrieval was carried out using pepsin (Golden Bridge International, Mukilteo, WA, USA) for 10 min at 37°C. The sections were blocked for 30 min in 10% normal goat serum and then incubated with primary antibodies for 1 h (room temperature). A253 cells and HSG cells were fixed (4% paraformaldehyde), permeabilized (0.5%

Triton X-100) for 10 min at 20–25°C, and then incubated for 1 h in blocking solution (1% BSA). Primary antibodies were challenged for 2 h (at room temperature) or overnight (at 4°C), and then incubated with secondary antibodies for 1 h (at room temperature). The following primary antibodies were used: Rabbit anti-human bradykinin B1, B2 receptors (1:50; Santa Cruz Biotechnology). Secondary antibody incubations were carried out for 1 h at room temperature using Alexa488-conjugated goat anti-rabbit IgG (1:700; Invitrogen) antibodies.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from primary culture human submandibular salivary gland cells, A253 cells and HSG cells with TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was treated with RNase-free DNase I for 30 min, and heat-inactivated. The 20-μl RT mixture contained 4 μg of the total RNA, 0.5 mM dNTP mix, 0.5 μg of oligo(dT), 5 U RNase inhibitor, 5 mM dithiothreitol and 5 U Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen, Carlsbad, CA). The expression of GAPDH, Bradykinin B1 receptor and B2 receptor mRNA levels was measured using the following PCR cycling protocols: 5 min at 94^{o}C, 35 cycles of 30 s at 94^{o}C, 30 s at 60^{o}C,

30 s at 72°C, and finally, 10 min at 72°C. The following primers were used as previously described[24] : GAPDH sense 5'-ACC ACA GTC CAT GCC ATC AC-3' and antisense 5'-TCC ACC ACC CTG TTG CTG TA-3', bradykinin B1 receptor sense 5'-CTC TGC CGT GTC ATC AAC GG-3' and antisense 5'-GAA GAC GAT CGC AGC CAG TG-3', bradykinin B2 receptor sense 5'-CAC CAT CTC CAA CAA CTT CG-3' and antisense 5'-GGT AGC TGA TGA CAC AAG CG-3'. The sizes of products were 336 bp (B1 receptor), 302 bp (B2 receptor) and 452 bp (GAPDH).

[^{3}H]cAMP Measurement

The cAMP concentration in cells was determined by a [^{3}H]cAMP competition assay evaluating binding to the cAMP binding protein (Lee et al., 2014). Cells were harvested and pre-incubated with Locke's solution containing 1 mM isobutylmethyl xanthine (IBMX) to inhibit phosphodiesterase. After stimulation with agonists for 15 min at 37°C, the reaction was terminated by addition of twice the volume ice-cold absolute ethanol. The supernatant fraction after centrifugation for 10 min at 4°C at 10,000 x g, was used for the cAMP binding assay, based on competition between [^{3}H]cAMP and unlabeled cAMP to crude cAMP-binding proteins.

The protein-bound cAMP were separated by charcoal (100 μl. The cAMP concentration was determined based on a standard curve and expressed as pmol/cell number.

Construction of Aquaporin-5 and Western blotting

The amount of membrane translocation of aquaporin-5 was determined by Western botting using HSG cells transfected with GFP-tagged aquaporin-5 (Ishikawa et al., 2000). HSG cells were transfected with the GFP-aquaporin-5 and incubated with bradykinin or carbachol for 20 min at 37°C. The apical membrane fraction was sonicated and centrifuged at 600×g (4 °C), and the supernatants were centrifuged at 20,000×g (4 °C). The pellets (P2 membrane fractions) were subjected to SDS/PAGE immunoblotting with anti-aquaporin-5 as the primary antibody (1:200, Santa Cruz, sc-9891). For normalization, the membranes were re-probed with antibody for anti-α1 Na^+/K^+-ATPase (1:1000, Abcam, ab7671).

Real-time RT-PCR

The mRNA expression levels of β-actin, TNF-α, and IL-10 were measured by real-time RT-PCR as previously described (Kim et al., 2010). Real-time PCR was performed using the SYBR Green reagent and an ABI Prism 7500 sequence detection system (Applied Biosystems, Warrington, UK), with the following PCR conditions: 50^{o}C for 2 min, 94^{o}C for 10 min, and 40 cycles of 95^{o}C for 30 sec, followed by 60^{o}C for 1 min. The primers used for PCR : human GAPDH forward: 5'-ACC ACA GTC CAT GCC ATC AC-3', human GAPDH reverse: 5'-TCC ACC ACC CTG TTG CTG TA-3', human TNF-α forward: 5'-ACC ACC ACT TCG AAA CCT GG-3', human TNF-α reverse: 5'-CTT CAC TGT GCA GGC CAC AC-3', human IL-10 forward: 5'-CAT CAA GGC GCA TGT GAA CTC-3', human IL-10 reverse: 5'-GCC TTG CTC TTG TTT TCA C-3'. The expression level of each gene was normalized to the level of the GAPDH gene and presented as the fold induction.

Data Analysis

All quantitative data are expressed as mean ± SEM. Differences were determined by one-way analysis of variance and considered to be significant only when $P < 0.05$.

RESULTS

Bradykinin B1 and B2 receptors are expressed in human submandibular gland cells

In order to test the expression of bradykinin receptors in human salivary glands, I first identified the subtypes of bradykinin receptors expressed in human submandibular gland tissue, HSG cells, and A253 cells with RT-PCR. Figure 3-1A clearly shows the expression of both B1 and B2 receptors in these cells. To confirm the expression of bradykinin receptors, I labeled human submandibular gland with bradykinin B1 and B2 receptor subtype-specific antibodies. Both bradykinin B1 (Figure 3-1B) and B2 receptors (Figure 3-1C) were distributed across most of the human submandibular gland, including both acinar and ducts. Notably, the bradykinin B1 and B2 receptors were also found in the human salivary gland cell lines, A253 and HSG cells (Figure 3-1B, 3-1C).

Bradykinin increases cytosolic Ca^{2+}

Next I examined bradykinin signaling in human submandibular gland cells. I found that bradykinin increases $[Ca^{2+}]_i$ in dissociated human submandibular

gland cells (Figure 3-2A). Based on the common distribution of bradykinin receptors in the human submandibular gland and in human salivary gland cell lines, I measured the bradykinin-induced $[Ca^{2+}]_i$ increase in A253 and HSG cells and found that bradykinin induced $[Ca^{2+}]_i$ increases in both A253 (Figure 3-2B) and HSG cells (Figure 3-2C). Because of limited availability of human submandibular tissues, I investigated the pharmacological characteristics of bradykinin action in human salivary gland cell lines, and found that bradykinin-induced $[Ca^{2+}]_i$ increases occurred in a concentration-dependent manner both in A253 cells (Figure 3-2B) and in HSG cells (Figure 3-2C).

Bradykinin signaling is independent from muscarinic signaling in human submandibular gland cells

Next I tested the interaction between bradykinin and muscarinic Ca^{2+} signaling, which share PLCβ for their common signaling pathway. Interestingly, pre-treatment of bradykinin did not affect the carbachol-evoked $[Ca^{2+}]_i$ increase in HSG cells (Figure 3-3A), even with saturated bradykinin Ca^{2+} signaling at micromolar concentration. Pre-treatment with carbachol did not inhibit a subsequent bradykinin-evoked $[Ca^{2+}]_i$ increase (Figure 3-3B). I

confirmed the results with A253 cells which lacks functional muscarinic receptor (Shin et al., 2015) and found bradykinin successfully increase a cytosolic Ca^{2+} increase even without carbachol-induced $[Ca^{2+}]_i$ increase (Figure 4-3C, 4-3D). These data suggest that bradykinin and muscarinic receptors are completely independent of one another without heterologous desensitization and an overlapping Ca^{2+} signaling pathway.

Prostaglandin receptors are not functional human submandibular gland cells

If salivary gland cells express bradykinin receptors and prostaglandin receptor together, bradykinin can act indirectly by the production of prostaglandins and subsequent activation of prostaglandin receptors (Kim et al., 2010). To investigate whether prostaglandin plays a role in the bradykinin effect after the bradykinin-mediated PLA_2 and COX activation, I tested for functional prostaglandin receptors in HSG cells. However, I could not detect any PGE_2-mediated Ca^{2+} increase, whereas bradykinin successfully evoked a Ca^{2+} increase (Figure 3-4A). In addition, PGE_2 did not generate cAMP, which was successfully generated by VIP, a neurohormone that stimulates G_s-coupled receptors in HSG cells, and forskolin, an activator

of adenylyl cyclase (Figure 3-4B). These results imply that the prostaglandin receptor is not expressed or functional in HSG cells, so bradykinin directly acts on salivary gland cells without the involvement of prostaglandin.

Bradykinin does not affect aquaporin translocation, but induces proinflammatory cytokine expression.

The translocation of aquaporin-5 into plasma membrane is one of cellular mechanism of water secretion in salivary gland cells, and GFP-aquaporin-5-transfected HSG cells have been used to analyze the amount of aquaporin-5 translocation with Na^+/K^+ ATPase as a membrane protein marker (Lee et al., 2015). I monitored the bradykinin-mediated modulation of aquaporin-5 translocation in GFP-aquaporin-5-transfected HSG cells with a membrane protein marker, Na^+/K^+ ATPase. However, bradykinin failed to trigger aquaporin-5 translocation, whereas carbachol successfully induced aquaporin-5 translocation (Figure 3-5A). In addition, bradykinin did not enhance but partially inhibit the carbachol-mediated aquaporin-5 translocation (Figure 3-5B). I next monitored the TNF-α and IL-10 expression by real-time PCR analyses with mRNA from HSG cells. Interestingly bradykinin increased expression of TNF-α like as poly(I:C), a

TLR-3 agonist (Figure 3-5C). The bradykinin marginally increased IL-10 expression (Figure 3-5D). The results suggest that bradykinin does not affect salivary secretion but modulate inflammation in human salivary glands.

Figure 3-1. Bradykinin receptors in human salivary gland cells.

(A) RNA extracted from human submandibular glands, A253 cells, and HSG cells were reverse-transcribed into cDNA and amplification reactions were performed using bradykinin B1, and B2 receptor-specific primers and Pfu polymerases. GAPDH was used as an internal loading control. (B-C) Cells or tissues were fixed and incubated with anti-human bradykinin B1 (B) or B2 receptor (C) antibodies. (top) human submandibular glands (Bars, 50 μm). (middle) A253 cells (Bars, 20 μm). (bottom) HSG cells (Bars, 20 μm).

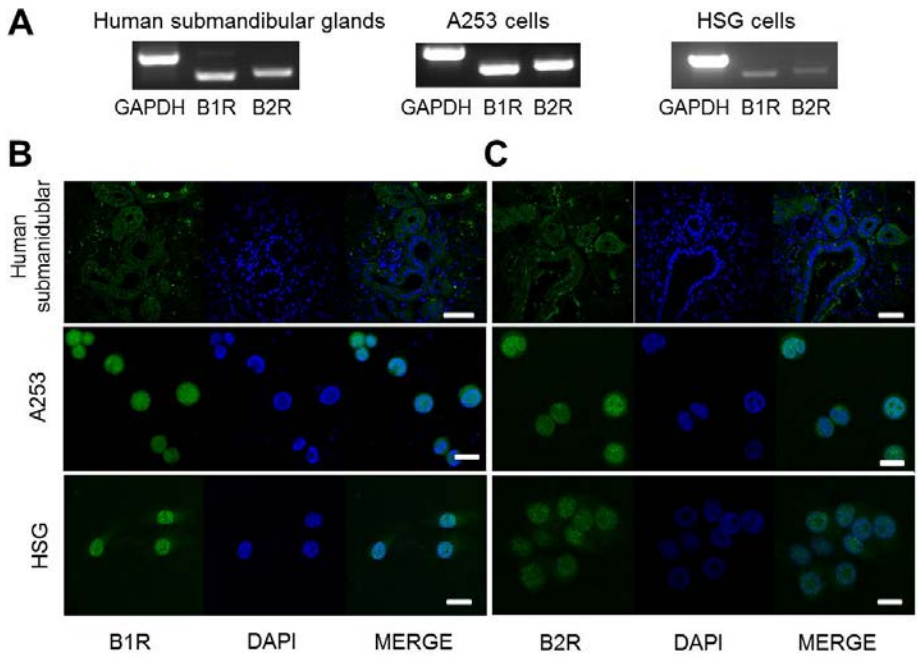

A
Human submandibular glands
A253 cells
HSG cells
GAPDH B1R B2R
GAPDH B1R B2R
GAPDH B1R B2R
B
C
Human submanidublar
A253
HSG
B1R
DAPI
MERGE
B2R
DAPI
MERGE

Figure 3-2. Bradykinin triggers the increase of intracellular Ca^{2+} concentration in human submandibular gland cells.

Fura-2-loaded human dissociated submandibular cells (A), A253 cells (B) or HSG cells (C) were challenged with bradykinin (300 nM, black; 30 nM, dark gray; 3 nM, light gray), and then monitored changes in F340/F380 fluorescence ratio. Typical Ca^{2+} transients are presented. Concentration-response relationships are also depicted by monitoring peak height of changes in cytosolic [Ca^{2+}] level. Each point is the mean ± SEM and all results were reproducible.

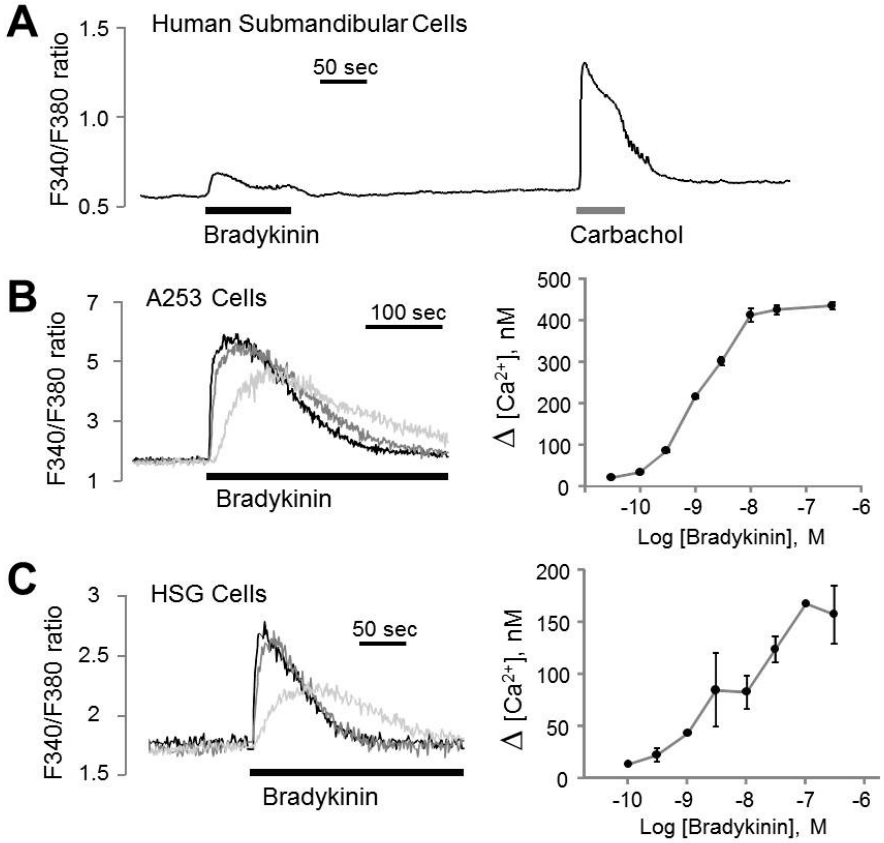

A
Human Submandibular Cells
F340/F380 ratio
1.5
1.0
0.5
50 sec
Bradykinin
Carbachol
B
A253 Cells
F340/F380 ratio
7
5
3
1
100 sec
Bradykinin
Δ [Ca2+], nM
500
400
300
200
100
0
-10
-9
-8
-7
-6
Log [Bradykinin], M
C
HSG Cells
F340/F380 ratio
3
2.5
2
1.5
50 sec
Bradykinin
Δ [Ca2+], nM
200
150
100
50
0
-10
-9
-8
-7
-6
Log [Bradykinin], M

Figure 3-3. Bradykinin receptor does not share its Ca^{2+} signaling with muscarinic receptors.

(A) Fura-2-loaded HSG cells were treated with 100 μM carbachol (CCh) with (black trace) or without (gray trace) the preincubated 300 nM bradykinin (BK). (B) HSG cells were treated with 300 nM bradykinin with (black trace) or without (gray trace) the pre-incubated 100 μM carbachol. (C-D) The same experiment of (A) and (B) were performed using fura-2-loaded A253 cells. All traces presented in the left panels are typical Ca^{2+} transients from more than three separate experiments. The peak height of Ca^{2+} increases at the points indicated as **a** (blank bar) and **b** (filled bar) are depicted in all the right panels with the % value of $[Ca^{2+}]_i$ increase without pretreatment. Each point is the mean ± SEM. All results were reproducible.

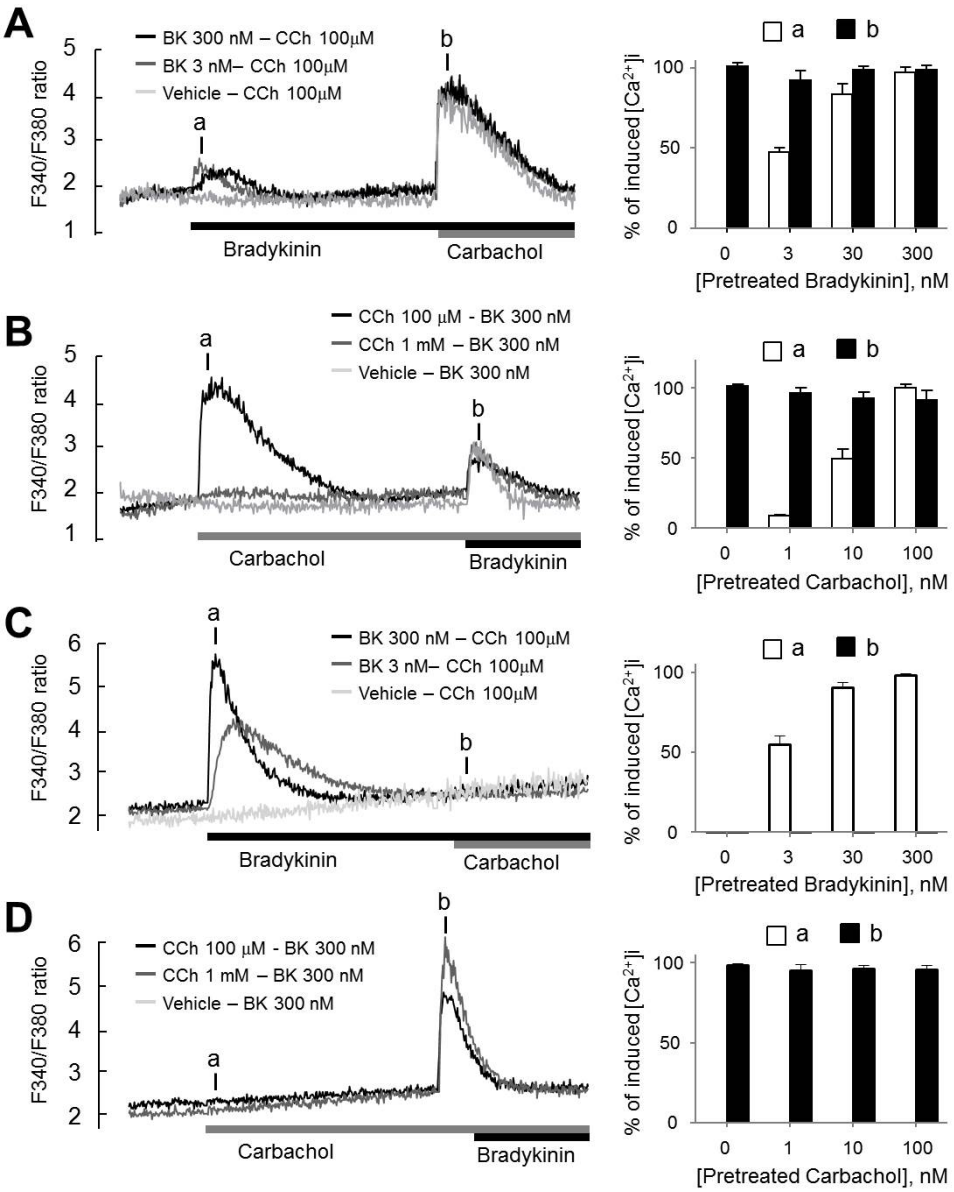

A
BK 300 nM – CCh 100μM
BK 3 nM– CCh 100μM
Vehicle – CCh 100μM
F340/F380 ratio
Bradykinin
Carbachol
a
b
% of induced [Ca2+]i
[Pretreated Bradykinin], nM
B
CCh 100 μM - BK 300 nM
CCh 1 mM – BK 300 nM
Vehicle – BK 300 nM
[Pretreated Carbachol], nM
C
D

Figure 3-4. Prostaglandin does not increase cytosolic Ca^{2+} or cAMP in HSG cells.

(A) Fura-2-loaded cells were challenged with 10 μM PGE_2, 300 nM bradykinin, and 100 μM carbachol (CCh). The fluorescence ratio of F_{340}/F_{380} was then monitored and typical Ca^{2+} transients are presented. (B) Cells were stimulated with 10 μM PGE_2, or 1 μM vasoactive intestinal peptide (VIP) or 3 μM forskolin, in the presence of 1 mM IBMX for 15 minutes, and then cAMP production was then monitored. The relative cAMP productions are depicted as percent of forskolin-induced cAMP production with the mean ± SEM of triplicate assays. $^{**}P < 0.01$.

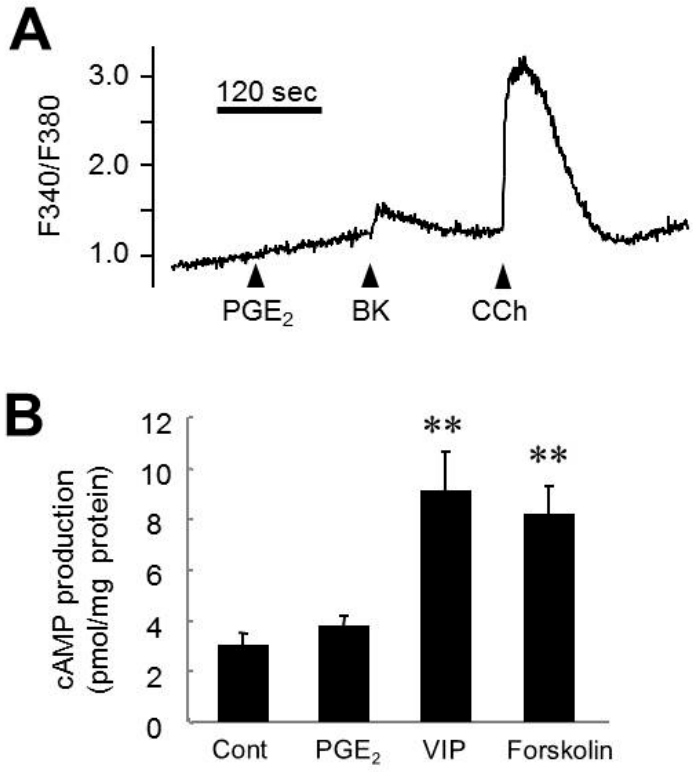
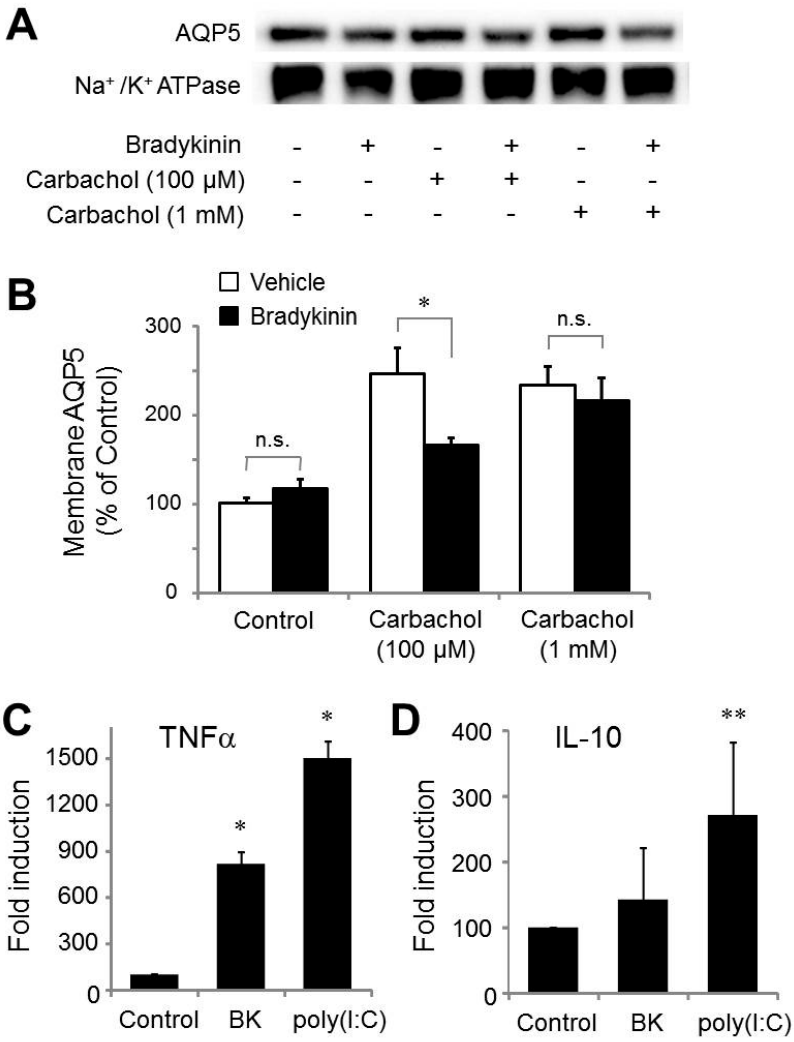

A
3.0
2.0
1.0
F340/F380
120 sec
PGE2
BK
CCh
B
12
10
8
6
4
2
0
cAMP production
(pmol/mg protein)
**
**
Cont
PGE2
VIP
Forskolin

Figure 3-5. Bradykinin does not modulate aquaporin-5 translocation but induce the expression of proinflammatory cytokines.

(A-B) GFP-tagged HSG cells transfected with aquaporin-5 were challenged with 300 nM bradykinin, 100 μM carbachol, or 100 μM carbachol for 20 min. (A) Western blots show membrane aquaporin-5 (AQP5) and Na^+-K^+-ATPase. (B) Quantification of membrane aquaporin-5 with Na^+-K^+-ATPase control (n = 3 - 6). Membrane aquaporin-5 levels were normalized to the levels of the membrane Na^+-K^+-ATPase subunit. (C-D) HSG cells were treated with bradykinin (BK) or 30 μg/ml poly(I:C) for 3 h. Total RNA was isolated, reverse transcribed and analyzed with real-time RT-PCR to determine the amount of TNF-α (C), and IL-10 expression (D). Each of the values (% of control) depicted is a mean ± SEM. n.s., not significant; *P < 0.05, **P < 0.01.


A
AQP5
Na+ /K+ ATPase
Bradykinin - + - + - +
Carbachol (100 μM) - - + + - -
Carbachol (1 mM) - - - - + +
B
Vehicle
Bradykinin
Membrane AQP5 (% of Control)
300
200
100
0
n.s.
*
n.s.
Control
Carbachol (100 μM)
Carbachol (1 mM)
C
TNFα
Fold induction
1500
1200
900
600
300
0
*
*
Control
BK
poly(I:C)
D
IL-10
Fold induction
400
300
200
100
0
**
Control
BK
poly(I:C)

DISCUSSION

In this study, I have shown that bradykinin receptors mediate Ca^{2+} signaling in human submandibular gland cells for the first time. I have shown that (1) the bradykinin B1 and B2 receptors are expressed in human submandibular gland cells, (2) bradykinin increases cytosolic Ca^{2+} independent of muscarinic Ca^{2+} signaling, (3) bradykinin does not affect aquaporin translocation, and (4) bradykinin induces proinflammatory cytokine expression. .

My results using subtype-specific antibodies for bradykinin receptors clearly show that human submandibular gland cells express both bradykinin B1 and B2 receptors (Figure 3-1). Both B1 and B2 receptors are commonly linked to PLC-β and subsequent Ca^{2+} signaling. Given that muscarinic receptors have been considered representative of GPCR-linked Ca^{2+} signaling, clarification of the relationship between bradykinin and muscarinic receptors is needed. I addressed this question with "heterologous desensitization," a decrease in signaling of a certain receptor by another neurotransmitter that shares the same downstream signal transduction pathway. I confirmed that bradykinin pre-treatment does not cause a muscarinic Ca^{2+} increase (Figure 3-3). Furthermore, A253 cells do not

express any muscarinic receptors (Sun et al., 1999) because of epigenetic transcriptional control (Shin et al., 2015). However, bradykinin successfully induced a $[Ca^{2+}]_i$ increase, confirming the independence of bradykinin from muscarinic receptor signaling. The action of bradykinin can be classified as (1) a direct effect with bradykinin-mediated Ca^{2+} signaling after the activation of PLCβ, and (2) an indirect effect via bradykinin-mediated PGE_2 or NO production after the activation of PLA_2, COX or NOS. The bradykinin-mediated PGE_2 production and subsequent physiological modulation is reported in rat submandibular gland cells (Genaro et al., 2000) and gingival fibroblast (Nakao et al., 2001). However, I could not find any PGE_2-mediated Ca^{2+} increase or cAMP production in HSG cells (Figure 3-4), implying a lack of functional prostaglandin receptors in HSG cells. Thus, it seems less likely that bradykinin acts on salivary glands in a paracrine manner via the PLA_2-COX-PGE_2 signaling cascade.

Then what is bradykinin's function in human salivary glands? my results showed that bradykinin does not trigger aquaporin-5 translocation and does not affect carbachol-induced aquaporin-5 translocation. This result complimented another finding, that pre-treatment with bradykinin did not affect muscarinic Ca^{2+} signaling. Thus it is plausible that bradykinin hardly

contributes salivation. In fact, xerostomia is very marginal or undetectable as a side effect with Icatibant, a bradykinin B2 receptor-specific antagonist prescribed for hereditary angioedema (Proud et al., 1995; Bas et al., 2007; Cicardi et al., 2010). It is suggested that bradykinin does not affect muscarinic Ca^{2+} signaling and salivary secretion directly, but modulates the pathological events by its own receptor signaling. Instead of salivation, my results show that bradykinin modulates proinflammatory cytokine expression. It is not surprising that bradykinin modulates inflammatory reactions, due to a large body of studies reporting the bradykinin's critical roles in inflammation (Marceau and Regoli, 2004). Proinflammatory cytokines including IL-1β and TNF-α can modulate the expression of the bradykinin receptor (Brechter et al., 2008; Souza et al., 2013), and also bradykinin modulates the expression of cytokines including IL-1b and TNF-alpha (Cunha et al., 2007; Kim et al., 2010), revealing tight crosstalk. However, it is interesting that bradykinin shows a similar proinflammatory effect in exocrine system. Salivary glands are involved in the local immune reactions with their innate immunity related receptors including TLRs (Kawakami et al., 2007; Li et al., 2010; Into et al., 2014). Thus, further investigation for the bradykinin-mediated salivary cytokine induction on the basis of my findings will eventually contribute to understanding the changes in salivary functions mediated by pathological conditions such as inflammation and angioedema.

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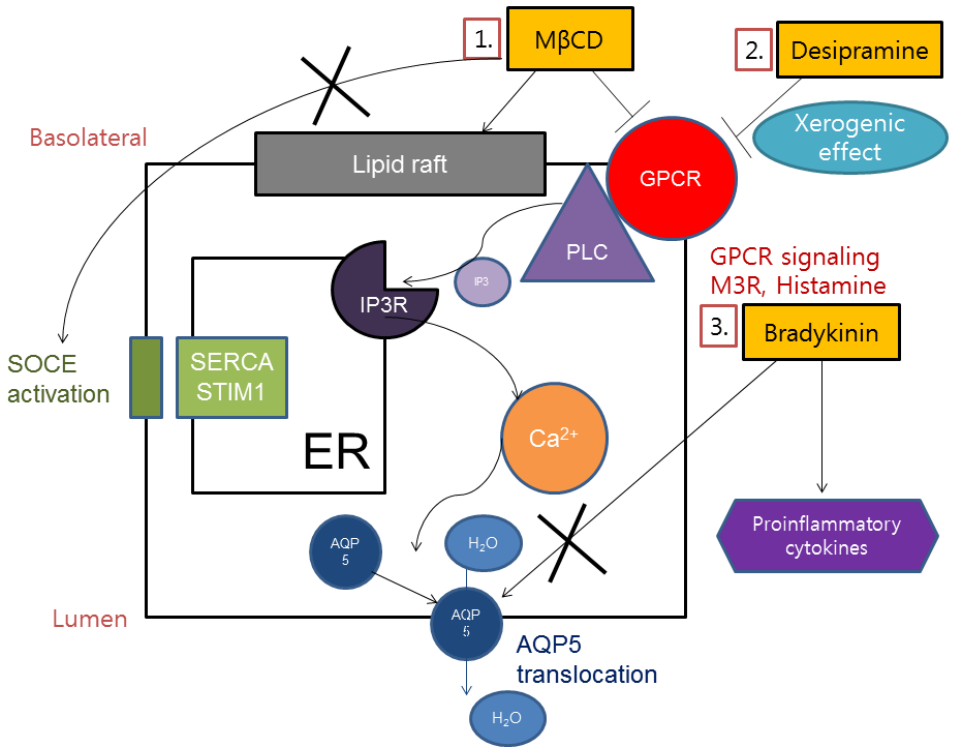
Conclusion

- Incubation of MβCD to remove the lipid raft through the depletion of cholesterol in the salivary gland cells inhibited muscarinic and histamine receptor-mediated Ca^{2+} signaling.
- On the other hand in the major salivary gland protein-lipid raft is coupled receptor Signaling SOCE or G AQP5 trafficking may have concluded andago effect.
- Incubation of MβCD was not suppressed thapsigargin-mediated Ca2 + signaling and aquaporin-5 translocation
- Desipramine inhibits muscarinic and histamine receptor-mediated Ca^{2+} signaling in human salivary gland cells.
- Desipramine inhibits muscarinic and histamine receptor-mediated translocation of aquaporin-5, a major water channel in human salivary gland cells.
- This finding contributes to a better understanding of the cellular and molecular pathological mechanisms of desipramine-induced dry mouth.
- Bradykinin receptors are expressed in human salivary gland cells
- Bradykinin increases cytosolic Ca^{2+} levels in human salivary gland cells

- Bradykinin-mediated Ca^{2+} signaling is independent from the muscarinic Ca^{2+} signaling
- My research is of biomedical significance because it contributes to the understanding of the cellular mechanisms of salivary secretion as well as its modulation.



The role of G-protein-coupled receptor induced Ca^{2+} signaling in Salivary gland cells

국문초록

타액선 G-protein-coupled receptor에 의한 세포내 칼슘신호의 역할

이 계 민

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타액은 구강 내 항상성 유지에 중요한 역할을 한다. 구강 내에서의 수분 및 삼투압, pH 등의 유지가 분비물질인 타액에 의해서 조절된다. 타액분비는 타액선의 중요한 기능이다. 타액선세포는 전압의존성 이온채널을 발현하지 않는 비흥분성 세포로서, 주로 G-protein coupled receptor(GPCR)를 통한 신호를 받아서

생리적 기능을 수행한다. 다른 세포에서처럼 타액선의 GPCR의 활성화는 세포내 칼슘 증가로 이어진다. 예를 들어 부교감신경말단에서 분비되는 아세틸콜린은 무스카린성 M3 수용체, Gq/11 단백질과 phospholipase C를 활성화 시켜 신호를 전달하며 이렇게 증가한 세포내 칼슘은 타액선에서 물의 이동을 일으킴으로써 타액 분비를 유도한다. 타액선의 분비가 신경세포를 비롯한 많은 외부 신호에 의해서 조절을 받는데 반해 몇 개의 GPCR을 제외하고는 알려져 있지 않다. 따라서 타액선 세포의 GPCR 신호의 역할을 규명하는 것은 외분비 생리학 차원에서와 임상 치의학적 차원에서 큰 의미가 있다고 할 수 있다. 이러한 점에 주목하여 타액선의 생리에 영향을 주고 타액분비의 변화를 가져오는 약물들이 어떤 작용과 기적을 가지며, 그 기전에 있어서 중요한 역할을 한다고 할 수 있는 GPCR의 신호전달 과정에 대해서 연구하였다. 먼저, 세포막 생리기능에 있어 중요한 역할을 한다고 알려져 있는 Lipid raft의 소멸에 의한 타액선 세포에 존재하는 GPCR의 생리학적 기능변화에 대해서 살펴보았다. 본 연구에서 콜레스테롤의 고갈을 통해서 lipid raft를 제거하는

MβCD의 처리가 미치는 타액선 세포내의 칼슘신호의 변화를 보여줌으로써 기존에 밝혀지지 않았던 Lipid raft의 타액선 세포 GPCR에 미치는 영향을 제시하였다. 다음으로, 장기간 복용했을 때 구강건조증을 야기한다고 알려져 있는 항우울제의 하나인 데시프라민을 타액선 세포에 처리 함으로써 그에 따른 기전을 살펴 봄으로써 외인성 약물이 타액선 세포의 GPCR에 미치는 영향을 밝혔다. 마지막으로, 기존에 밝혀지지 않았던 사람의 타액선에 존재하는 GPCR을 파악하고 그 역할을 밝히고자 사람의 타액선에서 브래디키닌 수용체의 발현과 그에 따른 기전을 살펴보았다. 이를 통해 종 특이적 차이와 타액선 세포에서의 브래디키닌 수용체의 기능을 제시할 수 있었다. 이상의 연구들은 타액선 세포에 존재하는 GPCR이 그 기능을 유지하기 위해 적절히 작용하고 있으며, 타액선의 질병 중 하나인 구강건조증 기전 연구에 가능성을 보여주고 있다.

종합해보면, 본 논문을 통해서 기존에 보고되지 않았던, 타액선 세포에서 lipid raft 고갈과 GPCR 신호의 관련성을 규명하였고, 항우울제 장기복용에 따른 구강건조증 유발의 예로써

데시프라민이 구강건조증을 야기시키는 매커니즘을 규명하였으며, 사람의 타액선에서 그 동안 보고 되지 않았던 브래디키닌 수용체의 기능에 대해서 밝힘으로써, 타액선 세포에서 GPCR에 의해 유도되는 칼슘 신호에 따른 타액선 기능을 연구하는데 방향성을 제시하였다.

주요어: 타액선, GPCR, 칼슘, Aquaporin5, Lipid raft, 구강건조증, 데시프라민, 브래디키닌

학번: 2010-22023

理學博士學位論文

The role of G-protein-coupled receptor induced Ca^{2+} signaling in salivary gland cells

타액선 G-protein-coupled receptor에 의한 세포내 칼슘신호의 역할

2016년 2월

서울대학교 대학원

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이 계 민

ABSTRACT

The role of G-protein-coupled receptor induced-Ca^{2+} signaling in salivary gland cells

Keimin Lee

Salivation is an important function of the salivary gland. The cells of salivary gland are non-excitable cells which lack the expression of voltage-sensitive channel but express G-protein coupled receptor (GPCR) which receives external signals and conducts physiological functions. In salivary gland, the function of GPCR is crucial for salivation. Therefore, a change in the signal transduction conducted by GPCR is thought to cause changes in salivation. The excretion of saliva from salivary gland is well known to be controlled by different external factors including neural input; however, not much research has been done on identifying the different types of GPCR that are involved in transferring these external signals. Also, the effect of different pharmaceutical drugs that cause xerostomia on the function of GPCR is not well established. Therefore, doing a research on identifying the role of GPCR signal transduction in salivary gland cell withholds a great

significance and it has the potential to improve the field of exocrine physiology and clinical dentistry. By observing the effect of lipid raft disruption in three form of membrane events involved in muscarinic transcellular water movement, I attempted to understand the contribution of lipid raft in each steps of water movement. Thus, it was focused on identifying the correlation between the underlying mechanism of salivation and antidepressant. Especially, the results were revealed the involvement of the signal transduction conducted by GPCR with salivation. Furthermore, I revealed many different receptors, ion channels and membrane transporters that are yet undiscovered and understand the external signal transmission mechanism to identify the connection between the salivation and GPCR signal transduction pathway. First, I examined for changes in physiology functions of the GPCR in salivary gland cells by disruption of the lipid raft which is known to play an important role in membrane physiology. In this study, through the depletion of cholesterol, I can explain that MβCD effects on the salivary GPCR. Next, I treated desipramine in the salivary gland cells and confirmed mechanisms accordingly. Desipramine is known to cause dry mouth when taken long term. However, because not yet clarified for its mechanism, my results can be explained due to dry mouth caused

desipramine. Finally, I found new GPCR in the human salivary gland cells. I was confirmed the expression of the bradykinin receptor, and my results suggested the role of bradykinin in human salivary gland cells.

Taken together, through this paper, the relationship between the lipid raft depletion and GPCR signaling were identified. And I described the mechanism of dry mouth caused by desipramine in salivary gland cells. Also I demonstrated the role of bradykinin in human salivary gland cells. So, I suggested that it is important to study the function of the salivary GPCR-induced calcium signals.

Key Words: Salivary glands, GPCR, Calcium, Aquaporin5, Lipid raft, Xerostomia, Desipramine, Bradykinin

Student Number: 2010-22023

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General Introduction

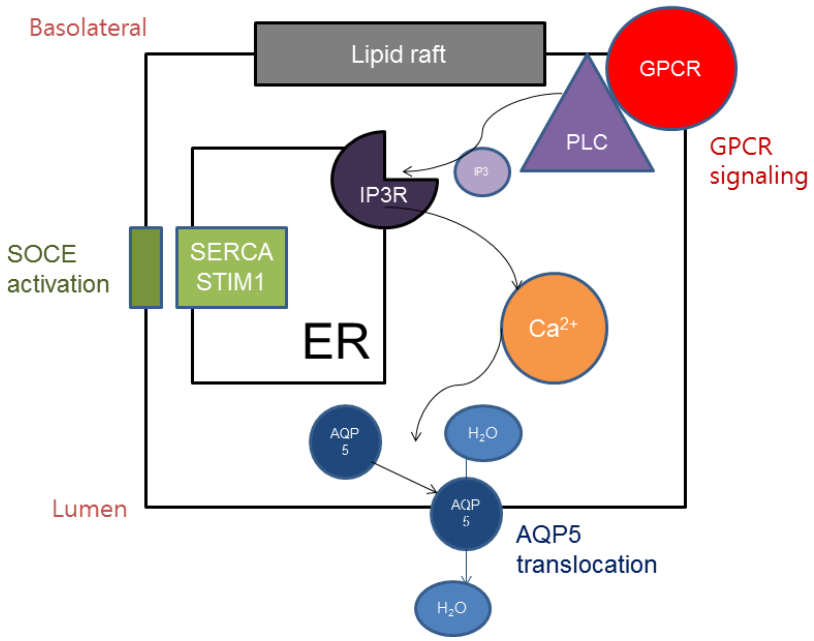
The salivary glands are exocrine organs formed saliva went to the inside of the epithelial surface is to be released into the oral cavity. The salivary glands are composed of acinar cell and ductal cell. The position can be divided into the parotid gland, submandibular gland, sublingual gland.

Saliva is known to play an important role in preserving homeostasis in the oral cavity. Homeostasis, including osmolarity, pH and hydration level, is controlled by the excretion of saliva. Salivation is an important function of the salivary gland. The cells of salivary gland are a non-excitable cells which lack the expression of voltage-sensitive channel but express G-protein coupled receptor (GPCR) which receive external signals and conduct physiological functions. The activation of GPCR in the salivary gland cells is already known to increase the intracellular Ca^{2+} level. As an example, acetylcholine released from parasympathetic nerve terminal activates muscarinic M3 receptors, Gq/11 protein and phospholipase C and cause an increase in intracellular Ca^{2+} level. This change in intracellular Ca^{2+} level causes movement of water in the salivary gland cell and eventually leads to excretion of saliva into the environment. There are two main types of water transportation in the salivary gland, transcellular pathway and paracellular

pathway. Transcellular pathway is involved with the movement of a water channel called aquaporin under external stimulus. Aquaporin is water-permeable transmembrane protein involved in the physiology of these secretory gland functions. The expression and roles of AQPs have been studied in secretory glands where their main function is in fluid secretion. When acetylcholine is present, AQP5 traffics in vitro from intracellular vesicles to the acinar plasma membrane. Paracellular pathway is involved with the release of Na^+ and water through intercellular space under particular condition produced by transporter mediated ion movement and movement of Cl^- to the luminal surface. These two pathways are known to be activated by the change in intracellular Ca^{2+} level. In salivary gland, the function of GPCR is crucial for salivation. Therefore, a change in the signal transduction conducted by GPCR is thought to cause changes in salivation. Xerostomia, also known as “dry mouth”, is a condition in which the level of salivation decrease consistently. The patients with xerostomia usually experience difficulty in maintaining homeostasis in their oral cavity and usually these patients end up with a secondary disease called the rampant caries. The excretion of saliva from salivary gland is well known to be controlled by different external factors including neural input; however, not much research has been done on identifying the different types of GPCR that are involved

in transferring these external signals. Also, the effect of different pharmaceutical drugs that cause xerostomia on the function of GPCR is not well established. Therefore, doing a research on identifying the role of GPCR signal transduction in salivary gland cell withholds a great significance and it has the potential to improve the field of exocrine physiology and clinical dentistry.

In the field of oral biology, many researchers have been focusing their experiments on the salivation of the patients with Sjogren's syndrome. In addition, more research has been conducted to explore the side effect of radiation therapy for Sjogren's syndrome which is also known to decrease salivation. With the aids of these researches, the clinical therapy for Sjogren's syndrome has improved with greater efficiency. Unlike the effect of Sjogren's syndrome and radiation therapy, not much experiment are being done on the effect of pharmaceutical drug on the salivary gland. In fact, drugs ingestion is a form of therapy which is more frequently practiced in my daily life and therefore, greater understanding of its side effect is urgent. In this thesis, the effect of different pharmaceutical drugs on the physiology of salivary gland and salivation is observed and greater emphasis is made on its linkage to the GPCR signaling transduction.



Ca^{2+} signaling and regulation of fluid secretion in salivary gland acinar cells.

CHAPTER 1:

Salivary G-protein coupled receptor mediated Ca^{2+} signaling and Lipid Rafts

Abstract

Lipid raft is located within the cell membrane and it is known to have an important physiological function; however, it is unclear whether the disruption of lipid raft has an impact on the function of the whole cell membrane or have only a partial impact. The functions that are influenced by the disruption of the lipid raft can have a constituent element that is either placed on top of the lipid raft or be regulated by the structure of the lipid raft. By observing the effect of lipid raft disruption in three form of membrane events involved in muscarinic transcellular water movement, I attempted to understand the contribution of lipid raft in each steps of water movement. In my lab, I demonstrated how MβCD incubation, which disrupts the lipid raft by depleting cholesterol**,** inhibits muscarinic and histamine receptor mediated Ca^{2+} signaling but do not effect thapsigargin-induced Ca^{2+} signaling. In addition, I also demonstrated how MβCD incubation inhibits all muscarinic histamine and thapsigargin mediated translocation of aquaporin.

Introduction

Lipid raft is known that many proteins together to send and receive signals. In particular, lipid raft in polarity cells play a critical role in the physiological function (Kai Simons and Derek Toomre, 2000). There are also a variety of lipid rafts in the receptor, and a platform that transfers the signal into the cell outside the cell. Lipid rafts have been mainly studied in epithelial and immune cell, it has recently been revealed about the functions of the exocrine. The function of the lipid raft has been reported in many types of cells, but little was known about its role in the salivary glands. Salivation is an important function of the salivary gland. The cells of salivary gland are a non-excitable cells which lack the expression of voltage-sensitive channel but express G-protein coupled receptor (GPCR) which receive external signals and conduct physiological functions. The activation of GPCR in the salivary gland cells is already known to increase the intracellular Ca^{2+} level. GPCR activation, store operated Ca^{2+} entry (SOCE) activation and AQP5 translocation are activated by membrane protein. However, it was unclear how lipid raft works with them. A recent study shows that activation of M3 mAChRs increases flotillin (lipid raft marker) and AQP5 co-localization (Ishikawa et al., 2005). However, there is not study about comparison GPCR

signaling with SOCE activation in lipid raft disruption. Therefore, I are going to study how lipid raft structure change after M3 receptor activation and AQP5 translocation in membrane. I demonstrate GPCR- Ca^{2+} signaling and AQP5 translocation relationship. In order to understand molecular mechanism of salivary lipid raft, I explain effect of MβCD incubation to GPCR signaling. In this study, I monitored the effect of MβCD on salivary Ca^{2+} signaling. Ca^{2+} signaling and aquaporin translocation in human salivary gland HSG cells have been used to study the pathological mechanisms of salivary impairment such as in Sjogren's syndrome (Ishikawa et al., 2005; Jin et al., 2012a; Lee et al., 2013). I tested the effect of MβCD on the salivary receptors expressed in salivary glands and HSG cells and known to induce Ca^{2+} signaling in an attempt to understand the correlation GPCR and lipid raft.

MATERIALS AND METHODS

Cell Preparation

Human submandibular salivary gland cells were prepared as previously described (Kim et al., 2009). HSG cells were grown in Modified Eagle's Medium (GIBCO, Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (GIBCO) and 1% (v/v) penicillin (5000 U/ml) + streptomycin (5000 μg/ml) solution (GIBCO). The cells were cultured in a humidified atmosphere of 95% air + 5% CO_2. The culture medium was changed every 2 days, and the cells were subcultured weekly.

$[Ca^{2+}]_i$ Measurement

Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was analyzed with fura-2/AM (Seo et al., 2010). Briefly, the cell suspension was incubated with 3 μM fura-2/AM at 37^{o}C for 60 minutes with continuous stirring. Sulfinpyrazone (250 μM) was added to prevent dye leakage. The cells were then washed with Locke's solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 2.2 mM $CaCl_2$, 5.0 mM HEPES, 10 mM glucose, pH 7.4 with NaOH). Changes in the fluorescence ratio were measured at dual excitation wavelengths of 340 and

380 nm with an emission wavelength of 500 nm.

Construction of Aquaporin-5 and Western blotting

Total RNA was isolated from HSG cells, and the full-length cDNA extracted from it was amplified with reverse-transcriptase PCR (RT-PCR). PCR utilized 5'-GAATTCTATGAAGAAGGAGGTGTGCTCC as the upstream primer and 5'-GGTACCGCGGGTGGTCAGCTCCATGGT as the downstream primer (the introduced EcoRI and KpnI restriction sites are underlined). The 798-bp human aquaporin-5 gene was then cloned into pEGFP-C1. HSG cells were transiently transfected with the aquaporin-5 gene using LipofectAMINE 2000 (Invitrogen). Aquaporin-5-transfected HSG cells were grown in a 60-mm dish, pre-incubated with MβCD for 30 min, and incubated with carbachol or histamine for 20 min at 37°C. The apical membrane fraction was sonicated in ice-cold 20 mM HEPES solution containing 1 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, and 0.3 mM phenylmethylsulfonyl fluoride (3×30 s, Branson Sonifer) at pH 7.4. The samples were centrifuged at 600×g at 4 °C, and the supernatants were further centrifuged at 20,000×g at 4 °C. The pellets (P2 membrane fractions) were subjected to SDS/PAGEβ immunoblotting. After electrophoresis in SDS-

PAGE (10 %) gel, the protein samples were transferred to polyvinylidene difluoride membranes (0.45 μm, Millipore). After blocking, the membrane was incubated with anti-aquaporin-5 as the primary antibody (1:200, Santa Cruz, sc-9891). The membrane was then washed and incubated with donkey anti-goat IgG-HRP. The membrane was finally subjected to an electrochemiluminescence assay. For normalization of the antibody signal, the membranes were stripped and re-probed with antibody for anti-α1 Na^+/K^+-ATPase (1:1000, Abcam, ab7671).

Fluorescence imaging

GFP-tagged cells transfected with aquaporin-5 were plated on glass coverslips coated with poly-L-ornithine. MβCD for 30 min, and incubated with carbachol or histamine or thapsigargin for 20 min at 37°C. The cells were fixed with 4% paraformaldehyde in PBS for 15 min. Slides were mounted and visualized using a laser scanning confocal microscopy (Zeiss LSM 700).

RESULT

MβCD preincubation inhibits muscarinic and histaminergic $[Ca^{2+}]_i$ increase without effect on thapsigargin-mediated $[Ca^{2+}]_i$

I monitored the effect of lipid raft depletion on salivary G-protein coupled receptor signaling in human salivary gland HSG cells, which have been broadly used for salivary Ca^{2+} studies. 10 μM MβCD inhibited the cytosolic increases in Ca^{2+} induced by carbachole (Figure 1-1A). MβCD also inhibited the histamine-induced increases in $[Ca^{2+}]_i$ (Figures 1-1B). However, MβCD did not affect the increase in $[Ca^{2+}]_i$ induced by thapsigargin, which mimics the downstream signaling of phospholipase C activation such as the depletion of the cytosolic Ca^{2+} pool and the induction of store-operated Ca^{2+} entry (Figure 1-1C). These results suggest that MβCD selectively inhibits GPCR Ca^{2+} signaling.

MβCD depletes cholesterol in HSG cells.

It is already well known that MβCD deplets cholesterol and eliminates lipid raft. But I do not have any information about the relevant MβCD incubating condition. Therefore, I need to check for a condition which can deplete cholesterol. After MβCD incubation, I can find cholesterol contents changes

and MβCD treat condition. In 10mM MβCD for 30min, cholesterol of HSG cell membrane decreased (Figure 1-2).

MβCD that inhibits trafficking of AQP5 is controlled by carbachol and histamine.

Trafficking of AQP5 in salivary gland cells is the result of increasing $[Ca^{2+}]_i$ activated by carbachol and histamine. My research is based on a method that has been used for the previous studies (Lee et al., 2015), I studied about the impact of MβCD the trafficking of AQP5. When using a Membrane fraction, the groups treated with histamine, carbachol showed that AQP5 translocation is reduced, the thapsigargin was no significant effect (Figure 1-3) When I use the AQP5-GFP were treated with the Carbachol Histamine, AQP5 was shifted to the plasma membrane, the MβCD was treated, it was confirmed that the tendency is reduced. However, the experimental group treated with thapsigargin was shown to be unaffected by MβCD (Figure 1-4). Through this result, MβCD blocks the calcium signal is controlled by the muscarinic and histamine and suggests that accordingly involved in the regulation AQP5 translocation.

Figure 1-1. The preincubation of MβCD inhibits carbachol- and histamine-induced Ca^{2+} increase.

(A-C) Fura-2-loaded HSG cells were treated by 100 μM carbachol (A), 100 μM histamine (B), or 1 μM thapsigargin (C) with (black trace) or without (gray trace) of preincubation of 10 mM MβCD for 30 min. Typical Ca^{2+} transients from more than five separate experiments are presented. (D) Cells were preincubated with the indicated MβCD concentrations, and then challenged with 100 μM carbachol, 100 μM histamine, or 1 μM thapsigargin. The peak height of Ca^{2+} elevation was monitored. Each point represents mean +/- SEM. All results were reproducible.

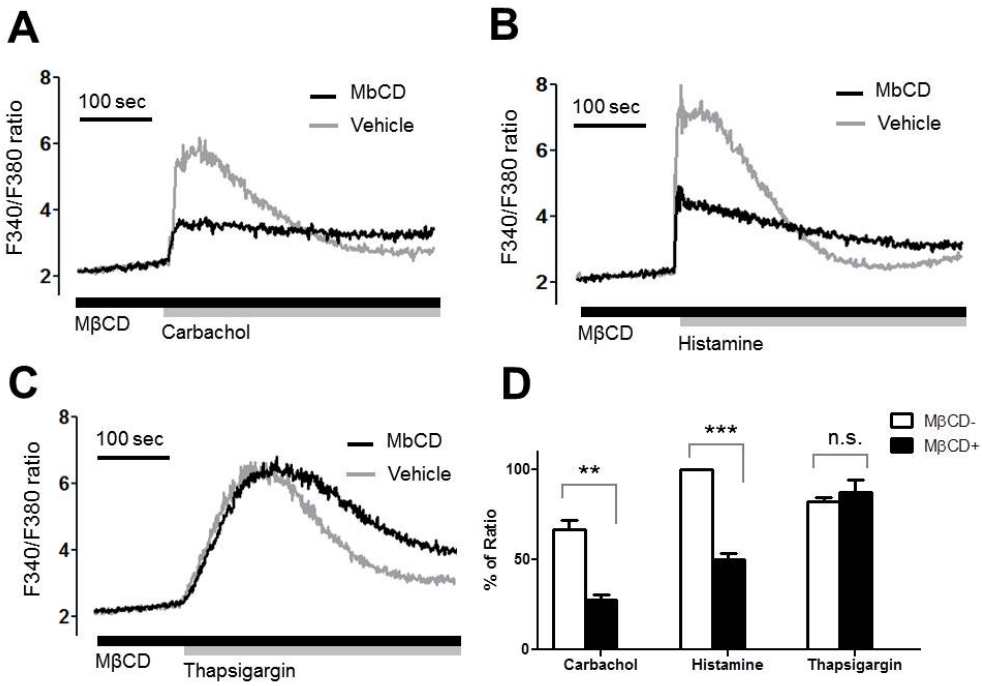

A
F340/F380 ratio
8
6
4
2
100 sec
MbCD
Vehicle
MβCD
Carbachol
B
F340/F380 ratio
100 sec
MbCD
Vehicle
MβCD
Histamine
C
F340/F380 ratio
100 sec
MbCD
Vehicle
MβCD
Thapsigargin
D
% of Ratio
100
50
0
**
***
n.s.
MβCD-
MβCD+
Carbachol
Histamine
Thapsigargin

Figure 1-2. MβCD depletes cholesterol in HSG cells.

(A) HSG cells were incubated with 10 mM MβCD for indicated preincubation time. (B) HSG cells were incubated with the indicated concentration of MβCD for 30 min. An Amplex Red Cholesterol Assay Kit was used to measure cholesterol contents as described in the Materials and Methods. The cholesterol contents of cell lysates are depicted as % of vehicle-treated control. The values represent the average ± SEM. **$p < 0.01$; one way repeated ANOVA test. ($n = 3 - 6$).

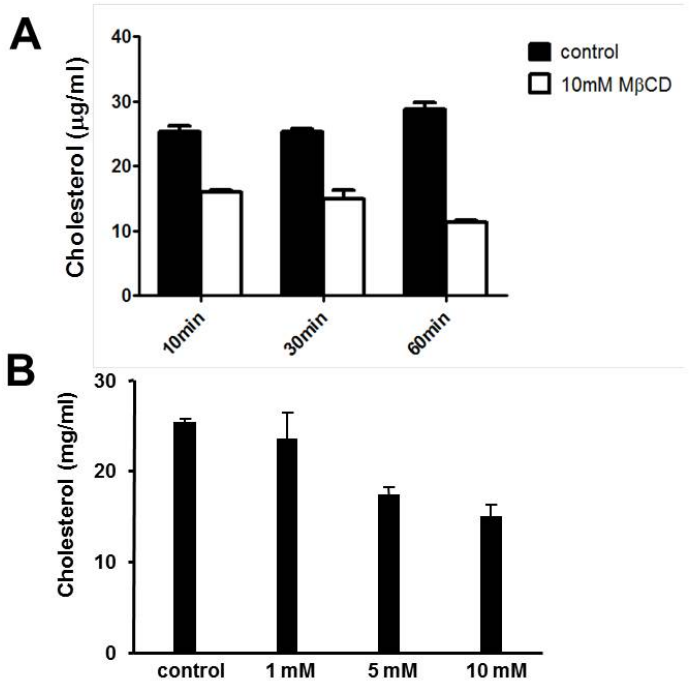

A
Cholesterol (µg/ml)
40
30
20
10
0
control
10mM MβCD
10min
30min
60min
B
Cholesterol (mg/ml)
30
20
10
0
control
1 mM
5 mM
10 mM

Figure 1-3. MβCD inhibits the carbachol- and histamine- mediated increase of AQP5 in the plasma membrane fraction, it does not affect the increasing in AQP5 by thapsigargin.

HSG cells transfected AQP5-GFP were pre-treated in the absence or presence of 10mM MβCD for 30min and then treated with (A) 100 μM carbachol, (B) 100 μM histamine and (C) 1 μM thapsigargin for 20 min. The western blots with membrane fractionation method is described in Materials and Methods. Each data quantification of the membrane AQP5 levels were normalized to the levels of the Na^+-K^+-ATPase subunit. Each values (% of control) was depicted as mean ± SEM. n.s., not significant; *$P < 0.05$.

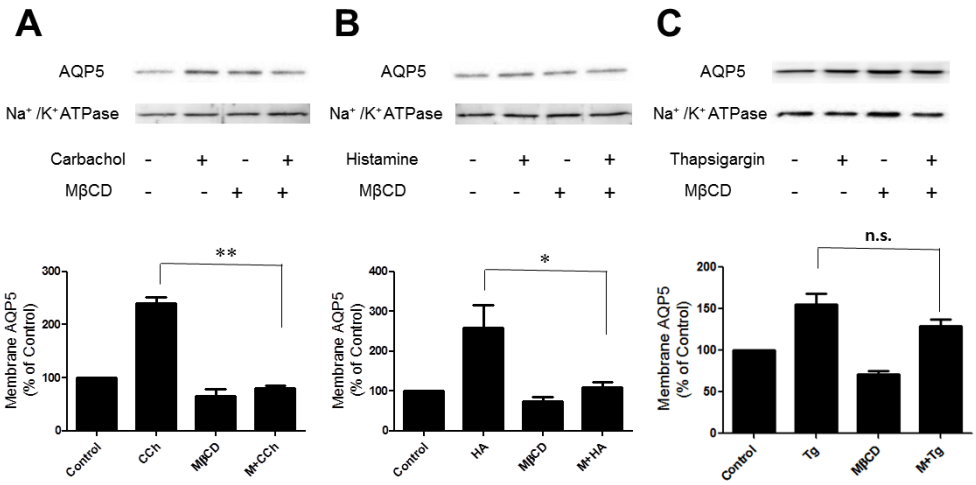
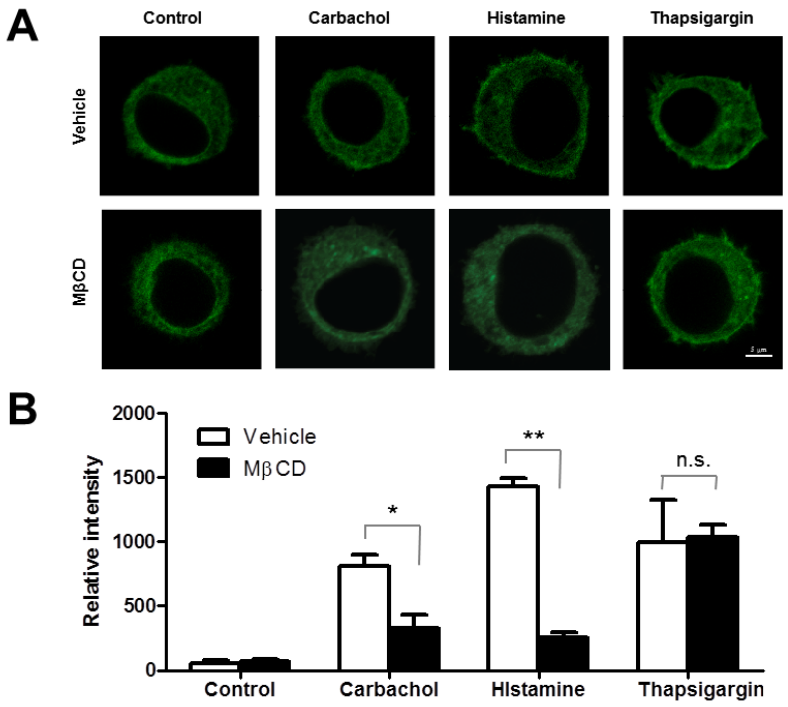

A
AQP5
Na+ /K+ ATPase
Carbachol - + - +
MβCD - - + +
Membrane AQP5 (% of Control)
300
200
100
0
**
Control
CCh
MβCD
M+CCh
B
AQP5
Na+ /K+ ATPase
Histamine - + - +
MβCD - - + +
Membrane AQP5 (% of Control)
400
300
200
100
0
*
Control
HA
MβCD
M+HA
C
AQP5
Na+ /K+ ATPase
Thapsigargin - + - +
MβCD - - + +
Membrane AQP5 (% of Control)
200
150
100
50
0
n.s.
Control
Tg
MβCD
M+Tg

Figure 1-4. MβCD blocks the carbachol- and histamine- induced AQP5 translocation, but it does not affect thapsigargin-mediated AQP5 translocation.

HSG Cells transfected AQP5-GFP were pre-incubated in the absence of presence of 10mM MβCD for 30min and then challenged with 100 μM carbachol, 100 μM histamine and 1 μM thapsigargin for 20 min. (A) the figure shows trend of trafficking AQP5-GFP. The images were made by a confocal microscope. (B) I quantified the AQP5 to location on the membrane statistically by measuring the fluorescence intensity.


A
Control
Carbachol
Histamine
Thapsigargin
Vehicle
MβCD
B
Relative intensity
2000
1500
1000
500
0
Vehicle
MβCD
*
**
n.s.
Control
Carbachol
HIstamine
Thapsigargin

DISCUSSION

I described to influence the AQP5 translocation as calcium signal is controlled by the muscarinic and histamine receptor lipid raft depletion by MβCD in salivary gland cells. The depletion of lipid raft not been reported to influence the function of saliva from the salivary gland cells. In salivary gland muscarinic receptor is known to influence in controlling the increase in intracellular calcium and salivation. In the salivary control, the movement of water can be divided into two main methods. One of the water movement in paracellular pathway due to ion exchange by cell membrane transporter, and the other is moved to the channel of AQP5 the plasma membrane to pass through the water there is a transcellular pathway by increasing the activity. My results suggest the lipid raft depletion inhibits the calcium signal of histamine and muscarinic receptor (Fig.1-1, 1-2) and shows that reduced translocation of AQP5 (Fig1-3, 1-4). In addition, lipid raft depletion does not affect thapsigargin induced $[Ca^{2+}]_i$ changes. And MβCD does not inhibit thapsigargin induced AQP5 translocation. Through these results, I suggest that lipid raft depletion does not affect SOCE.

I found that MβCD deplete lipid raft, and inhibit not only the muscarinic receptor, but also the histamine receptor. MβCD did not inhibit the $[Ca^{2+}]_i$ –

mediated increase in thapsigargin. Thapsigargin is a sarcoplasmic reticular Ca^{2+}-ATPase inhibitor that triggers Ca^{2+} release from the intracellular Ca^{2+} pool and store-operated calcium entry.

My results suggested that MβCD blocks salivary muscarinic and histamine – induced Ca^{2+} signaling and AQP5 translocation. But SOCE does not have any relationship with lipid raft depletion. These data will be helpful for understanding the cellular mechanism of lipid raft depletion in salivary gland and for studying effect of lipid raft depletion in dry mouth.

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CHAPTER 2*:

Desipramine-mediated inhibition of salivary Ca^{2+} signaling and aquaporin translocation

***This chapter has been largely reproduced from an article published Lee K, Choi S, Choi LM, Lee J, Kim JH, Chung G, Lee G, Choi SY, Park K. Oral Dis.2015 May;21(4):530-5**

ABSTRACT

Desipramine is a tricyclic antidepressant with a negative side effect of dry mouth. The Na^+/H^+ exchanger was suggested to be a target of desipramine in salivary gland cells. However, it is unclear whether desipramine has other targets in the salivary secretion pathway. Here I studied the effect of desipramine on salivary Ca^{2+} signaling. Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined with the fluorescent Ca^{2+} indicator fura-2/AM. Aquaporin translocation was analyzed by Western blotting and immunocytochemistry of confocal microscopy. Desipramine inhibited the carbachol- and histamine-mediated increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in a concentration-dependent manner. However, desipramine did not affect increases in $[Ca^{2+}]_i$ mediated by extracellular ATP, sphingosine-1-phosphate, or thapsigargin. The adrenergic receptor blockers prazosin and propranolol did not reverse the desipramine-mediated inhibition of carbachol- and histamine-induced increases in $[Ca^{2+}]_i$. I also found that desipramine inhibits the increase in membrane aquaporin-5 level triggered by carbachol and histamine treatments. These results imply that desipramine blocks muscarinic and histamine receptor-mediated Ca^{2+} signaling and the subsequent translocation of aquaporin-5 in human salivary gland cells, suggesting a novel mechanism for the xerogenic effects of desipramine.

INTRODUCTION

Xerostomia, or dry mouth, is one of the most common oral diseases, manifesting as decrease in salivation (Turner and Ship, 2007). The most common causes of dry mouth are extrinsic medicines, salivary gland damage caused by radiation therapy, and Sjögren's syndrome. To date, approximately 400-600 chemicals are reported to induce dry mouth (Sreebny and Schwartz, 1997; Scully, 2003; Guggenheimer and Moore, 2003). In particular, psychiatric drugs such as antidepressants, antipsychotics, and drugs for Parkinson's disease produce serious dry mouth.

Desipramine is an orally administrable tricyclic antidepressant that is widely prescribed for trigeminal neuropathic pain and major mood disorders (Potter et al., 1991). However, desipramine is reported to induce xerostomia (Scarpace et al., 1993; Koller et al., 2000; Galanter et al., 2002). With its inhibition of catecholamine reuptake in the nerve terminal, desipramine increases synaptic catecholamines and prolongs adrenergic signaling (Nelson et al., 1984). However, the relationship between adrenergic signaling and reduced salivation has not yet been proven in salivary glands. In a previous study, I reported that desipramine inhibits Na^+/H^+ exchanger activity (Choi et al., 2006), which serves an important role in salivary secretion (Turner and

Sugiya, 2002). However, it is unclear whether the Na^+/H^+ exchanger is the sole target of desipramine, or whether desipramine may have other critical targets such as members of the salivary Ca^{2+} signaling and aquaporin translocation pathways.

In this study, I monitored the effect of desipramine on salivary Ca^{2+} signaling. Ca^{2+} signaling and aquaporin translocation in human salivary gland HSG cells have been used to study the pathological mechanisms of salivary impairment such as in Sjögren's syndrome (Ishikawa et al., 2005; Jin et al., 2012a; Lee et al., 2013). I tested the effect of desipramine on the salivary receptors expressed in salivary glands and HSG cells and known to induce Ca^{2+} signaling (i.e., muscarinic M3 receptor, histamine H1 receptor, P2X7 purinoceptor, and sphingosine-1-phosphate S1P1 receptor) in an attempt to understand the xerogenic mechanism of desipramine.

MATERIALS AND METHODS

Chemicals

Desipramine, histamine, carbachol, desipramine, ATP, thapsigargin, propranolol, and prazosin were purchased from Sigma (St. Louis, MO, USA). Fura-2-acetoxymethyl ester (Fura-2-AM) was obtained from Molecular Probes (Eugene, OR, USA). Modified Eagle's Medium, bovine calf serum, and penicillin/streptomycin were purchased from GIBCO (Grand Island, NY, USA).

Cell Preparation

Human submandibular gland HSG cells were grown in Modified Eagle's Medium supplemented with 10% (v/v) heat-inactivated bovine calf serum and 1% (v/v) penicillin (5,000 U/ml) + streptomycin (5,000 μg/ml) solution. The cells were cultured in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was changed every two days, and the cells were sub-cultured weekly.

$[Ca^{2+}]_i$ *Measurement*

Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined with the

fluorescent Ca^{2+} indicator fura-2/AM. Briefly, a cell suspension was incubated with fresh Modified Eagle's Medium containing fura-2/AM (4 μM) for 40 min at 37°C with continuous stirring. The cells were then washed with a HEPES-buffered solution (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH adjusted to 7.4 with NaOH) or Locke's solution (154 mM NaCl; 5.6 mM KCl; 1.2 mM $MgCl_2$; 2.2 mM $CaCl_2$; 5.0 mM HEPES; 10 mM glucose, pH 7.4 with NaOH) and left at room temperature until use. Sulfinpyrazone (250 μM) was added to all solutions to prevent dye leakage. Cells were transferred to poly-L-lysine-coated 25-mm coverslips for cell attachment and held for 40 min in an incubator, and then the coverslips were mounted onto the chamber. Changes in the fluorescence ratio were monitored by a Ca^{2+} imaging machine with MetaFlour software (Molecular Devices, Sunnyvale, CA, USA) with dual excitation at 340 and 380 nm and emission at 500 nm. Experiments with suspended HSG cells were performed with a spectrofluorophotometer using the same principle.

Construction of Aquaporin-5 and Western blotting

Total RNA was isolated from HSG cells, and the full-length cDNA extracted from it was amplified with reverse-transcriptase PCR (RT-PCR).

PCR utilized 5’-GAATTCTATGAAGAAGGAGGTGTGCTCC as the upstream primer and 5’-GGTACCGCGGGTGGTCAGCTCCATGGT as the downstream primer (the introduced EcoRI and KpnI restriction sites are underlined). The 798-bp human aquaporin-5 gene was then cloned into pEGFP-C1. HSG cells were transiently transfected with the aquaporin-5 gene using LipofectAMINE 2000 (Invitrogen). Aquaporin-5-transfected HSG cells were grown in a 60-mm dish, pre-incubated with desipramine for 3 min, and incubated with carbachol or histamine for 20 min at 37°C. The apical membrane fraction was sonicated in ice-cold 20 mM HEPES solution containing 1 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, and 0.3 mM phenylmethylsulfonyl fluoride (3×30 s, Branson Sonifer) at pH 7.4. The samples were centrifuged at 600×g at 4 °C, and the supernatants were further centrifuged at 20,000×g at 4 °C. The pellets (P2 membrane fractions) were subjected to SDS/PAGE immunoblotting. After electrophoresis in SDS-PAGE (10 %) gel, the protein samples were transferred to polyvinylidene difluoride membranes (0.45 μm, Millipore). After blocking, the membrane was incubated with anti-aquaporin-5 as the primary antibody (1:200, Santa Cruz, sc-9891). The membrane was then washed and incubated with donkey anti-goat IgG-HRP. The membrane was finally subjected to an electrochemiluminescence assay. For normalization of the antibody signal,

the membranes were stripped and re-probed with antibody for Gβ (1:500, Santa Cruz, sc-25413) or anti-α1 Na^+/K^+-ATPase (1:1000, Abcam, ab7671).

Immunocytochemistry

GFP-tagged cells transfected with aquaporin-5 were plated on glass coverslips coated with poly-L-ornithine. Cells were pre-treated with desipramine for 3 min, followed by treatment with carbachol and histamine for 20 min. The cells were fixed with 4% paraformaldehyde in PBS for 15 min and permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature (20-25 ^oC). They were then incubated for 1 h at room temperature in blocking solution, 1% BSA in PBS. The samples were incubated with primary antibodies for 2 h at room temperature or overnight at 4°C and incubated with fluorescence-conjugated secondary antibodies for 1 h at room temperature The following antibodies were used in this study: anti-alpha1 Na^+-K^+-ATPase (1:100, Abcam, ab7671) and Cy3-conjugated secondary antibodies (1:200, Jackson Laboratories). Slides were mounted and visualized using a laser scanning confocal microscopy (Zeiss LSM 700).

Data Analysis

All quantitative data are expressed as mean ± SEM. We calculated the half-maximal inhibitory concentration (IC_{50}) with the Microcal Origin program. Differences were determined by one-way analysis of variance with post hoc tests and were considered to be significant when $P < 0.05$.

RESULT

Desipramine inhibits muscarinic and histamine Ca^{2+} signaling without an effect on extracellular ATP-, spingosine-1-phosphate-, or thapsigargin-mediated $[Ca^{2+}]_i$ increases

I monitored the effect of desipramine on salivary Ca^{2+} signaling in human salivary gland HSG cells, which have been broadly used for salivary Ca^{2+} studies. Desipramine inhibited the cytosolic increases in Ca^{2+} induced by carbachol, a strong salivary secretagogue (Figure 2-1A). The inhibitory effect of desipramine on carbachol-mediated increases in $[Ca^{2+}]_i$ showed concentration dependence with a 369 ± 95 nM IC_{50} (Figure 2-1B). Desipramine also inhibited the histamine-induced increases in $[Ca^{2+}]_i$ in a concentration-dependent manner (IC_{50}: 184 ± 35 nM) (Figures 2-1C, 2-1D). However, desipramine did not affect the ionotrophic P2X7 receptor-mediated increase in $[Ca^{2+}]_i$ (Hwang et al., 2009; Figure 2-2A). Desipramine also showed no inhibitory effect on the increase in $[Ca^{2+}]_i$ mediated by sphingosine-1-phosphate, another phospholipase C-linked G-protein coupled receptor in HSG cells (Seo et al., 2010; Figure 2-2B). In addition, desipramine did not affect the increase in $[Ca^{2+}]_i$ induced by thapsigargin, which mimics the downstream signaling of phospholipase C activation such

as the depletion of the cytosolic Ca^{2+} pool and the induction of store-operated Ca^{2+} entry (Figure 2-2C). These results suggest that desipramine selectively blocks muscarinic and histamine Ca^{2+} signaling.

The adrenergic receptor blockers propranolol and prazosin do not reverse desipramine-mediated inhibition

Desipramine inhibition of catecholamine reuptake is blocked by adrenergic receptor antagonists (Kleven and Koek, 1998). However, both prazosin, an alpha-adrenergic antagonist (Figures 2-3A), and propranolol, a beta-adrenergic antagonist (Figures 2-3C), failed to reverse the desipramine-mediated inhibition of carbachol-induced increases in $[Ca^{2+}]_i$. The same trend was observed with histamine-induced increases in $[Ca^{2+}]_i$ (Figures 2-3B, 2-3D), suggesting that the effect of desipramine is independent from the inhibition of catecholamine reuptake.

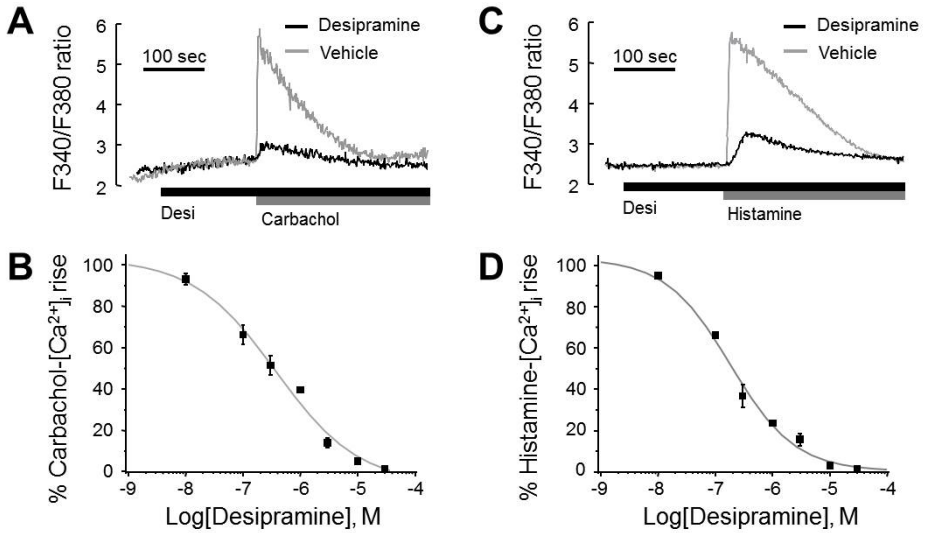
Desipramine inhibits carbachol and histamine-mediated aquaporin-5 translocation in HSG cells

Muscarinic and histamine Ca^{2+} signaling trigger the membrane trafficking of aquaporin-5, a ‘water channel’ protein in the salivary gland (Delporte and Steinfeld, 2006; Kim et al., 2009; Lee et al., 2013). To determine the effect of

desipramine on aquaporin, I analyzed the expression level of aquaporin-5 in the membrane fraction. I searched for optimal stimulation conditions for the quantification of AQP5 translocation and found that carbachol and histamine stimulation for 10 min and 20 min showed clear AQP5 translocation. With 20 min stimulation, carbachol and histamine increased membrane aquaporin-5 levels, while desipramine blocked both carbachol- and histamine-induced aquaporin-5 translocations (Figures 2-4). I confirmed this finding with GFP-tagged aquaporin-5, which moved to the plasma membrane with carbachol and histamine treatment. Cells treated with carbachol and histamine showed stronger fluorescence in the plasma membrane than in the cytosol, whereas cells co-treated with desipramine, carbachol, and histamine showed similar fluorescence intensities in both the cytosol and the plasma membrane (Figure 2-5). These results suggest that desipramine blocks muscarinic and histamine receptor-mediated Ca^{2+} signaling and subsequent aquaporin-5 translocation in human salivary gland cells.

Figure 2-1. Desipramine inhibits muscarinic and histamine-induced $[Ca^{2+}]_i$ increases in human salivary gland HSG cells.

(A) Fura-2-loaded HSG cells were pre-incubated with 10 μM desipramine (Desi, black) or vehicle (gray) for 3 min and treated with 300 μM carbachol. Changes in the fluorescence ratio of F340/F380 were monitored as described in Materials and Methods. Typical Ca^{2+} traces from more than five separate experiments are presented. (B) Cells were pre-incubated with desipramine with the indicated concentrations, and the inhibition of 300 μM carbachol-induced increases in Ca^{2+} was monitored. The peak height of Ca^{2+} elevation was analyzed, and each point represents the mean ± SEM. (C) Cells were pre-incubated with 10 μM desipramine (Desi, black) or vehicle (gray) for 3 min and then treated with 100 μM histamine. Changes in the fluorescence ratio of F340/F380 were monitored, and typical Ca^{2+} traces from more than four separate experiments are presented. (D) Cells were pre-incubated with desipramine at the indicated concentrations, and the inhibition of 100 μM histamine-induced increases in Ca^{2+} was monitored. The peak height of Ca^{2+} elevation was analyzed, and each point represents the mean ± SEM. All results were reproducible.


A
F340/F380 ratio
6
5
4
3
2
100 sec
Desipramine
Vehicle
Desi
Carbachol
C
F340/F380 ratio
6
5
4
3
2
100 sec
Desipramine
Vehicle
Desi
Histamine
B
% Carbachol-[Ca2+]i rise
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
Log[Desipramine], M
D
% Histamine-[Ca2+]i rise
100
80
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40
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0
-9
-8
-7
-6
-5
-4
Log[Desipramine], M

Figure 2-2. Desipramine does not inhibit extracellular ATP-, spingosine-1-phosphate-, or thapsigargin-mediated $[Ca^{2+}]_i$ increase in HSG cells.

(A-C) Fura-2-loaded HSG cells were pre-incubated with 10 μM desipramine (Desi, black) or vehicle (gray) for 3 min and treated with 300 μM ATP (B), 10 μM sphingosine-1-phosphate (S-1-P), or 3 μM thapsigargin (C). Changes in the fluorescence ratio of F340/F380 were monitored, and typical Ca^{2+} traces from more than five separate experiments are presented. All results were reproducible.

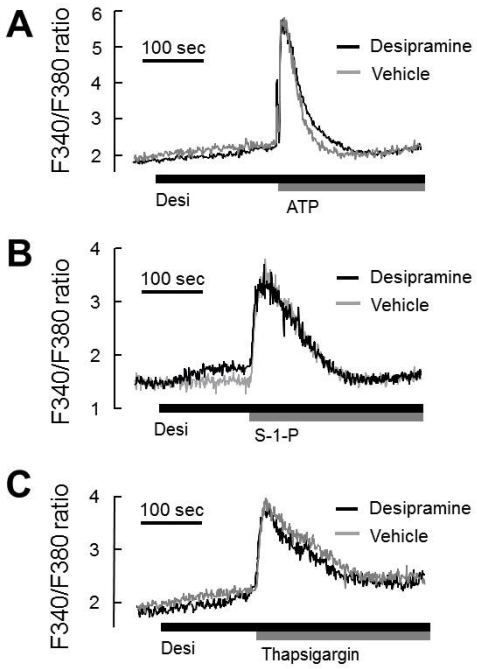

A
F340/F380 ratio
6
5
4
3
2
100 sec
Desipramine
Vehicle
Desi
ATP
B
F340/F380 ratio
4
3
2
1
100 sec
Desipramine
Vehicle
Desi
S-1-P
C
F340/F380 ratio
4
3
2
100 sec
Desipramine
Vehicle
Desi
Thapsigargin

Figure 2-3. The adrenergic receptor blockers propranolol and prazosin do not reverse desipramine-mediated inhibition.

(A-D) Fura-2-loaded HSG cells were pre-incubated with vehicle (light gray), 10 μM desipramine (Desi, dark gray), 10 μM desipramine with 3 μM prazosin (A, B) (Prazosin+Desi, black), or 10 μM desipramine with 3 μM propranolol (C, D) (Propranolol+Desi, black) for 3 min and treated with 300 μM carbachol (A, C) or 100 μM histamine (B, D). Changes in the fluorescence ratio of F340/F380 were monitored, and typical Ca^{2+} traces from more than five separate experiments are presented. (E-F) Cells were pre-incubated with desipramine with desipramine, prazosin, or propranolol, and then 300 μM carbachol (E)- or 100 μM histamine (F)-induced increases in Ca^{2+} were monitored. The peak height of Ca^{2+} elevation was analyzed, and each point represents the mean ± SEM.

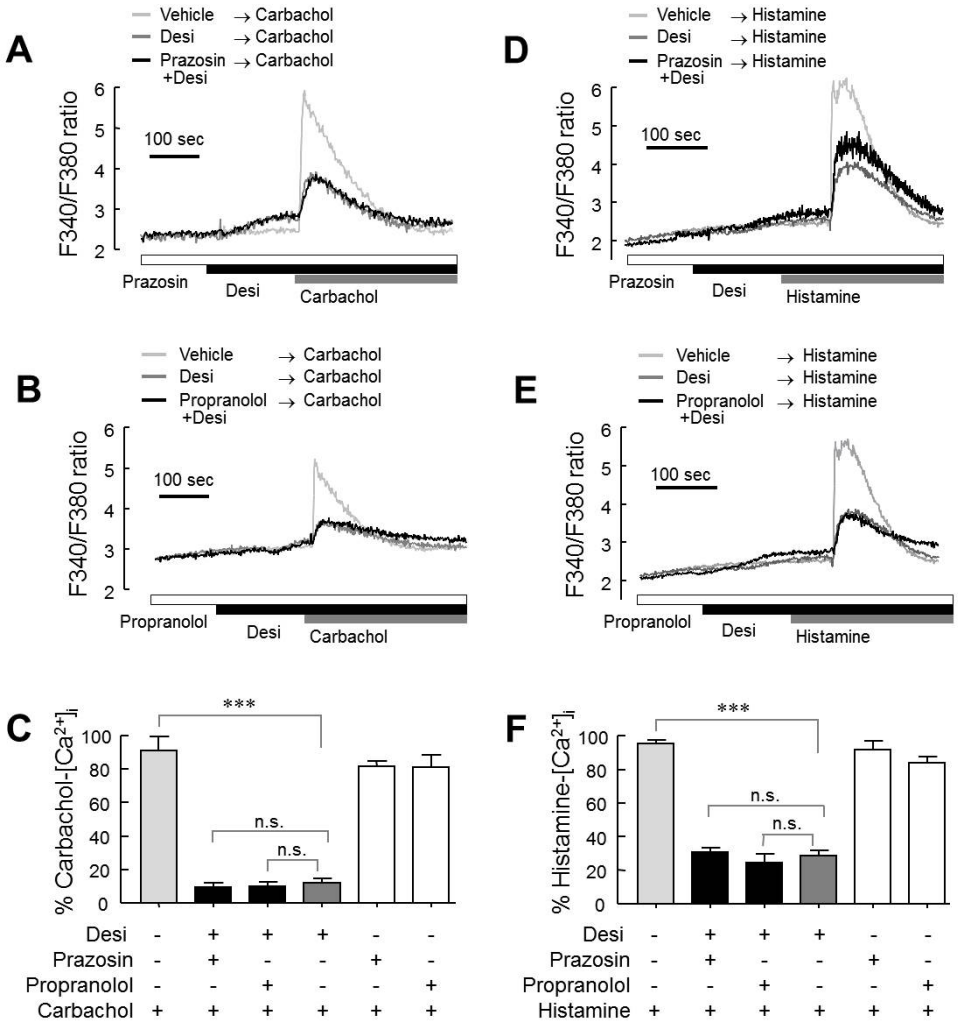

A
Vehicle → Carbachol
Desi → Carbachol
Prazosin +Desi → Carbachol
F340/F380 ratio
100 sec
Prazosin
Desi
Carbachol
D
Vehicle → Histamine
Desi → Histamine
Prazosin +Desi → Histamine
F340/F380 ratio
100 sec
Prazosin
Desi
Histamine
B
Vehicle → Carbachol
Desi → Carbachol
Propranolol +Desi → Carbachol
F340/F380 ratio
100 sec
Propranolol
Desi
Carbachol
E
Vehicle → Histamine
Desi → Histamine
Propranolol +Desi → Histamine
F340/F380 ratio
100 sec
Propranolol
Desi
Histamine
C
% Carbachol-[Ca2+]i
***
n.s.
n.s.
Desi - + + + - -
Prazosin - + - - + -
Propranolol - - + - - +
Carbachol + + + + + +
F
% Histamine-[Ca2+]i
***
n.s.
n.s.
Desi - + + + - -
Prazosin - + - - + -
Propranolol - - + - - +
Histamine + + + + + +

Figure 2-4. Desipramine blocks the carbachol- and histamine-mediated increase of aquaporin-5 in the plasma membrane fraction.

GFP-tagged HSG cells transfected with aquaporin-5 were pre-incubated in the absence or presence of 10 μM desipramine for 3 min and then challenged with 100 μM carbachol and 100 μM histamine for 20 min. (A) Western blots show the membrane aquaporin-5 (AQP5) and G-β subunit (Gβ). The preparation of the membrane fractionation is described in Materials and Methods. (B) Quantification of the membrane aquaporin-5 with G-β subunit control (n = 3 - 6). Membrane aquaporin-5 levels were normalized to the level of the membrane G-β subunit. (C) Western blots show membrane aquaporin-5 (AQP5) and Na^+-K^+-ATPase. (D) Quantification of the membrane aquaporin-5 with Na^+-K^+-ATPase (NKE) control (n = 3 - 6). Membrane aquaporin-5 levels were normalized to the levels of the membrane Na^+-K^+-ATPase subunit. Each values (% of control) was depicted as mean ± SEM. n.s., not significant; *$P < 0.05$.

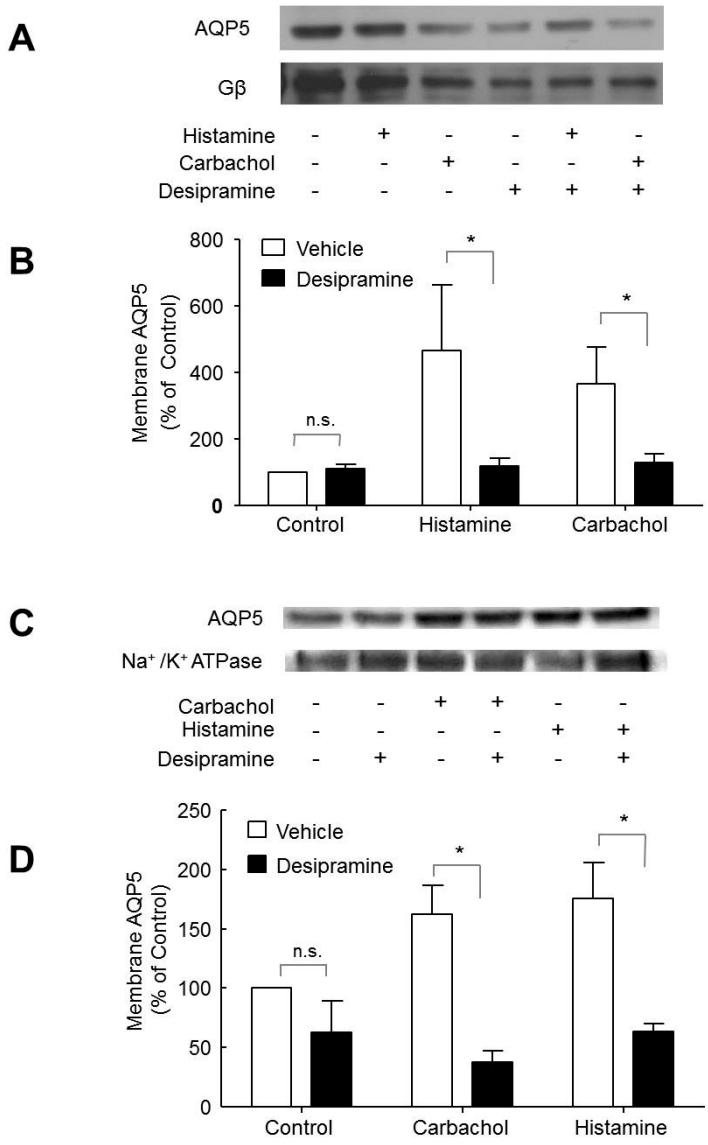
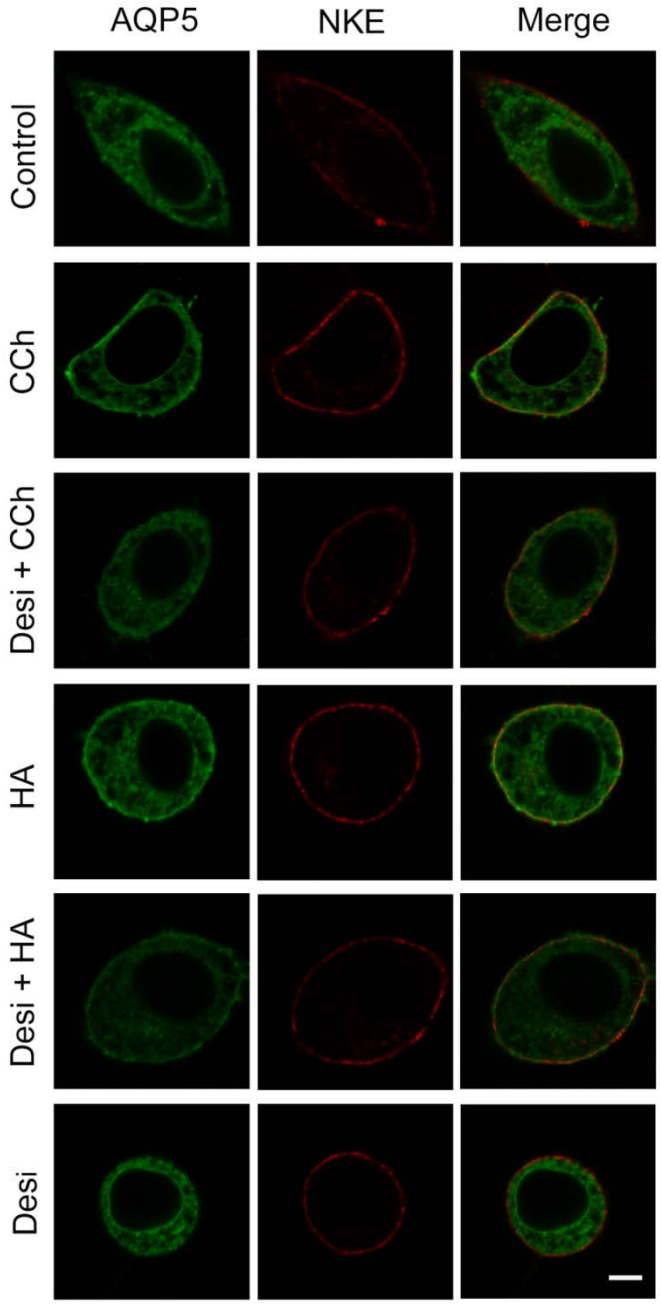

A
AQP5
Gβ
Histamine - + - - + -
Carbachol - - + - - +
Desipramine - - - + + +
B
Vehicle
Desipramine
Membrane AQP5 (% of Control)
800
600
400
200
0
n.s.
*
*
Control
Histamine
Carbachol
C
AQP5
Na+ /K+ ATPase
Carbachol - - + + - -
Histamine - - - - + +
Desipramine - + - + - +
D
Vehicle
Desipramine
Membrane AQP5 (% of Control)
250
200
150
100
50
0
n.s.
*
*
Control
Carbachol
Histamine

Figure 2-5. Desipramine inhibits carbachol- and histamine-mediated aquaporin-5 translocation.

Cells transfected with GFP-tagged aquaporin-5 were pre-incubated in the absence or presence of 10 μM desipramine (Desi) for 3 min and then challenged with 100 μM carbachol (CCh) or 100 μM histamine (HA) for 20 min. Na^+-K^+-ATPase (NKE, red) was used as the membrane marker and is depicted together with GFP-AQP5 (AQP5, green). The images were obtained with a confocal microscope as described in the Materials and Methods.


AQP5
NKE
Merge
Control
CCh
Desi + CCh
HA
Desi + HA
Desi

Discussion

I have shown that desipramine inhibits muscarinic and histamine receptor-mediated Ca^{2+} signaling and aquaporin-5 translocation in human salivary gland HSG cells, which I suggest is one of the mechanisms underlying desipramine-mediated decrease in salivation.

The inhibition of salivary muscarinic receptors is generally accepted as a causal mechanism of hyposalivation commonly seen in Sjögren's syndrome (Jin et al., 2012b; Lee et al., 2013). The activation of salivary muscarinic receptors is the most important mediator of the parasympathetic control of salivation and triggers increases in $[Ca^{2+}]_i$ (Turner and Sugiya, 2002; Li et al., 2006). Ca^{2+} signaling modulates salivary secretion via two pathways (Ambudkar, 2011). One pathway creates an osmotic gradient of anions using Ca^{2+}-activated anion channels, resulting in transepithelial secretion. The other increases the plasma membrane surface level of aquaporin, creating 'water channels' that result in transcellular secretion. Aquaporin-5 is the predominant water channel in salivary gland cells (Ishikawa et al., 2005). My results clearly demonstrate the desipramine-mediated inhibition of muscarinic and histamine Ca^{2+} signaling and subsequent aquaporin-5 translocation. My previous report showed that desipramine inhibits the

Na^+/H^+ exchanger in human primary salivary gland cells and HSG cells (Choi et al., 2006). Thus, desipramine affects both Ca^{2+} signaling-mediated transcellular salivary secretion and Na^+/H^+ exchanger-mediated transepithelial salivary secretion, thus producing a xerogenic effect.

I found that desipramine inhibits not only the muscarinic receptor, but also the histamine receptor. I previously reported that the salivary histamine receptor triggers Ca^{2+} signaling and aquaporin-5 translocation independent of the muscarinic receptor, suggesting an antihistamine-induced xerogenic mechanism (Kim et al., 2009). I believe that desipramine acts on both muscarinic and histamine receptors. The finding that desipramine did not inhibit the $[Ca^{2+}]_i$-mediated increase in sphingosine-1-phosphate receptor (another phospholipase C-linked receptor) and thapsigargin (a sarcoplasmic reticular Ca^{2+}-ATPase inhibitor that triggers Ca^{2+} release from the intracellular Ca^{2+} pool and store-operated calcium entry) strongly suggests that the inhibitory mechanism of desipramine acts at the receptor level before phospholipase C activation. Desipramine's lack of effect on the P2X7 receptor also supports this hypothesis. Finally, the results of the adrenergic antagonist experiment confirm that the effect of desipramine is not mediated by catecholamine reuptake inhibition or adrenergic potentiation, which are unique characteristics of tricyclic antidepressants. In fact, tricyclic

antidepressants including desipramine are reported to show a binding affinity to muscarinic and histamine receptors (Taylor and Richelson, 1980; Batra and Biörklund, 1986; Somogyi and Perel, 1989). Due to the similar characteristics of these receptors, some antihistamine drugs (i.e., diphenhydramine and promethazine) block muscarinic receptors (Liu et al., 2006; Brown and Eckberg, 1997), which possibly explains the inhibitory specificity of desipramine. Based on these findings, I believe there is potential in exploring the alternative prescription of antidepressants with fewer anti-cholinergic and anti-histamine side effects for patients with dry mouth and rampant caries caused by long-term desipramine treatment.

Taken together, my results demonstrate that desipramine inhibits salivary muscarinic and histamine receptor-induced Ca^{2+} signaling and aquaporin-5 translocation. These data will be informative for understanding the cellular and molecular mechanisms of desipramine-induced dry mouth and for assisting in the further understanding of the xerogenic mechanisms of extrinsic medicines.

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CHAPTER 3:

Human salivary gland cells modulation of the proinflammatory cytokine expression by bradykinin

ABSTRACT

Bradykinin is an important peptide modulator that affects the function of neurons, immune cells and exocrine cells. However, there is no evidence of the bradykinin receptors and their functions in human salivary glands. Here I have studied the identification and characterization of bradykinin receptors on human submandibular gland cells. In the human submandibular gland, A253 cells and HSG cells, both B1 and B2 receptors are expressed. Bradykinin increased cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in a concentration-dependent manner, implying that bradykinin receptors are functional in human salivary gland cells. Bradykinin did not affect a carbachol-induced $[Ca^{2+}]_i$ rise. Bradykinin did not modulate aquaporin-5 translocation but promote the expression of proinflammatory cytokines including TNF-α, implying the role of bradykinin in the salivary gland inflammation without any modulation of salivary secretion. These data suggest that bradykinin receptors serve GPCR-mediated functional modulation in human submandibular gland cells.

INTRODUCTION

The neurotransmitters and hormones produced by neurons and immune cells control exocrine functions. Salivary gland is one of exocrine glands communicating vigorously with other cells. Salivary gland cells are classified as non-excitable cells, as they lack voltage-sensitive channels, and they communicate with other cells through G-protein-coupled receptors (GPCRs). For example, salivary secretion, one of the essential functions of salivary glands, is mediated by the activation of GPCRs and the subsequent increase in cytosolic Ca^{2+} levels ($[Ca^{2+}]_i$) (Roussa, 2011; Lee et al., 2012; Ambudka, 2014). Therefore, impairment of GPCR-mediated signaling results in the dysfunction of salivary glands (Jin et al., 2012). In the human salivary gland, a series of GPCRs, including P2Y2, histamine, and S1P have been investigated, elucidating their unique functions (Baker et al., 2008; Kim et al., 2009; Seo et al., 2010).

Bradykinin is one of the neuropeptides of the kinin family, and is involved in many physiological functions such as neuronal function (pain), immune function (inflammation), and cardiovascular function (vasodilation and smooth muscle contraction) (Marceau and Regoli, 2004; Maurer et al., 2011). More specifically, bradykinin is known to play a significant role in the

pathological mechanisms of inflammation, angioedema, and ischemia (Kaplan and Joseph, 2014; Heusch et al., 2015). The effect of bradykinin is mediated by its GPCRs, B1 and B2 receptors, which induce phospholipase C activation and a subsequent $[Ca^{2+}]_i$ increase (Leeb-Lundberg et al., 2005). In oral tissues, bradykinin is reported to induce nitric oxide production in odontoblasts (Korkmaz et al., 2006), and modulate the expression of cyclooxygenase (COX) and toll-like receptors in gingival fibroblasts (Nakao et al., 2001; Gutiérrez-Venegas et al., 2012). Interestingly, heat reflex triggers the secretion of kallikrein from rat submaxillary glands and increases bradykinin levels, thereby inducing thermolytic salivation (Damas and Bourdon, 1994). Bradykinin also potentiates chorda tympani neuron-mediated salivation in cats (Stojic, 1999). Additionally, the bradykinin receptor mediates nitric oxide (NO) and PGE_2 production in the submandibular glands of rats (Genaro et al., 2000).

In human salivary glands, however, bradykinin receptors have not yet been identified nor have their functions and signaling mechanism been characterized. The study of bradykinin in human salivary gland cells is of significance because bradykinin signaling has often been demonstrated to function in a species-dependent manner (Gobeil et al., 1996; Décarie et al.,

1996; Sawada et al., 2004). In this study, my aim was to identify bradykinin receptors in human salivary gland cells and to characterize their functions and signaling mechanism.

MATERIALS AND METHODS

Cell and Tissue Preparation

Human submandibular salivary gland cells were prepared as previously described (Kim et al., 2009). Pieces of human submandibular glands were obtained from six malignant neoplasms from patients (males and females ranging from 26 to 81 years of age) who had provided informed consent. The glands did not contain histologically atypical cells. The experiments were performed according to the Declaration of Helsinki, the World Medical Association, and were approved by the Institutional Review Board (CRI06002) of Seoul National University Dental Hospital. The A253 cells and HSG cells were grown in Modified Eagle's Medium (GIBCO, Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (GIBCO) and 1% (v/v) penicillin (5000 U/ml) + streptomycin (5000 μg/ml) solution (GIBCO). The cells were cultured in a humidified atmosphere of 95% air + 5% CO_2. The culture medium was changed every 2 days, and the cells were subcultured weekly.

$[Ca^{2+}]_i$ Measurement

Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was analyzed with fura-2/AM (Seo et al., 2010). Briefly, the cell suspension was incubated with 3 μM fura-2/AM at 37^{o}C for 60 minutes with continuous stirring. Sulfinpyrazone (250 μM) was added to prevent dye leakage. The cells were then washed with Locke's solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 2.2 mM $CaCl_2$, 5.0 mM HEPES, 10 mM glucose, pH 7.4 with NaOH). Changes in the fluorescence ratio were measured at dual excitation wavelengths of 340 and 380 nm with an emission wavelength of 500 nm.

Immunofluorescent staining

Human submandibular glands were fixed in 10% paraformaldehyde overnight, embedded in paraffin, and sectioned. The 5-μm-thick paraffin sections were deparaffinized and antigen retrieval was carried out using pepsin (Golden Bridge International, Mukilteo, WA, USA) for 10 min at 37°C. The sections were blocked for 30 min in 10% normal goat serum and then incubated with primary antibodies for 1 h (room temperature). A253 cells and HSG cells were fixed (4% paraformaldehyde), permeabilized (0.5%

Triton X-100) for 10 min at 20–25°C, and then incubated for 1 h in blocking solution (1% BSA). Primary antibodies were challenged for 2 h (at room temperature) or overnight (at 4°C), and then incubated with secondary antibodies for 1 h (at room temperature). The following primary antibodies were used: Rabbit anti-human bradykinin B1, B2 receptors (1:50; Santa Cruz Biotechnology). Secondary antibody incubations were carried out for 1 h at room temperature using Alexa488-conjugated goat anti-rabbit IgG (1:700; Invitrogen) antibodies.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from primary culture human submandibular salivary gland cells, A253 cells and HSG cells with TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was treated with RNase-free DNase I for 30 min, and heat-inactivated. The 20-μl RT mixture contained 4 μg of the total RNA, 0.5 mM dNTP mix, 0.5 μg of oligo(dT), 5 U RNase inhibitor, 5 mM dithiothreitol and 5 U Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen, Carlsbad, CA). The expression of GAPDH, Bradykinin B1 receptor and B2 receptor mRNA levels was measured using the following PCR cycling protocols: 5 min at 94^{o}C, 35 cycles of 30 s at 94^{o}C, 30 s at 60^{o}C,

30 s at 72°C, and finally, 10 min at 72°C. The following primers were used as previously described[24] : GAPDH sense 5'-ACC ACA GTC CAT GCC ATC AC-3' and antisense 5'-TCC ACC ACC CTG TTG CTG TA-3', bradykinin B1 receptor sense 5'-CTC TGC CGT GTC ATC AAC GG-3' and antisense 5'-GAA GAC GAT CGC AGC CAG TG-3', bradykinin B2 receptor sense 5'-CAC CAT CTC CAA CAA CTT CG-3' and antisense 5'-GGT AGC TGA TGA CAC AAG CG-3'. The sizes of products were 336 bp (B1 receptor), 302 bp (B2 receptor) and 452 bp (GAPDH).

[^{3}H]cAMP Measurement

The cAMP concentration in cells was determined by a [^{3}H]cAMP competition assay evaluating binding to the cAMP binding protein (Lee et al., 2014). Cells were harvested and pre-incubated with Locke's solution containing 1 mM isobutylmethyl xanthine (IBMX) to inhibit phosphodiesterase. After stimulation with agonists for 15 min at 37°C, the reaction was terminated by addition of twice the volume ice-cold absolute ethanol. The supernatant fraction after centrifugation for 10 min at 4°C at 10,000 x g, was used for the cAMP binding assay, based on competition between [^{3}H]cAMP and unlabeled cAMP to crude cAMP-binding proteins.

The protein-bound cAMP were separated by charcoal (100 μl. The cAMP concentration was determined based on a standard curve and expressed as pmol/cell number.

Construction of Aquaporin-5 and Western blotting

The amount of membrane translocation of aquaporin-5 was determined by Western botting using HSG cells transfected with GFP-tagged aquaporin-5 (Ishikawa et al., 2000). HSG cells were transfected with the GFP-aquaporin-5 and incubated with bradykinin or carbachol for 20 min at 37°C. The apical membrane fraction was sonicated and centrifuged at 600×g (4 °C), and the supernatants were centrifuged at 20,000×g (4 °C). The pellets (P2 membrane fractions) were subjected to SDS/PAGE immunoblotting with anti-aquaporin-5 as the primary antibody (1:200, Santa Cruz, sc-9891). For normalization, the membranes were re-probed with antibody for anti-α1 Na^+/K^+-ATPase (1:1000, Abcam, ab7671).

Real-time RT-PCR

The mRNA expression levels of β-actin, TNF-α, and IL-10 were measured by real-time RT-PCR as previously described (Kim et al., 2010). Real-time PCR was performed using the SYBR Green reagent and an ABI Prism 7500 sequence detection system (Applied Biosystems, Warrington, UK), with the following PCR conditions: 50^{o}C for 2 min, 94^{o}C for 10 min, and 40 cycles of 95^{o}C for 30 sec, followed by 60^{o}C for 1 min. The primers used for PCR : human GAPDH forward: 5'-ACC ACA GTC CAT GCC ATC AC-3', human GAPDH reverse: 5'-TCC ACC ACC CTG TTG CTG TA-3', human TNF-α forward: 5'-ACC ACC ACT TCG AAA CCT GG-3', human TNF-α reverse: 5'-CTT CAC TGT GCA GGC CAC AC-3', human IL-10 forward: 5'-CAT CAA GGC GCA TGT GAA CTC-3', human IL-10 reverse: 5'-GCC TTG CTC TTG TTT TCA C-3'. The expression level of each gene was normalized to the level of the GAPDH gene and presented as the fold induction.

Data Analysis

All quantitative data are expressed as mean ± SEM. Differences were determined by one-way analysis of variance and considered to be significant only when $P < 0.05$.

RESULTS

Bradykinin B1 and B2 receptors are expressed in human submandibular gland cells

In order to test the expression of bradykinin receptors in human salivary glands, I first identified the subtypes of bradykinin receptors expressed in human submandibular gland tissue, HSG cells, and A253 cells with RT-PCR. Figure 3-1A clearly shows the expression of both B1 and B2 receptors in these cells. To confirm the expression of bradykinin receptors, I labeled human submandibular gland with bradykinin B1 and B2 receptor subtype-specific antibodies. Both bradykinin B1 (Figure 3-1B) and B2 receptors (Figure 3-1C) were distributed across most of the human submandibular gland, including both acinar and ducts. Notably, the bradykinin B1 and B2 receptors were also found in the human salivary gland cell lines, A253 and HSG cells (Figure 3-1B, 3-1C).

Bradykinin increases cytosolic Ca^{2+}

Next I examined bradykinin signaling in human submandibular gland cells. I found that bradykinin increases $[Ca^{2+}]_i$ in dissociated human submandibular

gland cells (Figure 3-2A). Based on the common distribution of bradykinin receptors in the human submandibular gland and in human salivary gland cell lines, I measured the bradykinin-induced $[Ca^{2+}]_i$ increase in A253 and HSG cells and found that bradykinin induced $[Ca^{2+}]_i$ increases in both A253 (Figure 3-2B) and HSG cells (Figure 3-2C). Because of limited availability of human submandibular tissues, I investigated the pharmacological characteristics of bradykinin action in human salivary gland cell lines, and found that bradykinin-induced $[Ca^{2+}]_i$ increases occurred in a concentration-dependent manner both in A253 cells (Figure 3-2B) and in HSG cells (Figure 3-2C).

Bradykinin signaling is independent from muscarinic signaling in human submandibular gland cells

Next I tested the interaction between bradykinin and muscarinic Ca^{2+} signaling, which share PLCβ for their common signaling pathway. Interestingly, pre-treatment of bradykinin did not affect the carbachol-evoked $[Ca^{2+}]_i$ increase in HSG cells (Figure 3-3A), even with saturated bradykinin Ca^{2+} signaling at micromolar concentration. Pre-treatment with carbachol did not inhibit a subsequent bradykinin-evoked $[Ca^{2+}]_i$ increase (Figure 3-3B). I

confirmed the results with A253 cells which lacks functional muscarinic receptor (Shin et al., 2015) and found bradykinin successfully increase a cytosolic Ca^{2+} increase even without carbachol-induced $[Ca^{2+}]_i$ increase (Figure 4-3C, 4-3D). These data suggest that bradykinin and muscarinic receptors are completely independent of one another without heterologous desensitization and an overlapping Ca^{2+} signaling pathway.

Prostaglandin receptors are not functional human submandibular gland cells

If salivary gland cells express bradykinin receptors and prostaglandin receptor together, bradykinin can act indirectly by the production of prostaglandins and subsequent activation of prostaglandin receptors (Kim et al., 2010). To investigate whether prostaglandin plays a role in the bradykinin effect after the bradykinin-mediated PLA_2 and COX activation, I tested for functional prostaglandin receptors in HSG cells. However, I could not detect any PGE_2-mediated Ca^{2+} increase, whereas bradykinin successfully evoked a Ca^{2+} increase (Figure 3-4A). In addition, PGE_2 did not generate cAMP, which was successfully generated by VIP, a neurohormone that stimulates G_s-coupled receptors in HSG cells, and forskolin, an activator

of adenylyl cyclase (Figure 3-4B). These results imply that the prostaglandin receptor is not expressed or functional in HSG cells, so bradykinin directly acts on salivary gland cells without the involvement of prostaglandin.

Bradykinin does not affect aquaporin translocation, but induces proinflammatory cytokine expression.

The translocation of aquaporin-5 into plasma membrane is one of cellular mechanism of water secretion in salivary gland cells, and GFP-aquaporin-5-transfected HSG cells have been used to analyze the amount of aquaporin-5 translocation with Na^+/K^+ ATPase as a membrane protein marker (Lee et al., 2015). I monitored the bradykinin-mediated modulation of aquaporin-5 translocation in GFP-aquaporin-5-transfected HSG cells with a membrane protein marker, Na^+/K^+ ATPase. However, bradykinin failed to trigger aquaporin-5 translocation, whereas carbachol successfully induced aquaporin-5 translocation (Figure 3-5A). In addition, bradykinin did not enhance but partially inhibit the carbachol-mediated aquaporin-5 translocation (Figure 3-5B). I next monitored the TNF-α and IL-10 expression by real-time PCR analyses with mRNA from HSG cells. Interestingly bradykinin increased expression of TNF-α like as poly(I:C), a

TLR-3 agonist (Figure 3-5C). The bradykinin marginally increased IL-10 expression (Figure 3-5D). The results suggest that bradykinin does not affect salivary secretion but modulate inflammation in human salivary glands.

Figure 3-1. Bradykinin receptors in human salivary gland cells.

(A) RNA extracted from human submandibular glands, A253 cells, and HSG cells were reverse-transcribed into cDNA and amplification reactions were performed using bradykinin B1, and B2 receptor-specific primers and Pfu polymerases. GAPDH was used as an internal loading control. (B-C) Cells or tissues were fixed and incubated with anti-human bradykinin B1 (B) or B2 receptor (C) antibodies. (top) human submandibular glands (Bars, 50 μm). (middle) A253 cells (Bars, 20 μm). (bottom) HSG cells (Bars, 20 μm).

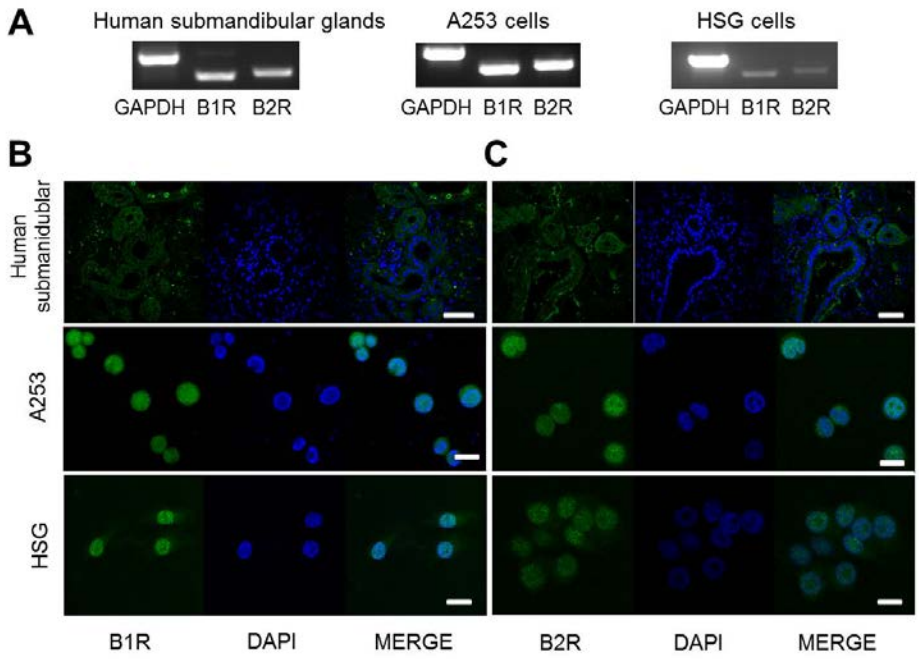

A
Human submandibular glands
A253 cells
HSG cells
GAPDH B1R B2R
GAPDH B1R B2R
GAPDH B1R B2R
B
C
Human submanidublar
A253
HSG
B1R
DAPI
MERGE
B2R
DAPI
MERGE

Figure 3-2. Bradykinin triggers the increase of intracellular Ca^{2+} concentration in human submandibular gland cells.

Fura-2-loaded human dissociated submandibular cells (A), A253 cells (B) or HSG cells (C) were challenged with bradykinin (300 nM, black; 30 nM, dark gray; 3 nM, light gray), and then monitored changes in F340/F380 fluorescence ratio. Typical Ca^{2+} transients are presented. Concentration-response relationships are also depicted by monitoring peak height of changes in cytosolic [Ca^{2+}] level. Each point is the mean ± SEM and all results were reproducible.

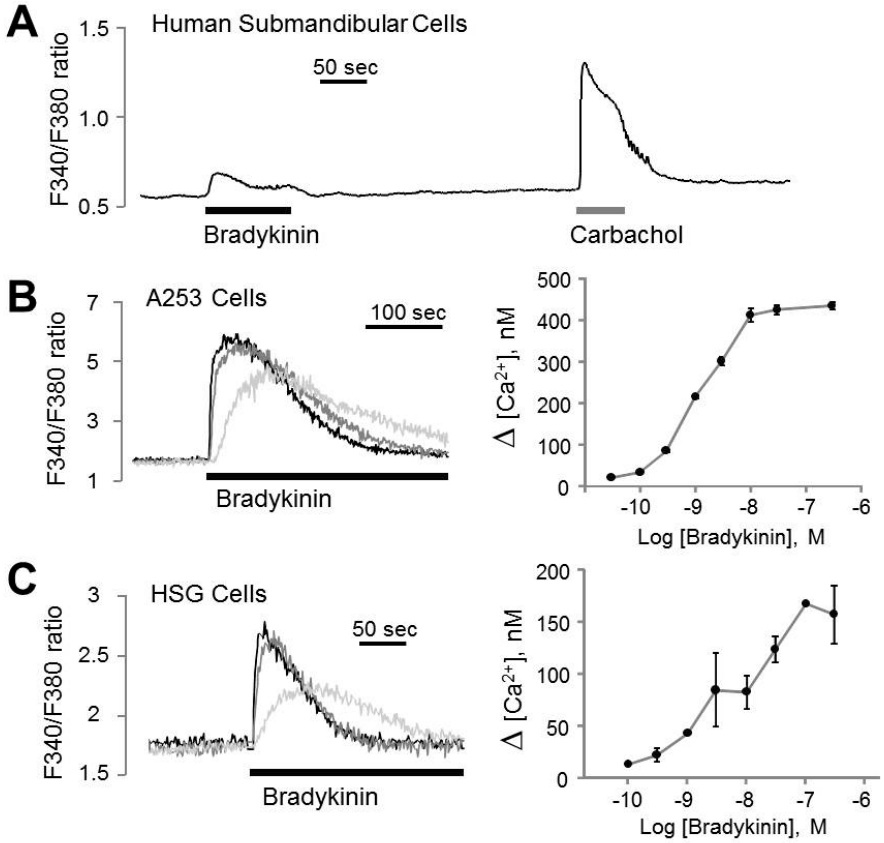

A
Human Submandibular Cells
F340/F380 ratio
1.5
1.0
0.5
50 sec
Bradykinin
Carbachol
B
A253 Cells
F340/F380 ratio
7
5
3
1
100 sec
Bradykinin
Δ [Ca2+], nM
500
400
300
200
100
0
-10
-9
-8
-7
-6
Log [Bradykinin], M
C
HSG Cells
F340/F380 ratio
3
2.5
2
1.5
50 sec
Bradykinin
Δ [Ca2+], nM
200
150
100
50
0
-10
-9
-8
-7
-6
Log [Bradykinin], M

Figure 3-3. Bradykinin receptor does not share its Ca^{2+} signaling with muscarinic receptors.

(A) Fura-2-loaded HSG cells were treated with 100 μM carbachol (CCh) with (black trace) or without (gray trace) the preincubated 300 nM bradykinin (BK). (B) HSG cells were treated with 300 nM bradykinin with (black trace) or without (gray trace) the pre-incubated 100 μM carbachol. (C-D) The same experiment of (A) and (B) were performed using fura-2-loaded A253 cells. All traces presented in the left panels are typical Ca^{2+} transients from more than three separate experiments. The peak height of Ca^{2+} increases at the points indicated as **a** (blank bar) and **b** (filled bar) are depicted in all the right panels with the % value of $[Ca^{2+}]_i$ increase without pretreatment. Each point is the mean ± SEM. All results were reproducible.

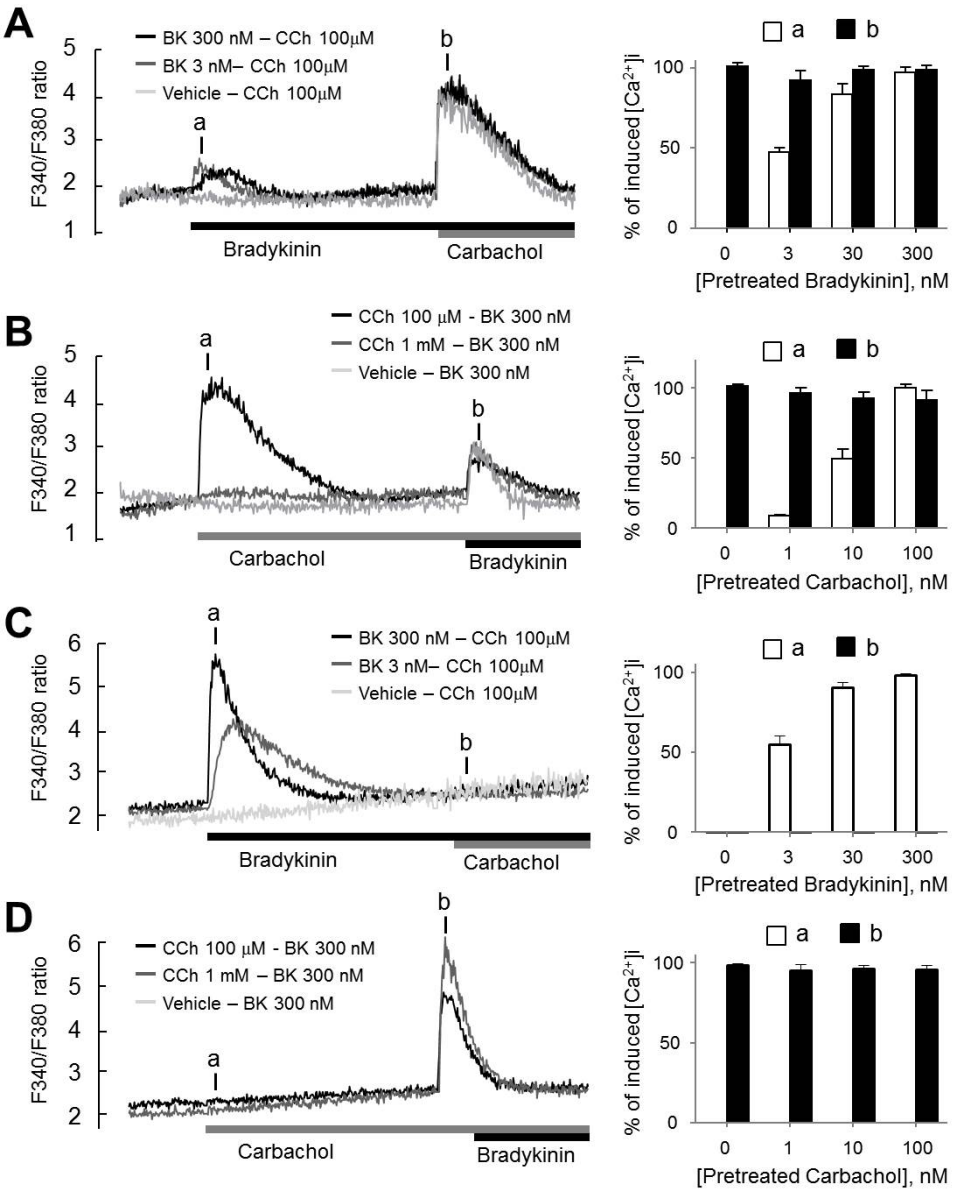

A
BK 300 nM – CCh 100μM
BK 3 nM– CCh 100μM
Vehicle – CCh 100μM
F340/F380 ratio
Bradykinin
Carbachol
a
b
% of induced [Ca2+]i
[Pretreated Bradykinin], nM
B
CCh 100 μM - BK 300 nM
CCh 1 mM – BK 300 nM
Vehicle – BK 300 nM
[Pretreated Carbachol], nM
C
D

Figure 3-4. Prostaglandin does not increase cytosolic Ca^{2+} or cAMP in HSG cells.

(A) Fura-2-loaded cells were challenged with 10 μM PGE_2, 300 nM bradykinin, and 100 μM carbachol (CCh). The fluorescence ratio of F_{340}/F_{380} was then monitored and typical Ca^{2+} transients are presented. (B) Cells were stimulated with 10 μM PGE_2, or 1 μM vasoactive intestinal peptide (VIP) or 3 μM forskolin, in the presence of 1 mM IBMX for 15 minutes, and then cAMP production was then monitored. The relative cAMP productions are depicted as percent of forskolin-induced cAMP production with the mean ± SEM of triplicate assays. $^{**}P < 0.01$.

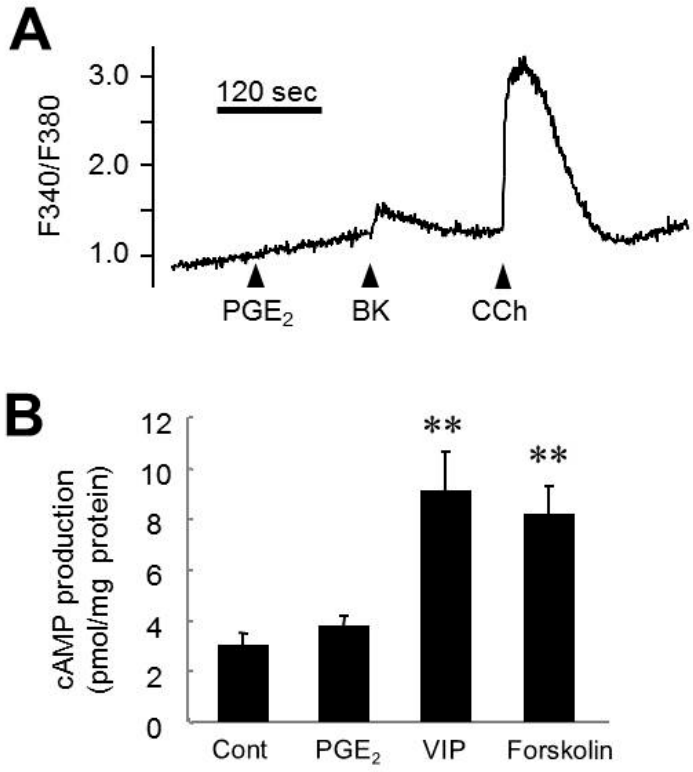
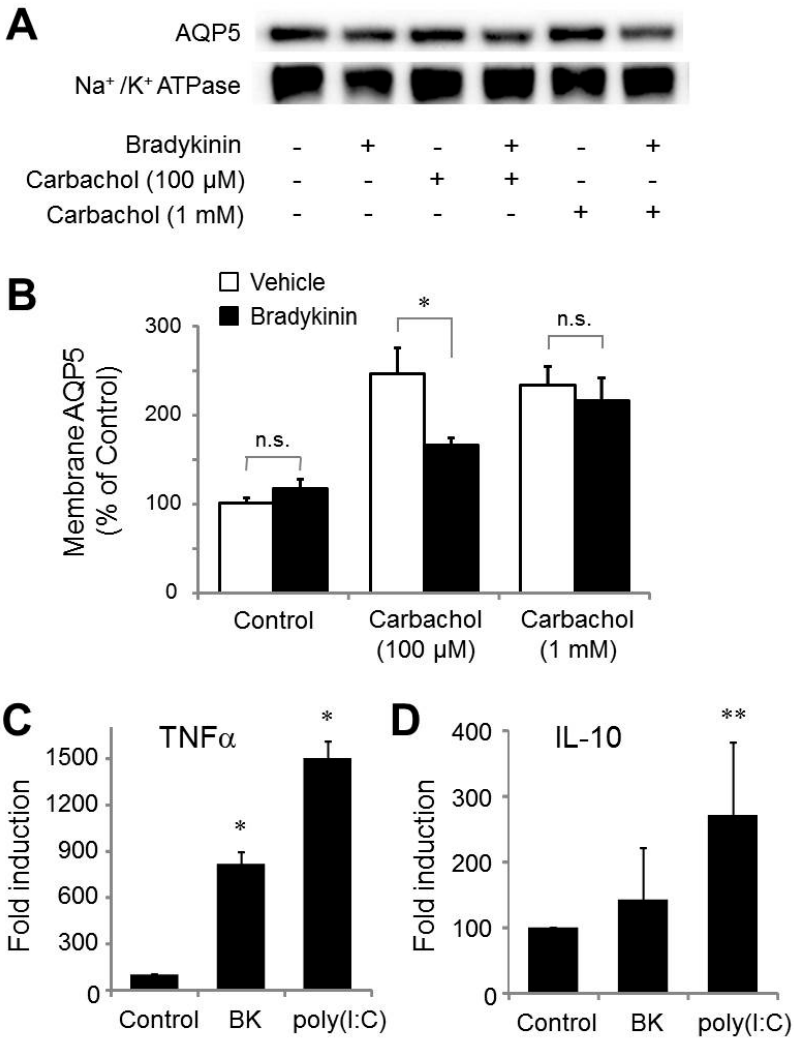

A
3.0
2.0
1.0
F340/F380
120 sec
PGE2
BK
CCh
B
12
10
8
6
4
2
0
cAMP production
(pmol/mg protein)
**
**
Cont
PGE2
VIP
Forskolin

Figure 3-5. Bradykinin does not modulate aquaporin-5 translocation but induce the expression of proinflammatory cytokines.

(A-B) GFP-tagged HSG cells transfected with aquaporin-5 were challenged with 300 nM bradykinin, 100 μM carbachol, or 100 μM carbachol for 20 min. (A) Western blots show membrane aquaporin-5 (AQP5) and Na^+-K^+-ATPase. (B) Quantification of membrane aquaporin-5 with Na^+-K^+-ATPase control (n = 3 - 6). Membrane aquaporin-5 levels were normalized to the levels of the membrane Na^+-K^+-ATPase subunit. (C-D) HSG cells were treated with bradykinin (BK) or 30 μg/ml poly(I:C) for 3 h. Total RNA was isolated, reverse transcribed and analyzed with real-time RT-PCR to determine the amount of TNF-α (C), and IL-10 expression (D). Each of the values (% of control) depicted is a mean ± SEM. n.s., not significant; *P < 0.05, **P < 0.01.


A
AQP5
Na+ /K+ ATPase
Bradykinin - + - + - +
Carbachol (100 μM) - - + + - -
Carbachol (1 mM) - - - - + +
B
Vehicle
Bradykinin
Membrane AQP5 (% of Control)
300
200
100
0
n.s.
*
n.s.
Control
Carbachol (100 μM)
Carbachol (1 mM)
C
TNFα
Fold induction
1500
1200
900
600
300
0
*
*
Control
BK
poly(I:C)
D
IL-10
Fold induction
400
300
200
100
0
**
Control
BK
poly(I:C)

DISCUSSION

In this study, I have shown that bradykinin receptors mediate Ca^{2+} signaling in human submandibular gland cells for the first time. I have shown that (1) the bradykinin B1 and B2 receptors are expressed in human submandibular gland cells, (2) bradykinin increases cytosolic Ca^{2+} independent of muscarinic Ca^{2+} signaling, (3) bradykinin does not affect aquaporin translocation, and (4) bradykinin induces proinflammatory cytokine expression. .

My results using subtype-specific antibodies for bradykinin receptors clearly show that human submandibular gland cells express both bradykinin B1 and B2 receptors (Figure 3-1). Both B1 and B2 receptors are commonly linked to PLC-β and subsequent Ca^{2+} signaling. Given that muscarinic receptors have been considered representative of GPCR-linked Ca^{2+} signaling, clarification of the relationship between bradykinin and muscarinic receptors is needed. I addressed this question with "heterologous desensitization," a decrease in signaling of a certain receptor by another neurotransmitter that shares the same downstream signal transduction pathway. I confirmed that bradykinin pre-treatment does not cause a muscarinic Ca^{2+} increase (Figure 3-3). Furthermore, A253 cells do not

express any muscarinic receptors (Sun et al., 1999) because of epigenetic transcriptional control (Shin et al., 2015). However, bradykinin successfully induced a $[Ca^{2+}]_i$ increase, confirming the independence of bradykinin from muscarinic receptor signaling. The action of bradykinin can be classified as (1) a direct effect with bradykinin-mediated Ca^{2+} signaling after the activation of PLCβ, and (2) an indirect effect via bradykinin-mediated PGE_2 or NO production after the activation of PLA_2, COX or NOS. The bradykinin-mediated PGE_2 production and subsequent physiological modulation is reported in rat submandibular gland cells (Genaro et al., 2000) and gingival fibroblast (Nakao et al., 2001). However, I could not find any PGE_2-mediated Ca^{2+} increase or cAMP production in HSG cells (Figure 3-4), implying a lack of functional prostaglandin receptors in HSG cells. Thus, it seems less likely that bradykinin acts on salivary glands in a paracrine manner via the PLA_2-COX-PGE_2 signaling cascade.

Then what is bradykinin's function in human salivary glands? my results showed that bradykinin does not trigger aquaporin-5 translocation and does not affect carbachol-induced aquaporin-5 translocation. This result complimented another finding, that pre-treatment with bradykinin did not affect muscarinic Ca^{2+} signaling. Thus it is plausible that bradykinin hardly

contributes salivation. In fact, xerostomia is very marginal or undetectable as a side effect with Icatibant, a bradykinin B2 receptor-specific antagonist prescribed for hereditary angioedema (Proud et al., 1995; Bas et al., 2007; Cicardi et al., 2010). It is suggested that bradykinin does not affect muscarinic Ca^{2+} signaling and salivary secretion directly, but modulates the pathological events by its own receptor signaling. Instead of salivation, my results show that bradykinin modulates proinflammatory cytokine expression. It is not surprising that bradykinin modulates inflammatory reactions, due to a large body of studies reporting the bradykinin's critical roles in inflammation (Marceau and Regoli, 2004). Proinflammatory cytokines including IL-1β and TNF-α can modulate the expression of the bradykinin receptor (Brechter et al., 2008; Souza et al., 2013), and also bradykinin modulates the expression of cytokines including IL-1b and TNF-alpha (Cunha et al., 2007; Kim et al., 2010), revealing tight crosstalk. However, it is interesting that bradykinin shows a similar proinflammatory effect in exocrine system. Salivary glands are involved in the local immune reactions with their innate immunity related receptors including TLRs (Kawakami et al., 2007; Li et al., 2010; Into et al., 2014). Thus, further investigation for the bradykinin-mediated salivary cytokine induction on the basis of my findings will eventually contribute to understanding the changes in salivary functions mediated by pathological conditions such as inflammation and angioedema.

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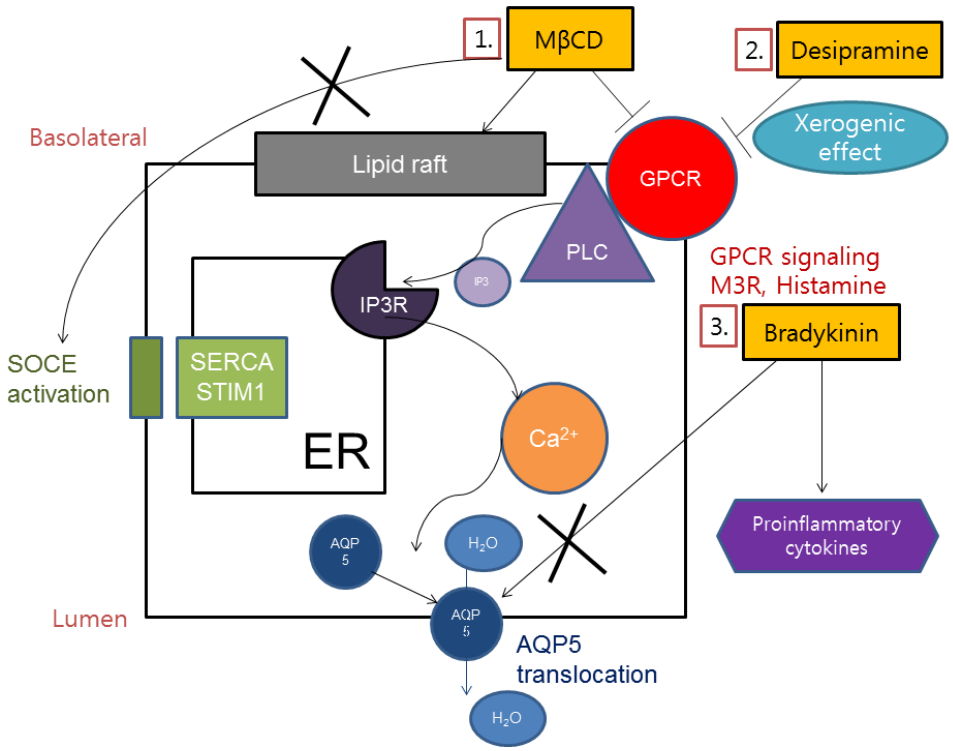
Conclusion

- Incubation of MβCD to remove the lipid raft through the depletion of cholesterol in the salivary gland cells inhibited muscarinic and histamine receptor-mediated Ca^{2+} signaling.
- On the other hand in the major salivary gland protein-lipid raft is coupled receptor Signaling SOCE or G AQP5 trafficking may have concluded andago effect.
- Incubation of MβCD was not suppressed thapsigargin-mediated Ca2 + signaling and aquaporin-5 translocation
- Desipramine inhibits muscarinic and histamine receptor-mediated Ca^{2+} signaling in human salivary gland cells.
- Desipramine inhibits muscarinic and histamine receptor-mediated translocation of aquaporin-5, a major water channel in human salivary gland cells.
- This finding contributes to a better understanding of the cellular and molecular pathological mechanisms of desipramine-induced dry mouth.
- Bradykinin receptors are expressed in human salivary gland cells
- Bradykinin increases cytosolic Ca^{2+} levels in human salivary gland cells

- Bradykinin-mediated Ca^{2+} signaling is independent from the muscarinic Ca^{2+} signaling
- My research is of biomedical significance because it contributes to the understanding of the cellular mechanisms of salivary secretion as well as its modulation.



The role of G-protein-coupled receptor induced Ca^{2+} signaling in Salivary gland cells

국문초록

타액선 G-protein-coupled receptor에 의한 세포내 칼슘신호의 역할

이 계 민

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타액은 구강 내 항상성 유지에 중요한 역할을 한다. 구강 내에서의 수분 및 삼투압, pH 등의 유지가 분비물질인 타액에 의해서 조절된다. 타액분비는 타액선의 중요한 기능이다. 타액선세포는 전압의존성 이온채널을 발현하지 않는 비흥분성 세포로서, 주로 G-protein coupled receptor(GPCR)를 통한 신호를 받아서

생리적 기능을 수행한다. 다른 세포에서처럼 타액선의 GPCR의 활성화는 세포내 칼슘 증가로 이어진다. 예를 들어 부교감신경말단에서 분비되는 아세틸콜린은 무스카린성 M3 수용체, Gq/11 단백질과 phospholipase C를 활성화 시켜 신호를 전달하며 이렇게 증가한 세포내 칼슘은 타액선에서 물의 이동을 일으킴으로써 타액 분비를 유도한다. 타액선의 분비가 신경세포를 비롯한 많은 외부 신호에 의해서 조절을 받는데 반해 몇 개의 GPCR을 제외하고는 알려져 있지 않다. 따라서 타액선 세포의 GPCR 신호의 역할을 규명하는 것은 외분비 생리학 차원에서와 임상 치의학적 차원에서 큰 의미가 있다고 할 수 있다. 이러한 점에 주목하여 타액선의 생리에 영향을 주고 타액분비의 변화를 가져오는 약물들이 어떤 작용과 기적을 가지며, 그 기전에 있어서 중요한 역할을 한다고 할 수 있는 GPCR의 신호전달 과정에 대해서 연구하였다. 먼저, 세포막 생리기능에 있어 중요한 역할을 한다고 알려져 있는 Lipid raft의 소멸에 의한 타액선 세포에 존재하는 GPCR의 생리학적 기능변화에 대해서 살펴보았다. 본 연구에서 콜레스테롤의 고갈을 통해서 lipid raft를 제거하는

MβCD의 처리가 미치는 타액선 세포내의 칼슘신호의 변화를 보여줌으로써 기존에 밝혀지지 않았던 Lipid raft의 타액선 세포 GPCR에 미치는 영향을 제시하였다. 다음으로, 장기간 복용했을 때 구강건조증을 야기한다고 알려져 있는 항우울제의 하나인 데시프라민을 타액선 세포에 처리 함으로써 그에 따른 기전을 살펴 봄으로써 외인성 약물이 타액선 세포의 GPCR에 미치는 영향을 밝혔다. 마지막으로, 기존에 밝혀지지 않았던 사람의 타액선에 존재하는 GPCR을 파악하고 그 역할을 밝히고자 사람의 타액선에서 브래디키닌 수용체의 발현과 그에 따른 기전을 살펴보았다. 이를 통해 종 특이적 차이와 타액선 세포에서의 브래디키닌 수용체의 기능을 제시할 수 있었다. 이상의 연구들은 타액선 세포에 존재하는 GPCR이 그 기능을 유지하기 위해 적절히 작용하고 있으며, 타액선의 질병 중 하나인 구강건조증 기전 연구에 가능성을 보여주고 있다.

종합해보면, 본 논문을 통해서 기존에 보고되지 않았던, 타액선 세포에서 lipid raft 고갈과 GPCR 신호의 관련성을 규명하였고, 항우울제 장기복용에 따른 구강건조증 유발의 예로써

데시프라민이 구강건조증을 야기시키는 매커니즘을 규명하였으며, 사람의 타액선에서 그 동안 보고 되지 않았던 브래디키닌 수용체의 기능에 대해서 밝힘으로써, 타액선 세포에서 GPCR에 의해 유도되는 칼슘 신호에 따른 타액선 기능을 연구하는데 방향성을 제시하였다.

주요어: 타액선, GPCR, 칼슘, Aquaporin5, Lipid raft, 구강건조증, 데시프라민, 브래디키닌

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