Cevimeline induced monophasic salivation from the mouse submandibular gland: decreased Na\(^+\) content in saliva results from specific and early activation of Na\(^+\)/H\(^+\) exchange

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Abbreviations:

CVL: cevimeline, PLC: pilocarpine, CCh: carbachol, ENaC: epithelial Na⁺ channel, [Ca²⁺]: intracellular calcium concentration, pHi: intracellular pH, CFTR: cystic fibrosis transmembrane conductance regulator, NHE: Na⁺/H⁺ exchanger, BCECF: 3′-0-acetyl-2′,7′-bis(carboxyethyl)-4 or 5-carboxyfluorescein, CA: carbonic anhydrase, AE: Cl⁻/HCO₃⁻ exchanger (anion exchanger), mAchR: muscarinic acetylcholine receptor

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Abstract

Cevimeline and pilocarpine are clinically used muscarinic agonists to rescue dry mouth. In this study, we explored fluid secretion from mouse submandibular glands to determine the mechanism of cevimeline, pilocarpine and an experimentally used agent carbachol. Cevimeline evoked almost the same amount of secretion from 30 µM to 1 mM. Pilocarpine also induced secretion at a concentration as low as 1 µM and was the most powerful secretagogue at 10 µM. Secretion was induced by carbachol at 0.1 µM, with maximum secretion at 1.0 µM. Cevimeline induced monophasic secretion at all concentrations tested, whereas higher concentrations of pilocarpine and carbachol induced secretion with variable kinetics, i.e., an initial transient high flow rate, followed by decreased secretion after 2-3 minutes. In the presence of an epithelial Na+ channel blocker amiloride, neither carbachol nor pilocarpine affected the Na+ level of secreted saliva; however, it significantly increased the Na+ content of cevimeline-induced saliva. The intracellular Ca2+ response of acinar cells was almost identical among all three agents, although recovery after drug removal was slower for cevimeline and pilocarpine. A profound decrease in intracellular pH was observed during pilocarpine and carbachol treatment, whereas intracellular acidification induced by
cevimeline was only seen in the presence of a Na⁺/H⁺-exchange inhibitor. When external HCO₃⁻ was removed, cevimeline-induced saliva significantly decreased. These findings suggest that cevimeline specifically activates Na⁺/H⁺ exchange and may promote Na⁺ reabsorption by stabilizing epithelial sodium channel activity.
Introduction

Salivary gland hypofunction induces dry mouth, which results in loss of not only oral health-related quality of life, but also of general health. The salivary glands are innervated by both sympathetic and parasympathetic pathways, with parasympathetic muscarinic receptors being the most important for control of salivary fluid secretion (Melvin et al., 2005). Systemic muscarinic drugs for severe dry mouth patients, such as those with Sjögren’s syndrome and those who have undergone irradiation, are used in addition to supportive therapy to rescue dry mouth patients.

Cevimeline (CVL) is a rigid analog of acetylcholine and is used clinically for the treatment of dry mouth. CVL induces salivation mainly through the activation of muscarinic M3 acetylcholine receptors (AchRs) on salivary acinar cells. In animal studies, CVL evoked salivation in a dose-dependent manner. This secretion was completely inhibited by atropine, a muscarinic AchR antagonist, and 4-diphenylacetoxy-N-methyl-piperidine methiodide, an M3 AchR antagonist (Iwabuchi and Masuhara, 1994). The binding affinity of M3 receptors for CVL is thought to be the same as or higher than affinities for other muscarinic drugs (Iga et al., 1998). Furthermore, CVL has been shown to increase salivary secretion in both healthy
subjects and patients being treated for dry mouth, including patients with Sjögren’s syndrome and patients after radiotherapy for head and neck cancer (Fife et al., 2002; Chambers et al., 2007; Braga et al., 2009). CVL is reported to be effective with oral administration as well as with gargling before swallowing, to directly activate minor and major salivary glands (Takagi et al., 2004).

Another muscarinic agonist used clinically is pilocarpine (PLC), an alkaloid imidazole obtained from the leaves of *Pilocarpus jaborandi*. PLC activates M3 AchRs and enhances fluid secretion from salivary tissue in a dose-dependent manner (Omori et al., 2003). It is used clinically to induce saliva secretion in patients with Sjögren’s syndrome, patients who are post-radiotherapy, and patients with graft-versus-host disease-induced dry mouth (Greenspan and Daniels, 1987; Rieke et al., 1995; Berk, 2008).

Clinical and animal studies comparing these two muscarinic drugs have revealed that although CVL requires a higher dose, it has minimal adverse effects and a longer-lasting salivation effect than PLC (Masunaga et al., 1997; Braga et al., 2009). However, most reports on CVL lack mechanistic details, and the differences between these agonists have not been well characterized at the cellular or glandular level. At the cellular level, intracellular free
Ca²⁺ plays a critical role in salivation. Within 1 s of stimulation, [Ca²⁺]ᵢ activates the opening of Cl⁻ channels located at the luminal surface of salivary acinar cells, resulting in the rapid loss of Cl⁻ and water. Concomitant with the loss of Cl⁻ is the loss of HCO₃⁻, which results in intracellular acidification and the consequent extrusion of H⁺ by the Na⁺/H⁺ exchanger (NHE) to compensate for the pH decrease (Nauntofte, 1992). It has been reported that pHᵢ affects epithelial Na⁺ channel (ENaC) activity, which regulates Na⁺ reabsorption (Reddy et al., 2008).

In the present study, we focused on the effects of CVL and PLC, as well as those of carbachol (CCh), a muscarinic agonist used experimentally, on the secretion and ion composition of saliva, the alteration of intracellular pH, and the mechanism of cellular signaling.
Methods

*Ex vivo* mouse submandibular gland analysis

All experiments were approved by the Animal Committee of Kyushu Dental College.

C57BL/6J mice aged 7-9 weeks, purchased from Kyushu Animal Laboratory, were

habituated, and experiments were performed with the mice at 8-10 weeks of age. In the

mouse facility, mice were exposed to a 12-h light/dark cycle and fed *ad libitum*. To eliminate

neurological input, we used an *ex vivo* vascular perfusion technique for glandular fluid

secretion analysis. The surgical procedure was described previously (Romanenko et al.,

2007; Nakamoto et al., 2008). In brief, mice were anesthetized with chloral hydrate

(400 mg/kg body weight), and the submandibular glands were dissected out under a

dissecting microscope (Olympus SZX7). The main artery was cannulated with a 31-32 gauge

blunt-end cannula, and perfusion solution was applied with a peristaltic pump (Ismatec IPC4)

at a flow rate of 1 ml/min. The perfusion solution was composed of 120 mM NaCl, 4.3 mM

KCl, 25 mM NaHCO₃, 1.0 mM MgCl₂, 1.0 mM CaCl₂, 5 mM glucose, and 10 mM HEPES

and was equilibrated with 95% O₂/5% CO₂. NaHCO₃ was replaced with the same

concentration of NaCl to prepare a HCO₃⁻-removal solution and was equilibrated with 100%
O₂. Unlike CCh, CVL and PLC stimulated very little saliva secretion at 25°C (room temperature) in our preliminary experiments. Consequently, the solutions and the glands were warmed at 37 ± 0.5°C with a custom-made water jacket heating system. Fluid secretion was stimulated by adding each agonist at the indicated concentrations. Secreted saliva was collected by a pre-calibrated glass capillary tube, and the saliva flow inside the capillary was collected every 30 s for the first 3 min, and then every 1 min for the duration; the collected saliva was stored in 500-μL tubes at −80°C until analyzed. The Na⁺ and Cl⁻ concentrations in the saliva were analyzed with an electrode chip using Dri Chem 7000 (Fuji Film Medical, Tokyo, Japan).

Intracellular signaling measurements: Intracellular Ca²⁺ and pH

For cellular signaling, intracellular Ca²⁺ ([Ca²⁺]ᵢ) and intracellular pH (pHᵢ) were measured. Submandibular glands were removed from anesthetized mice and placed in Krebs-Henseleit Ringer’s solution (103 mM NaCl, 4.7 mM KCl, 25 mM NaHCO₃, 2.8 mM glucose, 12.5 mM HEPES, 1.2 mM NaH₂PO₄, 4.9 mM Na-pyruvate, 2.7 mM Na₂-fumarate, 2.7 mM Na-glutamate, 2.6 mM CaCl₂, and 1.1 mM MgCl₂ supplemented with 1 mg/ml bovine serum albumin), minced about 100 times with fine scissors, and then digested with 520 U/ml
collagenase L (Nitta Gelatin, Osaka, Japan) for 20 min at 37°C with continuous shaking at 100 cycles/min. The digested tissue was washed three times and dispersed into 5 ml of Ringer’s solution. The cells were incubated for 20 min with 1-2 μM Fura-2 AM to detect [Ca\textsuperscript{2+}], or with 1 μM 3’-0-Acetyl-2’, 7’-bis(carboxyethyl)-4 or 5-carboxyfluorescein, diacetoxyethyl ester (BCECF AM) to detect pH\textsubscript{i}. Fluorescence was detected under a microscope (Olympus IX71) equipped with a fluorescence analysis system (Argus/Aquacosmos; Hamamatsu Photonics, Hamamatsu, Japan), with excitation and emission wavelengths of 340 or 380 nm and 510 nm, respectively, for Fura-2, and 440 or 495 nm and 535 nm, respectively, for BCECF. The [Ca\textsuperscript{2+}]; was determined as the ratio of the emission at 380 nm excitation and emission at 340 nm excitation; the pH\textsubscript{i} was determined similarly for emissions at 495 nm and 440 nm excitation.

**Drugs and statistical analysis**

Pilocarpine was purchased from Nacalai Tesque (Kyoto, Japan). Collagenase L was from Nitta Gelatin (Osaka, Japan). Fura-2 AM and BCECF AM were from Dojindo (Kumamoto, Japan). All other chemicals were purchased from Sigma Japan (Tokyo, Japan). Cevimeline was a kind gift from Nippon Kayaku (Tokyo, Japan).
The collected data were shown as mean ± standard error (S. E.). Student’s t-test was used for a comparison between two group means. For multiple comparisons, one way ANOVA and then the Tukey post hoc test was applied to detect statistically significant differences using SPSS ver. 14.0J (SPSS, Inc., Chicago, IL). Results were deemed significant at $p$-values $\leq 0.05$. 
Results

Cevimeline- and pilocarpine-induced salivation in mouse submandibular glands ex vivo

Cevimeline (CVL) and pilocarpine (PLC) are commonly used to induce fluid secretion in vivo, but few studies have examined the function of these agents ex vivo or in vitro. We examined the effects of these agonists in an in vitro system. At concentrations as low as 1 μM, CVL was able to induce salivation in perfused mouse submandibular glands ex vivo. Stable salivation was observed using 10 μM CVL [as well as 30 μM (126.5 ± 2.5 μL/10 min, mean ± S.E.)], and the kinetics of secretion during agonist stimulation was unchanged with up to nearly 1 mM CVL (Fig. 1A). PLC at a concentration as low as 0.1 μM induced salivation (1.5 ± 0.8 μL/10 min), and stable secretion was observed with about 3 μM PLC (170.5 ± 7.5 μL/10 min; Fig. 1B). In addition, PLC was the most effective secretagogue during the 10 minutes of stimulation tested in this study (187.9 ± 13.7 μL/10 min, at 10 μM). Carbachol (CCh), a muscarinic agonist used experimentally, showed moderate secretion induction at 0.1 μM, and stable secretion was observed at 0.3 μM CCh (148.4 ± 3.2 μL/10 min; Fig. 1C). Higher concentrations of PLC induced salivation for a longer time period after removal of the
agonist at 10 min, giving a greater total amount of secretion over a collection time of 20 min (Fig. 1E) compared with CVL (Fig. 1D) or CCh (Fig. 1F).

The kinetics of fluid secreted in response to PLC and CCh varied depending on concentration. At higher concentrations, secretion was biphasic, i.e., an initial rapid phase, followed by a lower plateau or gradual decrease (Fig. 1B, 1C). However, CVL lacked the initial peak phase (Fig. 1A). Both PLC and CCh induced a rapid high flow rate in less than 1 min with strong stimulation at most concentrations tested, whereas CVL required about 3 min to reach the highest flow rate at all concentrations tested. Higher concentrations of PLC and CVL required 3–5 min to fully wash out their effects (Fig. 1D, 1E).

The analysis of Na⁺ and Cl⁻ concentrations in secreted saliva revealed clear differences among the agonists. Compared with CVL, both PLC and CCh induced significantly higher Na⁺ concentrations in saliva (CVL vs. PLC, \( p < 0.05 \) at 10 μM and 30 μM, \( p < 0.01 \) at 100 μM; CVL vs. CCh, \( p < 0.05 \) at 10 μM) and showed significantly higher Cl⁻ concentrations (CVL vs. PLC, \( p < 0.01 \) at 10 μM) (Fig. 1G, 1H, 1I). Whereas Na⁺ and Cl⁻ content changed depending on the PLC and CCh agonist concentration, they were unchanged in CVL saliva.
Effect of β-agonist and contribution of epithelial Na\(^+\) channels in CVL-, PLC-, and CCh-induced fluid secretion

The Na\(^+\) and Cl\(^-\) concentrations in mouse submandibular saliva are lower than those in parotid saliva, owing mainly to epithelial Na\(^+\) channels in the apical membrane of ductal cells (Romanenko et al., 2008). The cystic fibrosis transmembrane conductance regulator (CFTR) Cl\(^-\) channel is critical for Cl\(^-\) reabsorption in ductal cells (Catalan et al., 2010). Both mechanisms are up-regulated by phosphodiesterase, which can be pharmacologically activated by β-stimulation (Romanenko et al., 2008). Here, when 10 μM isoproterenol (IPR) was added in the presence of a muscarinic agonist (30 μM CVL, 3 μM PLC, or 0.3 μM CCh), the flow rate decreased slightly (Fig. 2A, 2C, 2E), and the Na\(^+\) and Cl\(^-\) concentrations decreased by ~50% for all agonists (Fig. 2B, 2D, 2F).

We next examined whether the CVL-induced decrease in Na\(^+\) in saliva was attributable to specific activation of ENaC by using low concentrations of the inhibitor amiloride to specifically block ENaC. To ensure thorough inhibition of ENaCs, the glands were perfused with 10 μM amiloride for 30 min prior to muscarinic stimulation (Catalan et al., 2010). Only the saliva of CVL-treated glands showed significantly increased Na\(^+\) levels in the presence of
amiloride. Amiloride also blocked the IPR-induced increase in Na\(^+\) and Cl\(^-\) absorption observed in the presence of all muscarinic agonists.

**The effect of muscarinic agonists on \([Ca^{2+}]_i\) and pH\(_i\)**

As an increase in intracellular Ca\(^{2+}\) is central to regulating fluid secretion, we hypothesized that differences in activities, especially initial secretion, among the agonists stem from differences in the ability to modulate cellular signaling. The induction of fluid secretion by CVL was much slower than that for the other agonists, but increases in Ca\(^{2+}\) in the sustained phase did not differ significantly among the agonists (Fig. 3A). Both CVL and PLC showed a slower decrease in \([Ca^{2+}]_i\) after removal of the agonist, and this was completely blocked by a muscarinic antagonist (0.5 \(\mu\)M atropine; Fig. 3B).

With regard to pH\(_i\), both PLC and CCh induced a sustained decrease in pH\(_i\) (Fig. 4B, 4C), whereas CVL at 30 \(\mu\)M and higher concentrations (1 mM) produced only a minor change in pH\(_i\) (Fig. 4A). CVL showed a statistically significant, but minor decrease in pH at maximal concentration (CVL vs. PLC: \(p < 0.05\), CVL vs. CCh: \(p < 0.01\)).

Melvin et al. revealed that stimulation with CCh acidified salivary cells and then the resting pH level recovers, which was accomplished by Na\(^+\)/H\(^+\) exchanger (Melvin et al., 1988). We
hypothesized that CVL produces only a minor change in pH, because it specifically activates this exchanger. Several types of NHEs are expressed in salivary glands. NHE1 is the primary form, and it controls proton concentration in exocrine glands in both acinar and ductal cells (Evans et al., 1999; Brown et al., 2003). Lower doses of ethyl isopropyl amiloride (EIPA) have been shown to specifically inhibit NHE1 (Orlowski and Kandasamy, 1996; Praetorius et al., 2000). Perfusion of glands with 5 μM EIPA for 2 min prior to muscarinic stimulation in the present study unmasked intracellular acidification, confirming that the reduced effect of CVL stimulation was due to its specific activation of NHE1 (Fig. 5A). With the maximum drop in pH in the presence of EIPA, the differences among the agonists disappeared (Fig. 5A, 5B, 5C).

Anion accumulation from basolateral membranes and anion release at apical membranes are two main initiating components of saliva secretion. For continuous saliva flow, both the Na⁺–K⁺–2Cl⁻ transporter and Cl⁻/HCO₃⁻ exchanger (AE) work together, coupled with NHE at the basolateral membrane (Evans et al., 2000, Melvin et al., 2005). We confirmed the effect of NHE on fluid secretion by removing HCO₃⁻ from external solution, which was accomplished by replacing NaHCO₃ with the same concentration of NaCl. The fluid in
response to CVL decreased significantly ($p < 0.01$); however, that in PLC and CCh was not significantly different (Fig. 6).
Discussion

We explored the mechanistic differences among two clinically used muscarinic agonists, cevimeline and pilocarpine, and the experimentally used muscarinic agonist carbachol (Fig. 1). Both carbachol and pilocarpine showed different properties depending on concentrations. At higher concentrations, they show a biphasic response, i.e., an initial transient high flow rate followed by decreased secretion. By comparison, cevimeline demonstrated slower induction of secretion at all concentrations tested and achieved stable secretion levels at higher doses. During the 10-min drug application, CVL and PLC induced sustained secretion over a very broad range of concentrations. CCh was capable of inducing fluid secretion over only a very narrow concentration range (0.3–1.0 μM). We identified the lowest concentration at which we could detect stable secretion in response to 10 min of stimulation and confirmed stable secretion in response to several 10-minute pulse stimulations (data not shown). Based on these findings, we used 30 μM CVL, 3 μM PLC, and 0.3 μM CCh for further experiments. Glands continued to secrete for several minutes after stimulation with CVL or PLC (and with higher concentrations of CCh), suggesting that these agents bind strongly to muscarinic receptors. This was confirmed by measuring [Ca^{2+}]_{i} in vitro (Fig. 3), which gave results
consistent with ex vivo fluid secretion. The slow decay in [Ca\textsuperscript{2+}]\textsubscript{i} levels that was observed after removal of CVL or PLC was completely inhibited by atropine, producing a rapid return to resting [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 3B). Furthermore, perfusion with 0.5 μM atropine on muscarinic stimulation completely blocked ex vivo saliva secretion, indicating that CVL, PLC, and CCh induce salivation by activating muscarinic receptors (data not shown).

Compared with PLC and CCh, CVL treatment resulted in a significantly lower Na\textsuperscript{+} level in ex vivo saliva. There are at least three possible explanations for the significant Na\textsuperscript{+} decrease in ex vivo saliva in CVL-treated glands. First, Na\textsuperscript{+} absorption is flow rate dependent; thus, the lower saliva flow rate observed with CVL relative to PLC and CCh may account for this difference. However, this possibility was eliminated by our tests on the effects of perfusion flow rate (data not shown). Decreasing the perfusion flow rate to 0.5 mL/min during exposure to 0.3 μM CCh decreased the fluid secretion rate to approximately 20% less than that observed during perfusion with 30 μM CVL at 1 mL/min, yet the Na\textsuperscript{+} concentration remained significantly lower in the CVL-induced saliva (42.6 ± 2.1 mM Na\textsuperscript{+} for CVL vs. 53.5 ± 1.8 mM Na\textsuperscript{+} for CCh; p = 0.002, Student’s t-test). Second, CVL may activate a Na\textsuperscript{+} reabsorption pathway, presumably NHE3, which is highly expressed on the apical membrane.
of salivary duct cells (Park et al., 2001). However, no change was observed in the Na\(^+\) levels of ex vivo parotid (Park et al., 2001) or in vivo submandibular (Catalan et al., 2010) saliva from NHE3-gene disrupted mice, suggesting that NHE3-mediated Na\(^+\) absorption is probably not important at these flow rates. Finally, CVL may specifically activate ENaCs.

We examined this possibility using amiloride, which inhibits ENaCs (Lingueglia et al., 1996), but it also has other broad effects on membrane proteins in epithelial cells. We used amiloride at lower concentration (10 µM) because it can inhibit not only ENaC but also NHEs at higher concentrations (Orlowski and Kandasamy, 1996; Praetorius et al., 2000). Furthermore, the Na\(^+\)-dependent Ca\(^{2+}\) transport mechanism (presumably the Na\(^+\)/Ca\(^{2+}\) exchanger) shown to be functionally expressed in the rat submandibular gland (Morris et al., 1987, Gallacher and Morris, 1987) and may be effected by amiloride. However, replacing external Na\(^+\) with Li\(^+\) to inhibit Na\(^+\)/Ca\(^{2+}\)-exchanger activity (Blaustein and Santiago, 1977, Laskowski and Medler, 2009) failed to show any significant change in [Ca\(^{2+}\)]\(_i\) in response to CCh, suggesting that it may present in mouse submandibular gland, but it is not functionally important during muscarinic stimulation (data not shown). In glands infused with amiloride starting 30 min prior to and during muscarinic stimulation, Na\(^+\) absorption was blocked.
significantly, and the differences among the agonists disappeared, indicating that ENaC activity increased in CVL-stimulated glands. The interaction between CFTR and ENaC, which is discussed extensively elsewhere (Reddy and Quinton, 2003), was made evident by treatment with a β-agonist (Fig. 2B, 2D, 2F), but the Na⁺ was still significantly lowest in CVL, and ENaC inhibition abolished the differences in ion compositions among agonists.

This suggests that the decreased Na⁺ concentration in CVL resulted from activation of ENaC, which was not associated with mechanisms involving β-agonists.

Unlike PLC and CCh, CVL produced minor intracellular acidification. Intracellular acidification during muscarinic receptor stimulation has been linked to up-regulation of NHE1, which is thought to be coupled with enhanced anion exchanger activity and increased fluid secretion. To determine whether CVL activated NHE, acinar cells were treated with 5-(N-ethyl-N-isopropyl) amiloride (EIPA), a specific inhibitor of NHE, particularly NHE1 (Orlowski and Kandasamy, 1996; Praetorius et al., 2000). This maneuver revealed that in the presence of EIPA, CVL produced a drop in pH to a level similar to that induced by PLC and CCh. This suggests that CVL rapidly enhanced the activity of NHE (presumably NHE1) during fluid secretion.
Removing the HCO$_3^-$ from the external solution did not affect PLC and CCh ex vivo saliva, which was consistent with previously reported in rats (Martinez, 1987). Contrary, HCO$_3^-$ removal decreased CVL saliva significantly (Fig. 6), suggesting that a HCO$_3^-$-dependent Cl/HCO$_3^-$ exchange mechanism, which was coupled with NHE transport, was more active in the presence of CVL. Putative mechanisms discussed here are summarized in Figure 7. There are a lot of reports on NHE activation, [Ca$^{2+}$]$_i$ increase activates NHE exchange in salivary gland (Mangnel and Turner, 1990, 1991). Later, the Ca$^{2+}$–calmodulin complex has been proposed to activate NHE, as no additional phosphorylation occurred in response to muscarinic stimulation in salivary gland (Robertson et al., 1997). Otherwise, it has been discussed about intracellular Na$^+$ concentration sensing mechanisms (Ishibashi et al., 1999). In this study, it is likely that [Ca$^{2+}$]$_i$ alone is not involved in NHE upregulation because we observed almost identical increase both 30μM CVL and 3 μM PLC(Fig. 3-5). In addition, we show HCO$_3^-$ may be involved in NHE activation mechanisms (Fig. 6), consistent with previous reports (Yao et al., 1999). However, HCO$_3^-$ involved mechanisms are extremely complex. HCO$_3^-$ can pass through anion channels, it is transported by AE and Na$^+$-HCO$_3^-$ cotransporters, and it can be produced by cellular metabolism by oxygen consumption and
carbonic anhydrase (CA) (Li et al., 2006). In addition, HCO$_3^-$ transport is regulated as part of a so-called metabolon, where NHE, CA and AE interact (Gonzalez et al., 2007). The activation mechanism by which CVL regulates the HCO$_3^-$ dependent pathway will be addressed in future studies.
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Authorship Contributions

Participated in research design: Kondo, Nakamoto, Masaki and Hosokawa

Conducted experiments: Kondo, Nakamoto, Mukaibo and Kidokoro

Performed data analysis: Kondo, Mukaibo and Kidokoro

Wrote or contributed to the writing of the manuscript: Kondo and Nakamoto
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maxi-K (K$_{Ca}$.1.1) channels mediate K$^+$ secretion by the mouse submandibular

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Footnotes

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Legends for Figures

Figure 1. *Ex vivo* fluid secretion in response to muscarinic agonists.

Fluid secretion in response to cevimeline (CVL), pilocarpine (PLC), and carbachol (CCh) at the concentrations indicated is shown in Panels a, b and c, respectively. Each drug was applied for 10 min, and saliva was collected for an additional 10 min. Both the 10-min and 20-min amounts are shown in Panels D-F. The concentrations of Na\(^+\) and Cl\(^-\) in secreted saliva samples are shown in Panels G-I. For each condition, n = 5 or more. * p < 0.05, ** p < 0.01, *** p < 0.001; one way ANOVA followed by Tukey post hoc test.

Figure 2. Effect of muscarinic agonists on epithelial Na\(^+\) channel activity, submandibular gland fluid secretion, and ion levels in secreted saliva.

The effects of pharmacological activation of Na\(^+\) re-absorption via β-adrenergic stimulation by 10 μM isoproterenol (IPR), and inhibition the epithelial Na\(^+\) channel by 10 μM amiloride (AML), on mouse submandibular saliva secretion and ion composition was assessed *ex vivo*. Agonist concentrations were 30 μM CVL, 3 μM PLC, and 0.3 μM CCh. For each condition,
n = 6 or more. Values are means ± S.E. * p < 0.05, ** p < 0.01, *** p < 0.001 according to one way ANOVA with Tukey post hoc test.

Figure 3. Changes in intracellular Ca^{2+} in response to CVL, PLC, and CCh.

The changes in the fluorescence emission ratio of Fura-2 in collagenase-digested mouse submandibular gland acinar cell preparations during stimulation with CVL, PLC, or CCh are shown. The fluorescence ratio (380 nm/340 nm) at time 0 was set as 0. Agonist was applied for 2 min, and then the cells were perfused with physiological solution (Panel A) or atropine-containing solution (Panel B). At least four different preparations were used. Values are means ± S.E.

Figure 4. Changes in intracellular pH in response to CVL, PLC, and CCh.

Intracellular pH (pHi) from submandibular acinar clumps was measured as the BCECF fluorescence emission ratio at maximal agonist concentrations (30 μM CVL, 3 μM PLC, or 0.3 μM CCh) and agonist concentrations on the descending arm of the dose-response curve (1 mM CVL, 100 μM PLC, or 10 μM CCh). At least four different preparations were used.
Values are means ± S. E. Maximum pH decreases were (in ratio units): CVL, −0.591 ± 0.090 (30 μM) and −0.405 ± 0.052 (1 mM); PLC, −1.512 ± 0.279 (3 μM) and −1.656 ± 0.150 (100 μM); CCh, −1.771 ± 0.213 (0.3 μM) and −2.267 ± 0.137 (10 μM).

Figure 5. Effect of NHE1 inhibition on muscarinic agonist-induced pH change.

Intracellular pH (pHi) from submandibular acinar clumps was measured as the BCECF fluorescence emission ratio. The NHE inhibitor EIPA was applied for 1 min prior to the addition of each agonist. Dashed lines are cells in the presence of 30μM CVL, 3μM PLC, 0.3 μM CCh shown in Fig. 4A, B and C, respectively. Solid lines with filled symbols (mean value) and error bars (S.E.) are cells in presence of EIPA. Inhibition of NHE1 significantly lowered the pH, especially in the presence of CVL (n=5). In contrast, only slight change was observed in the presence of EIPA when the glands were perfused with PLC (n=4) and CCh (n=4). Maximum pH decreases were (in ratio units): CVL + EIPA, -2.297 ± 0.195; PLC + EIPA, -2.210 ± 0.104; CCh + EIPA, -2.112 ± 0.119.

Figure 6. Effect of removing HCO₃⁻ from perfusion solution on ex vivo fluid secretion.
Saliva secretion in response to 30µM CVL, 3µM PLC, and 0.3 µM CCh over 10 min stimulation were measured from *ex vivo* mice submandibular gland in the absence of HCO$_3^-$ in perfusion solution. To remove HCO$_3^-$, 25mM NaHCO$_3$ was replaced with 25mM NaCl.

Agonist concentrations were 30µM CVL, 3µM PLC and 0.3µM CCh. Filled bar are secreted saliva in the presence of HCO$_3^-$ as shown in Fig. 1D, 1E, 1F. For each condition, n=5 or more.

Values are means ±S.E. ** p < 0.01; Student’s t-test.

Figure 7. Schematic mechanisms of CVL-induced specific effects in salivary cells.

NHE, AE and CA complex was activated by CVL through HCO$_3^-$ dependent mechanism. In ductal cells, CVL might also control pH$_i$ by activating NHE to maintain ENaC activity.

However, PLC and CCh did not effectively activate this complex and ENaC.
Figure 3

A

B
Figure 4

A

\[ \Delta \text{Ratio (495/440nm)} \]

\[ \text{Time (s)} \]

CVL

- CVL 30\( \mu \)M
- CVL 1mM

B

\[ \Delta \text{Ratio (495/440nm)} \]

\[ \text{Time (s)} \]

PLC

- PLC 3\( \mu \)M
- PLC 100\( \mu \)M

C

\[ \Delta \text{Ratio (495/440nm)} \]

\[ \text{Time (s)} \]

CCh

- CCh 0.3\( \mu \)M
- CCh 10\( \mu \)M
Figure 6

![Bar graph showing saliva secretion levels](image-url)