Physiological cAMP-raising secretagogues differentially regulate fluid and protein secretions in mouse submandibular and sublingual glands.

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Running head: cAMP-dependent signaling pathways in mouse salivary glands

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Abstract

The mechanisms underlying the functional differences in sympathetic and parasympathetic regulation of the major salivary glands have received little attention. The acute effects of parasympathetic muscarinic (carbachol)-dependent and combined parasympathetic-dependent + cAMP-dependent pathways on fluid secretion rates, ion composition and protein content were assessed using a newly developed *ex vivo* preparation that allows the simultaneous perfusion of the mouse submandibular (SMG) and sublingual (SLG) glands. Our results confirm that the muscarinic dependent pathway accounts for the bulk of salivation in SMG and SLG while co-stimulation with a cAMP-increasing agent (forskolin, isoproterenol, or vasoactive intestinal peptide) did not increase the flow rate. Co-stimulation with carbachol + the β-adrenergic agonist isoproterenol decreased the concentration of NaCl and produced a substantial increase in the protein and Ca^{2+} content of SMG but not SLG saliva, consistent with a sparse sympathetic innervation of the SLG. On the other hand, Forskolin, which bypasses receptors to increase intracellular cAMP by directly activating the enzyme adenylate cyclase, enhanced the secretion of protein and Ca^{2+} by both the SMG and SLG. In contrast, isoproterenol and vasoactive intestinal peptide specifically stimulated protein secretion in SMG and SLG salivas, respectively. In summary, cAMP-dependent signaling does not play a major role in the stimulation of fluid secretion in SMG and SLG while each cAMP-increasing agonist behaves differently in a gland-specific manner suggesting differential expression of G-protein coupled receptors in the epithelial cells of SMG and SLG.

Introduction

Saliva is a complex fluid mainly composed of secretions from the major glands and hundreds of minor salivary glands that drain into the oral cavity where it plays many important roles such as lubrication of oral mucosal surfaces, demineralization and remineralization of teeth, food digestion and control of the oral microbiome (26). The importance of saliva is clearly inferred from patients suffering from diseases that are associated with salivary gland hypofunction such as Sjögren's syndrome or as the consequence of radiotherapy in head and neck cancers (22).

In humans, the major salivary glands (parotid, submandibular and sublingual glands) secrete between 0.5-1 liter of saliva per day. This efficiency is achieved because acinar secretory cells express ion-transporting proteins in their apical and basolateral domains that allow vectorial secretion of solutes and fluid into the luminal space (25).

Salivary gland function is regulated by the parasympathetic and sympathetic branches of the autonomic nervous system. It is known that sympathetic, adrenergic stimulation of parotid and submandibular glands results in the secretion of low amounts of a protein-rich fluid with a very low NaCl content. Conversely, parasympathetic, cholinergic stimulation of parotid and submandibular glands is associated with copious amounts of fluid containing comparatively low amounts of proteins and a relatively higher NaCl concentration (3, 28).

Although the molecular mechanism underlying fluid secretion is well conserved among salivary glands (15), the regulation of ion transport and fluid secretion processes apparently differ between glands. It is known that parotid and submandibular glands receive inputs from sympathetic and parasympathetic fibers, where they modulate ion transport as well as fluid and protein secretion (22, 27). In contrast, mucous-secreting minor and sublingual glands receive extensive inputs from the parasympathetic system but the innervation by the sympathetic system is sparse (26).

Protein secretion by the rat sublingual gland is modulated by the parasympathetic activation of cholinergic muscarinic receptors in a PKC-dependent manner (8). The role of the sympathetic system on protein secretion by the sublingual gland is contradictory. It has been reported that sympathetic adrenergic agonists do not stimulate mucin secretion by isolated rat sublingual gland cells (7), while the amount and concentration of protein in the saliva from rat sublingual glands increased upon intraperitoneal injection of cathecolamines compared to cholinergic agonists (1).

On the other hand, little is known about the modulation of protein and fluid secretion of mucous-secreting glands by cAMP-dependent pathways. Vasoactive intestinal peptide (VIP) is a parasympathetic-associated neurotransmitter that raises intracellular cAMP levels and have been reported to modulate the exocytosis of protein-storing granules in human major salivary gland acinar cells (9). Furthermore, intravenous injections of VIP induced saliva secretion by the rat parotid and submandibular glands (11). Of note, increased levels of VIP are observed in the parotid and submandibular glands of sympathectomized rats, suggesting a compensatory mechanism mediated by these cAMP-raising secretagogues in the absence of β-adrenergic receptor-mediated, cAMP cell signaling (10). Nonetheless, the negligible sympathetic innervation of mucous-secreting glands raises the question of which, if any, physiological secretagogues that increase intracellular cAMP levels might be involved in the regulation of fluid and protein secretion in the mucus-secreting sublingual salivary glands.

Taken together, we hypothesize that cAMP-dependent pathways might regulate the activity of mucus-secreting salivary glands. To test this model, we developed an *ex vivo* assay for simultaneously assessing the role of cAMP-raising secretagogues in mouse serous-secreting submandibular (SMG) and mucous-secreting sublingual (SLG) glands. Our results show that cAMP-dependent mechanisms differentially regulate serous- and mucous-secreting glands.

Materials and Methods

Ex vivo perfused SLG and SMG

Ex vivo SLG and SMG salivas were simultaneously collected by modifying the *ex vivo* mouse SMG perfusion technique (30). In brief, mice were anesthetized by chloral hydrate injection (400 mg/kg i.p.) and all branches of the common carotid artery were ligated, except for the artery supplying blood to SLG and SMG. Both glands were then removed and perfused at 37°C via the common artery, which was cannulated with a 31G cannula. SLG and SMG ducts were placed in different calibrated glass capillary tubes (Figure 1). Salivation was stimulated by perfusion with the muscarinic receptor agonist carbachol (CCh, 0.3 μM) or CCh (0.3 μM) + cAMP-increasing agents (5 μM isoproterenol, 100 nM vasoactive intestinal peptide or 10 μM forskolin). The perfusion solution was (mM): 4.3 KCl, 120 NaCl, 25 $NaHCO_3$, 1 $CaCl_2$, 1 $MgCl_2$, 5 glucose, and 10 Hepes, pH 7.4 (gassed with 95% O_2/5% CO_2). Saliva samples were collected and stored at -80°C until analyzed.

Ion and protein concentrations in SLG- and SMG-secreted fluids

Na^+, K^+ and Ca^{2+} concentrations in saliva were measured by atomic absorption spectrometry using the appropriate lamps (AAnalyst 200 spectrometer, PerkinElmer). The Cl^- concentration was analyzed with an Orion™ Cl^- electrode (Thermo Fisher Scientific). To insure enough sample for measuring the ion composition of saliva secreted in response to cAMP-raising secretagogues, data shown in Figures 3 and 4 were obtained from SMG and SLG stimulated for 15 minutes.

Protein concentrations were measured according to the manufacturer's instructions by the micro-BCA assay (Thermo Fisher Pierce, Rockford, IL, USA) using a DU530 spectrophotometer (Beckman) at 562 nm. Bovine serum albumin was used as standard for calibrations. Protein concentrations were calculated from SMG and SLG stimulated for 5 minutes with agonist. This time window was chosen for the protein measurements shown in Figures 5 and 6 as 75% of amylase (one of the most abundant protein in saliva) is secreted within the first 5 minutes in response to stimulation in rat parotid slices (14).

Gene expression analysis

Gene expression analysis of β-adrenergic and VIP receptors and the $K_{Ca1.1}$ (Kcnma1) K^+ channel was performed using publicly available RNA-sequencing data of mouse major salivary glands (SRA databases SRX2648311 to SRX2648322; n=12) (13). Threshold for positive gene expression was 0.1 FPKM (fragments per kilobase of transcript per million of mapped reads). Gene-sequencing data from salivary glands from 3 females and 3 male mice were used for comparing gene expression levels between submandibular and sublingual glands.

1) The *Kcnma1* mRNA expression levels for SMG and SLG from RNA-Seq data reported as FPKM values were 4.47 ± 0.55 vs. 1.21 ± 0.08 (average ± SEM; SMG vs. SLG, respectively; n=6 each gland, p value < 0.005, Student's t test).
2) The β-adrenergic receptor expression levels (FPKM) were 5.15 ± 1.16 vs. 0.41 ± 0.12 for *Adrb1* and 3.61 ± 0.50 vs. 1.30 ± 0.41 for *Adrb2*, SMG vs. SLG, respectively (n=6 each gland, p value < 0.005, Student's t test).
3) VIP receptor mRNA levels (FPKM) of type 1 VIP receptor (*Vipr1)* for SMG vs. SLG were 0.49 ± 0.06 vs. 3.31 ± 0.18; n=6 each gland, p value < 0.005, Student's t test. Transcript levels for type 2 VIP receptors encoded by *Vipr2* were very low in both SMG and SLG (FPKM values <0.1).

Statistical Analysis

Results are presented as the mean ± standard deviation (SD). Individual data points are shown in box graphs. Statistical significance was determined using Student's t test and one-way ANOVA analysis followed by Bonferroni's *post hoc* test. P values of less than 0.05 were considered statistically significant. Origin 8.0 Software was used for statistical calculations (OriginLab, Northampton, MA, USA).

Animals and Reagents

Mice (BS/129Svj background) were housed in cages with access to laboratory chow and water *ad libitum* with a 12-hour light/dark cycle. BS/129Svj background mice was generated in house by crossing BS (Charles River Laboratories) and 129Svj (The Jackson Laboratory) mice. Equal numbers of sex- and age-matched (from 6 to 12 wk old) mice were utilized. All animal procedures were approved by the Animal Care and Use Committee of the National Institute of Dental and Craniofacial Research, National Institutes of Health (ASP 13-686). All reagents were from Sigma-Aldrich (St.Louis, MO, USA), unless otherwise indicated.

Results

Saliva secretion by simultaneous ex vivo perfusion of SMG and SLG

Saliva formation is a two-step process where a NaCl-rich, plasma-like fluid is secreted by acinar cells (stage 1). The ion composition of this primary fluid is subsequently modified (mainly NaCl reabsorption and $KHCO_3$ secretion) as it passes through the ductal epithelium (stage 2) (22, 25). Given that ductal function in salivary glands is regulated by ß-adrenergic receptors (5), we evaluated the effect of muscarinic (CCh) plus ß-adrenergic (Isoproterenol, IPR) receptor stimulation on SMG and SLG flow rates and ion composition. As seen in Figure 2, a slight but significant reduction in flow rates was observed in SMG (Figures 2A-B) and SLG (Figures 2E-F) secretion rates when the ß-adrenergic agonist IPR (5 μM) was co-perfused with the muscarinic agonist CCh (0.3 μM). In terms of duct function, IPR and CCh co-stimulation induced a substantial NaCl reabsorption in SMG (Figure 2C; 38% decrease in $[Na^+] + [Cl^-]$) but not in SLG-secreted fluid (Figure 2G). Moreover, the K^+ concentration of SMG saliva did not differ between CCh- and CCh/IPR-stimulated glands (Figure 2C) while the K^+ content of SLG saliva was slightly reduced upon β-adrenergic receptor stimulation with IPR (Figure 2G). Of note, the Ca^{2+} concentration of SMG saliva increased ~140% but did not significantly change in SLG upon β-adrenergic receptor activation by isoproterenol (Figures 2D and 2H, respectively).

We then evaluated the effects of other physiological secretagogues that raise intracellular cAMP levels on SMG and SLG function. To assess if cAMP secretagogues affect the secretory (acinar) function of SMG (Figures 3A-B) or SLG (Figures 3C-D), the flow rates were measured in response to 0.3 μM CCh or combined stimulation with CCh (0.3 μM) plus forskolin or VIP. As seen in Figures 3A-B and 3C-D, comparison of SMG and SLG secretion rates during 15-minute stimulation showed that forskolin, which directly activates adenylate cyclase, reduced CCh-mediated flow rates in both SMG and SLG, respectively.

To evaluate if ductal function in SMG and SLG are modulated by cAMP-raising secretagogues, we then evaluated the ion composition of the SMG- and SLG-secreted fluids collected in response to the experimental conditions shown in Figure 3. As seen in Figure 4, the Na^+, K^+ and Cl^- compositions of SMG- and SLG-secreted salivas were differentially affected by different cAMP secretagogues: *i.* NaCl content in saliva from SMGs stimulated with by forskolin or VIP were statistically equal to NaCl content from SMG-secreted saliva in response to CCh (Figures 4A-B); *ii.* K^+ content in saliva collected from SMG co-stimulated with CCh and forskolin was statistically less from all the other groups tested (Figure 4C); *iii.* Reduction in Na^+ (Figure 4E) and K^+ (Figure 4G) content in SLG saliva co-stimulated with VIP was statistically different from SLG fluids stimulated with CCh or co-stimulation with forskolin; *iv.* K^+ content in saliva from SLG glands stimulated with CCh + forskolin secreted a fluid with lower K^+ content; and, *v.* none of the cAMP-inducing agonists tested in this study modified the Cl^- content compared to its control (0.3 μM CCh; Figures 4A and 4F). Regarding the Ca^{2+} content of saliva, co-stimulation with CCh + forskolin resulted in higher [Ca^{2+}] in the salivas of both SMG and SLG. In contrast, VIP did not induce a significant increase in Ca^{2+} content in SMG or SLG saliva (Figures 4D and 4H, respectively).

Protein secretion in saliva from perfused SMG and SLG

It has been shown that a higher protein concentration in saliva is associated with sympathetic activity in parotid and SMG glands. Given that the SLG gland receives a sparse innervation of sympathetic fibers,

180 little is known about the regulation of protein secretion in SLG by agonists that raise intracellular cAMP
181 levels (7, 26, 27). Taking advantage of the simultaneous SMG and SLG perfusion technique described
182 here, we measured the protein concentration in SMG and SLG salivas obtained by perfusion of both
183 glands with solutions containing different agonists.

184 We first compared protein concentrations and calculated the total amount of protein secreted from both
185 SMG and SLG stimulated for 5 minutes with CCh and CCh + IPR, respectively. Figures 5A-C show that
186 the protein concentration and the amount of protein secreted by the SMG gland was substantially
187 stimulated nearly 3-fold upon β-adrenergic receptor stimulation. In contrast, no difference was observed
188 in the protein concentration and the amount of protein secreted from SLG glands stimulated with CCh
189 compared to CCh+ IPR (Figure 5D-F).

190 We then evaluated the protein concentration and amount of protein secreted by SMG and SLG co-
191 stimulated with CCh and different cAMP-raising secretagogues. Figures 6A-C show that forskolin and
192 VIP increased the amount and concentration of protein secreted compared to SMG stimulated with CCh.
193 Similarly, the amount and concentration of protein in SLG saliva increased when SLG glands were co-
194 stimulated with CCh and forskolin or VIP (Figure 6D-F).

Discussion

The molecular mechanism underlying fluid secretion in mammalian salivary glands is well conserved (20, 22, 25). Briefly, the basolateral domain of secretory acinar cells express Na^+/K^+ ATPase (21), Na^+-K^+-2 Cl^- cotransporter (12, 21) and K^+ channels (3, 24, 28, 30), which promote the intracellular accumulation of Cl^- above its equilibrium potential. Apical Ca^{2+}-activated Cl^- channels allow Cl^- efflux across the luminal membrane, which creates a negative transepithelial potential difference that drives Na^+ secretion into the luminal space via the paracellular pathway (4, 29, 32). Finally, NaCl accumulation generates an osmotic gradient that energizes the water movement in the secretory direction via transcellular (aquaporin 5-dependent) and paracellular pathways (16, 19). Key proteins involved in fluid secretion have been found in acinar cells from all major salivary glands (15). However, the major salivary glands display distinct secretory properties, i.e. the contribution of each gland to whole saliva differs in terms of ion and protein content and the stimuli that trigger secretion.

In this study, we evaluated the dependence of acinar cell fluid secretion on β-adrenergic receptor stimulation in SMG and SLG. As seen in Figure 2, co-stimulation with CCh + isoproterenol slightly decreased the flow rates of both SMG and SLG (<15%). In contrast, the physiological cAMP-raising agonist VIP had no effect on the flow rates displayed by SMG and SLG in response to muscarinic receptor stimulation. Our results contrast with those reported by Larsson et al. where CCh-induced *in vivo* salivation by rat parotid gland is potentiated by forskolin and VIP (18). We do not have a clear explanation for such differences but the *in vivo* pro-stimulatory effect of forskolin and VIP might be due to systemic effects that are not present in the isolated perfused-gland preparation.

Sympathetic fibers innervate the ducts of the parotid and SMG, where stimulation enhances NaCl reabsorption, while the sympathetic innervation in SLG is sparse (26). Consistent with these anatomical observations, reabsorption of NaCl was strongly activated upon β-adrenergic receptor stimulation in SMG but not in SLG. On the other hand, the NaCl content of SMG saliva produced by CCh co-stimulation with

VIP did not differ from stimulation with only CCh (Figure 4A-B). This finding suggests that VIP receptors are not expressed at significant levels in the SMG duct cells responsible for NaCl reabsorption. Alternatively, we cannot rule out that VIP-mediated signals can inhibit ductal function in SMG ducts.

Co-stimulation of SLGs with CCh + VIP strongly reduced Na^+ but not Cl^- content in saliva, indicating that VIP receptors are functionally expressed in SLG ducts. This finding revealed that Na^+ and Cl^- reabsorption are independent processes in SLG. Such a non-coupled NaCl reabsorption mechanism clearly differs from that observed in mouse SMG gland (5). Moreover, forskolin did not increase Na^+ reabsorption, which clearly differs from the effect mediated by the cAMP-increasing agonists isoproterenol (SMG) and VIP (SLG). The cAMP-mediated signals that originate from receptor-mediated or adenylate cyclase directly stimulated by forskolin might result in different responses from ion transporting proteins involved in Na^+ reabsorption.

In this study, we found that the K^+ content in SMG saliva was higher than in SLG saliva. According to the current salivary gland secretion model, the final K^+ concentration of saliva is mostly determined by active electro-diffusive K^+ secretion via the $K_{Ca1.1}$ (Kcnma1) channel (23). Consistent with this model, higher levels of *Kcnma1* transcript levels were observed in mouse SMG compared to SLG tissue (13) supporting our findings that the $[K^+]$ of saliva is higher in SMG (see Materials and Methods section for more details).

Given that Na^+ reabsorption in SMG via ENaC is activated by an increase in intracellular cAMP levels (5), we expected a higher K^+ content in SMG saliva stimulated with CCh + isoproterenol due to the depolarization caused by ENaC-mediated Na^+ influx. However, co-stimulation with isoproterenol did not increase the K^+ content of SMG saliva. We do not have an explanation for this finding, but it is tempting to speculate that K^+ secretion might take place in a subset of duct cells that express $K_{Ca1.1}$ but do not express ENaC.

Little is known regarding K^+ secretion by SLG, but it is assumed that K^+ secretion occurs in duct cells. Given that K^+ content in saliva is inversely proportional to the flow rate, higher K^+ content is expected

when SLG were co-stimulated with CCh + isoproterenol (which reduces flow rate). In contrast, K^+ content in SLG saliva was significantly reduced when SLG was stimulated with CCh + isoproterenol compared to that obtained from SLG stimulated with only CCh. The same effect was also observed when SLGs were stimulated with CCh + VIP. Furthermore, K^+ content in both SMG and SLG salivas was significantly reduced when co-stimulated with forskolin. Our results differ from those reported by Larsson et al. where they found that VIP and forskolin potentiated the CCh-mediated K^+ efflux in rat parotid fragments (17). The differences between the studies could be due to 1) the different experimental approaches (K^+ efflux measurements vs. K^+ content in saliva). In this case, a higher K^+ efflux caused by the activation of a basolateral K^+ channel mediated by VIP and forskolin cannot be ruled out. 2) Different species (rat vs. mouse) or gland (parotid vs. SMG-SLG).

Together, the data presented in this study show that VIP and IPR decrease K^+ secretion in the mouse SLG. Additionally, the reduction in K^+ content in SMG and SLG in response to co-stimulation with forskolin strongly suggests that a non-described, cAMP-dependent signaling pathway might directly inhibit K^+ secretion in both glands.

Alternatively, given that K^+ and HCO_3^- secretion are coupled in salivary glands, we could assume a lower HCO_3^- content in SLG saliva produced in response to CCh + IPR or CCh + VIP. Interestingly, there is compelling evidence linking the structure of mucins with HCO_3^- ions, where lower HCO_3^- secretion is associated with more viscous mucous in the airways (6). Together, a lower $KHCO_3$ secretion would result in lower flow rates due to the higher viscosity of saliva.

Na^+ (and K^+ content) in SLG saliva was reduced when co-stimulated with CCh + VIP. Given that the Cl^- was unaffected when co-stimulated with CCh + VIP and it is assumed that $[Na^+] + [K^+] = [Cl^-] + [HCO_3^-]$, it is tempting to speculate that such co-stimulation might reduce the content of HCO_3^- in SLG saliva. Further experiments are required to address this important topic.

Regarding calcium content, we found that the [Ca^{2+}] of saliva was dramatically increased when β-adrenergic receptors were co-stimulated with muscarinic receptors in SMG, but as expected, the β-adrenergic receptor agonist IPR had no effect in SLG. Calcium concentration in SMG saliva increased by 140% when stimulated with carbachol + isoproterenol. On the other hand, SMG flow rate was decreased by 12.4% when stimulated with carbachol + isoproterenol. Therefore, the increase in calcium content in saliva collected in response to carbachol + isoproterenol cannot be explained by the lower flow rates observed during carbachol + isoproterenol treatment.

Little is known regarding the mechanism underlying Ca^{2+} transport in salivary glands, but it has been suggested that the final Ca^{2+} content in saliva is the result of two competing mechanisms, Ca^{2+} secretion by acinar cells and Ca^{2+} reabsorption by duct cells (2). Based on our findings, the higher Ca^{2+} content measured in SMG saliva stimulated with CCh+ IPR could be due to: 1) enhanced Ca^{2+} secretion by the acinar epithelium; 2) decreased Ca^{2+} reabsorption by the duct epithelium; or, 3) activation of both Ca^{2+} secretion and inhibition of Ca^{2+} reabsorption. Given that the Ca^{2+} content increased in SMG saliva when glands were co-stimulated with CCh + isoproterenol, it was not surprising that a robust increase in Ca^{2+} content was observed in SMG and SLG salivas when the glands were co-stimulated with CCh + forskolin. Together, our results show that the mechanism(s) underlying Ca^{2+} transport by SMG and SLG strongly depend on the intracellular levels of cAMP. However, VIP did not increase Ca^{2+} content in SMG and SLG salivas, suggesting that other physiological secretagogues might be required to sufficiently raise cAMP to affect Ca^{2+} transport by SMG and SLG.

The salivary mucins, which are mainly synthesized by the seromucous acinar cells of submandibular and mucous cells of sublingual glands, form an adherent layer that coats the oral cavity where it plays a key role in modulating the oral microbiome, hydration and lubrication of oral structures and teeth remineralization and demineralization (31). In addition to the mucins, the oral mucus also consists of other proteins and lipids secreted by mucous-secreting glands. Taking advantage of the *ex vivo* perfused SMG/SLG preparation, which allows the evaluation of the direct effects of secretagogues on the SMG

and SLG function, we found that the activation of β-adrenergic receptors stimulates protein secretion by SMG but not SLG (Figure 5). In contrast, vasoactive intestinal peptide (VIP) induced protein secretion in both SMG and SLG. In summary, isoproterenol selectively activated protein secretion by SMG while VIP induced protein secretion in both glands. Similar results to those displayed by VIP were obtained when glands were co-stimulated with forskolin, which bypasses receptors to directly activate cAMP production. Together, protein secretion by SMG and SLG are activated by increasing intracellular cAMP levels, which suggest a similar molecular secretion mechanism in both glands albeit through different receptors. Based on the activation profile of protein secretion by mouse salivary glands, the secretory acinar cells responsible for the bulk of protein secretion express robust levels of β-adrenergic receptors in SMG. In fact, detailed analysis of transcript levels revealed that the expression is higher for genes encoding β1- and β2-adrenergic receptors (*Adrb1* and *Adrb2*) in SMG compared to SLG (See Material and Methods Section) (13).

VIP induced protein secretion in both SMG and SLG saliva, suggesting that VIP receptors are expressed in secretory cells from SMG and SLG. Considering that VIP also regulates duct function in SLG (stimulates Na^+ reabsorption, see Figure 4E), transcript levels of *Vipr1* (which encodes for a type 1 VIP receptor) were higher in SLG compared to SMG. On the other hand, transcript levels for type 2 VIP receptors encoded by *Vipr2* were very low in both SLG and SMG (FPKM values <0.1) (See Material and Methods Section).

In summary, in the current study we describe the development of a modification of the mouse SMG-perfused preparation where both SMG and SLG are simultaneously perfused. Using this experimental approach, we found that cAMP-mobilizing agents modulate diverse aspects of salivary gland function such as flow rate, as well as the ion and protein compositions of saliva.

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Disclosures

The authors declare that they have no conflicts of interest with the contents of this article.

Footnote

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Figure Legends.

Figure 1. Simultaneous perfusion of mouse submandibular and sublingual glands. Image depicting SMG and SLG perfused via common carotid artery by a 31G cannula. SMG and SLG main ducts were separated and connected to different calibrated glass capillary tubes for collecting saliva to quantify flow rates and performing ion and protein analysis.

Figure 2. *Ex vivo* flow rates and ion composition of SMG and SLG saliva upon combined muscarinic acetylcholine and β-adrenergic receptor activation. A. Flow rates for SMGs (10 minutes of stimulation) perfused with 0.3 μM CCh (black circles, n=14 glands) or 0.3 μM CCh + 5 μM IPR (grey circles, n=13 glands) bicarbonate-containing solutions. **B.** Total amount of saliva secreted by SMGs stimulated with 0.3 μM CCh (black diamonds) or 0.3 μM CCh + 5 μM IPR (grey diamonds). Boxes correspond to the average (line) ± standard deviation (SD) of experiments shown in panel **A. C-D.** Na^+, K^+ and Cl^- **(C)** and Ca^{2+} **(D)** concentrations (mM) in SMG saliva stimulated with 0.3 μM CCh (black diamonds, n=14 except for Ca^{2+} measurements where the n=8) or 0.3 μM CCh + 5 μM IPR (grey diamonds, n=13 except for Ca^{2+} measurements where the n=8). **E**. Flow rates for SLGs (10 minutes of stimulation) perfused with 0.3 μM CCh (black circles, n=14 glands) or 0.3 μM CCh + 5 μM IPR (grey circles, n=14 glands) bicarbonate-containing solutions. **F.** Total amount of saliva secreted by SMGs stimulated with 0.3 μM CCh (black diamonds) or 0.3 μM CCh + 5 μM IPR (grey diamonds). Boxes correspond to the average (line) ± SD of experiments shown in panel **E. G-H.** Na^+, K^+ and Cl^- **(G)** and Ca^{2+} **(H)** concentrations (mM) in SLG saliva stimulated with 0.3 μM CCh (black diamonds, n=14 except for Ca^{2+} measurements where the n=5) or 0.3 μM CCh + 5 μM IPR (grey diamonds, n=14 except for Ca^{2+} measurements where the n=4). * and ** p <0.05 and <0.01, respectively (Student's t test). Box graphs correspond to the average (line) ± SD for each experimental condition.

Figure 3. Effect of cAMP-raising secretagogues on the e*x vivo* secretions by SMG and SLG. A. Flow rates for SMGs (15 minutes of stimulation) stimulated with 0.3 μM CCh (white circles, n=8 glands) or 0.3

µM CCh + 10 µM forskolin (grey circles, n=11 glands) or + 100 nM VIP (black circles, n=6 glands). **B.** Total amount of saliva secreted by SMGs stimulated with the secretagogues shown in panel **A**. **C.** Flow rates for SLGs (15 minutes of stimulation) stimulated with 0.3 µM CCh (white circles, n=11) or 0.3 µM CCh + 10 µM forskolin (grey circles, n=15) or + 100 nM VIP (black circles, n=11). **D.** Total amount of saliva secreted by SMGs stimulated with the secretagogues shown in **C**. Box graphs correspond to the average (line) ± SD for each experimental condition. * $p<0.05$ (ANOVA followed by Bonferroni's *post hoc* test).

Figure 4. Effect of cAMP-raising secretagogues on the ion composition of SMG and SLG salivas. Na^+ **(A)**, Cl^- **(B)**, K^+ **(C)** and Ca^{2+} **(D)** concentrations of SMG saliva (from Figure 3) stimulated with 0.3 µM CCh (white circles, n=14) or 0.3 µM CCh + 10 µM forskolin (grey circles, n=10) or + 100 nM VIP (black circles, n=6). Na^+ **(E)**, Cl^- **(F)**, K^+ **(G)** and Ca^{2+} **(H)** concentrations of SLG saliva (from Figure 3) stimulated with 0.3 µM CCh (white circles; Na^+: n=6, Cl^-: n=6, K^+: n=6, Ca^{2+}: n=5) or 0.3 µM CCh + 10 µM forskolin (grey circles; Na^+: n=8, Cl^-: n=9, K^+: n=8, Ca^{2+}: n=4) or + 100 nM VIP (black circles; Na^+: n=7, Cl^-: n=7, K^+: n=7, Ca^{2+}: n=4). Box graphs correspond to the average (line) ± SD for each experimental condition. * $p<0.05$ (ANOVA followed by Bonferroni's *post hoc* test).

Figure 5. Effect of β-adrenergic agonist on the protein secretion by SMG and SLG. A-C. Protein concentration **(A)**, amount of saliva secreted **(B)** and total amount of protein secreted **(C)** by SMG (5 minutes of stimulation) in response to 0.3 µM CCh (black circles, n=9) or 0.3 µM CCh + 5 µM IPR (grey circles, n=6). **D-F.** Protein concentration **(D)**, amount of saliva secreted **(E)** and total amount of protein secreted **(F)** by SLG (5 minutes of stimulation) in response to 0.3 µM CCh (black circles, n=9) or 0.3 µM CCh + 5 µM IPR (grey circles, n=7). Total amount of protein was calculated by multiplying the protein concentration by the secreted saliva volume. Box graphs correspond to the average (line) ± SD of experiments. ** $p<0.01$ (Student's *t* test).

Figure 6. Effect of cAMP-raising secretagogues on the protein secretion by SMG and SLG. A-C. Protein concentration **(A)**, amount of saliva secreted **(B)** and total amount of protein secreted **(C)** by SMG (5 minutes of stimulation) in response to 0.3 µM CCh (white circles, n=9) or 0.3 µM CCh + 10 µM forskolin (grey circles, n=12) or + 100 nM VIP (black circles, n=8). **D-F.** Protein concentration **(D)**, amount of saliva secreted **(E)** and total amount of protein secreted **(F)** by SLG (5 minutes of stimulation) in response to 0.3 µM CCh (white circles, n=9) or 0.3 µM CCh + 10 µM forskolin (grey circles, n=10) or + 100 nM VIP (black circles, n=7). Box graphs correspond to the average (line) ± SD of experiments. * $p<0.05$ (ANOVA followed by Bonferroni's *post hoc* test).

Figure 1

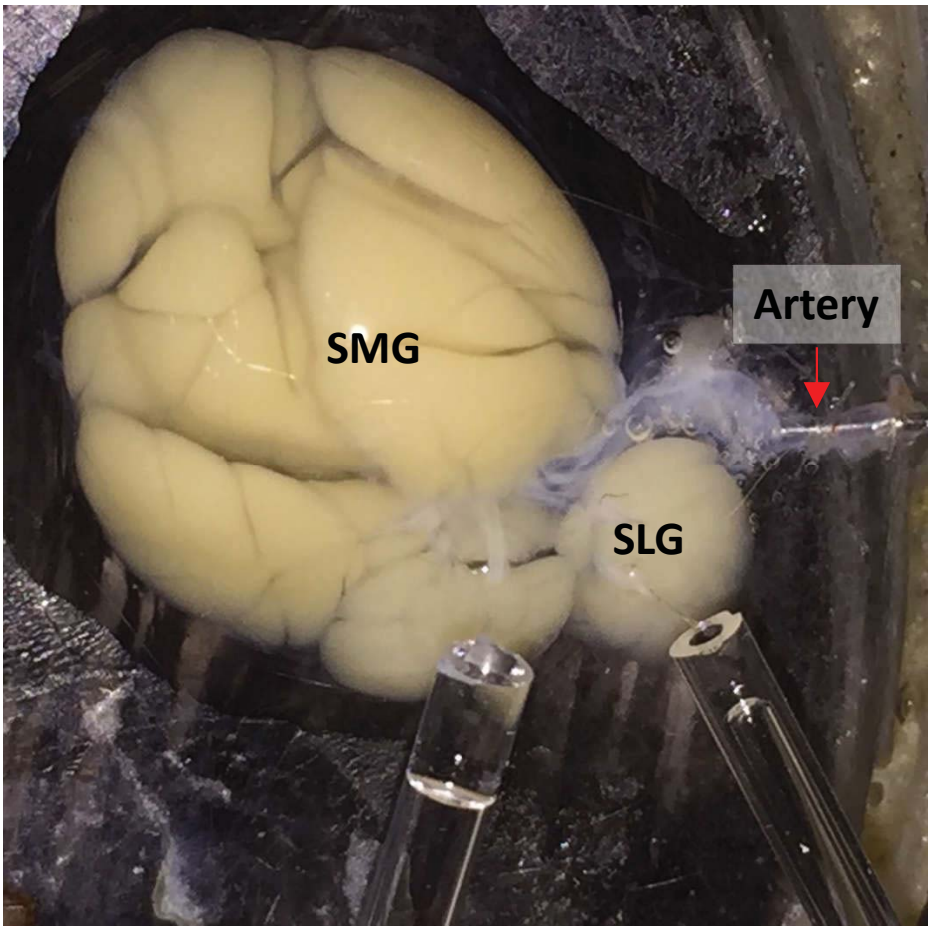


Figure 2

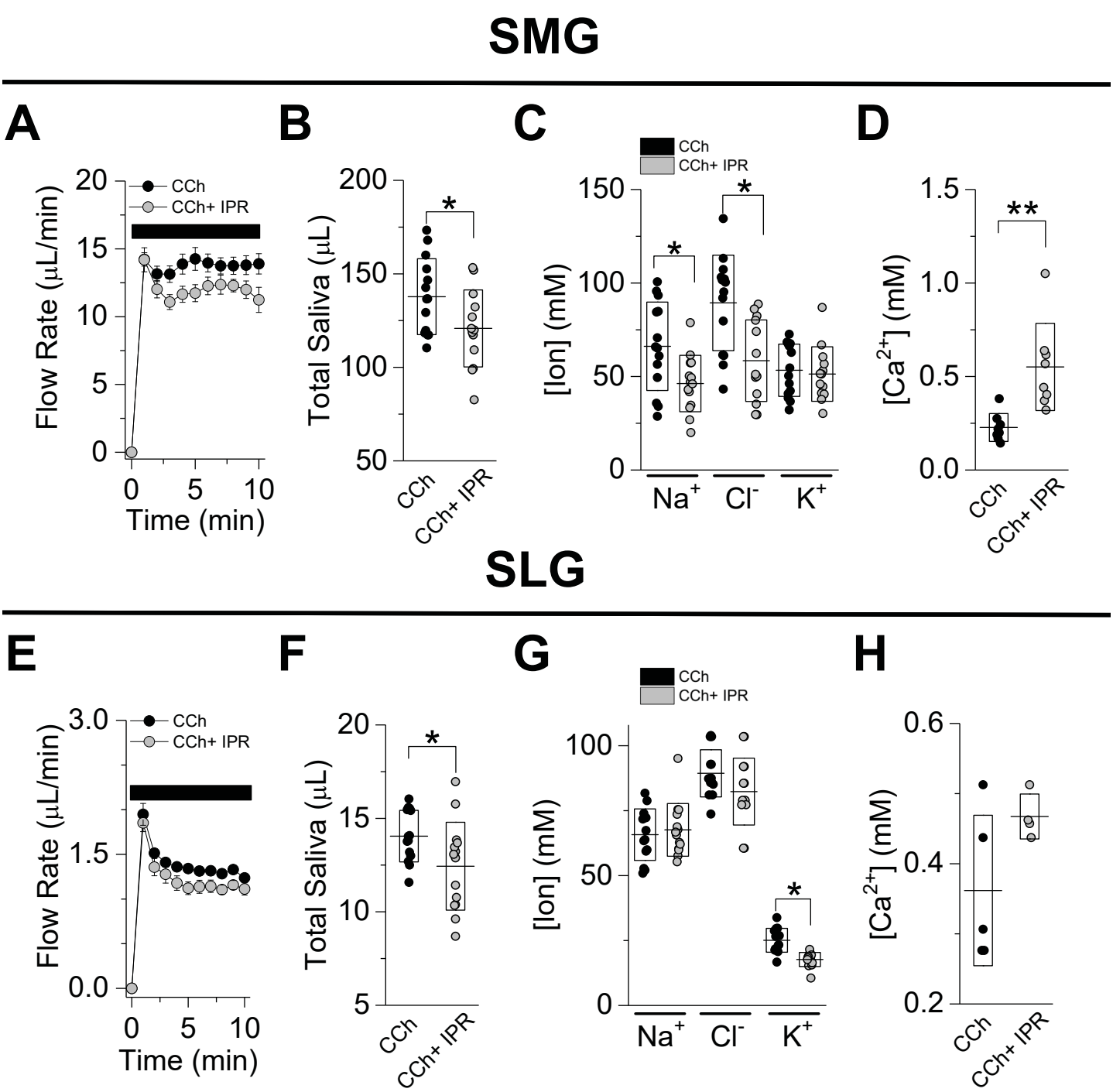
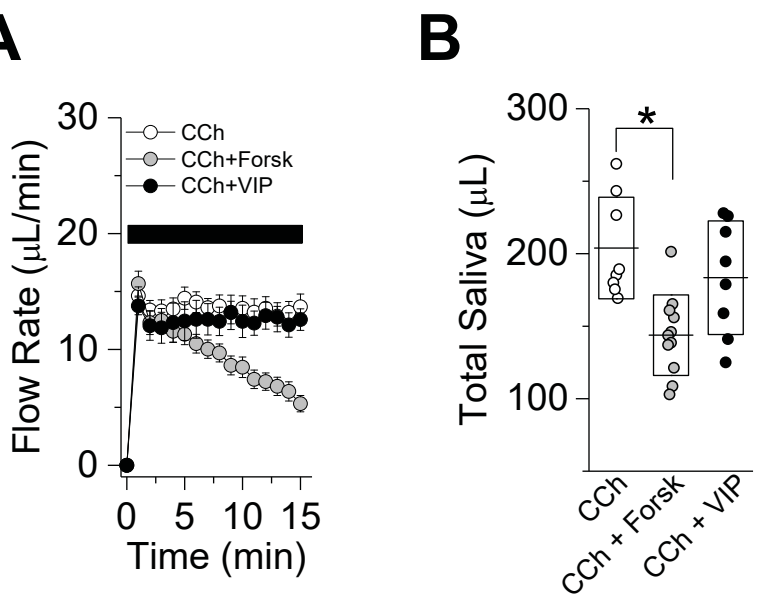

SMG
A
B
C
D
CCh
CCh+ IPR
Flow Rate (µL/min)
Time (min)
Total Saliva (µL)
[Ion] (mM)
Na+
Cl-
K+
[Ca2+] (mM)
SLG
E
F
G
H

Figure 3

SMG



SLG

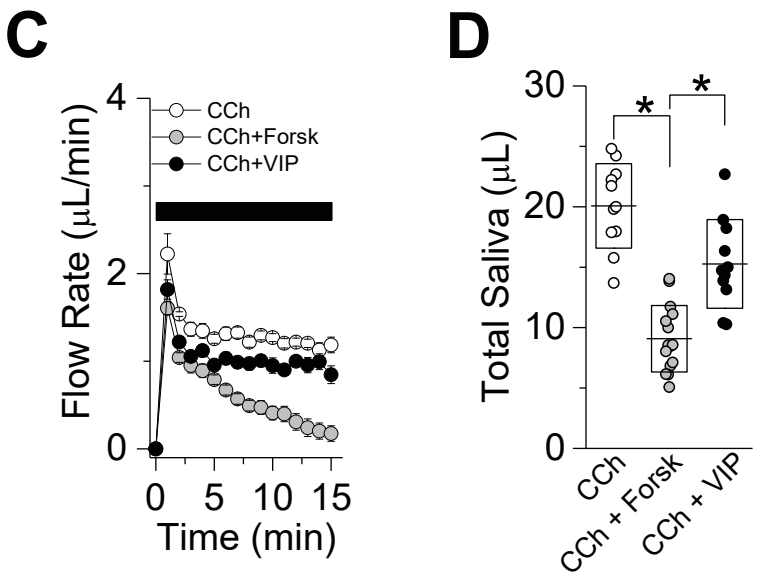


Figure 4

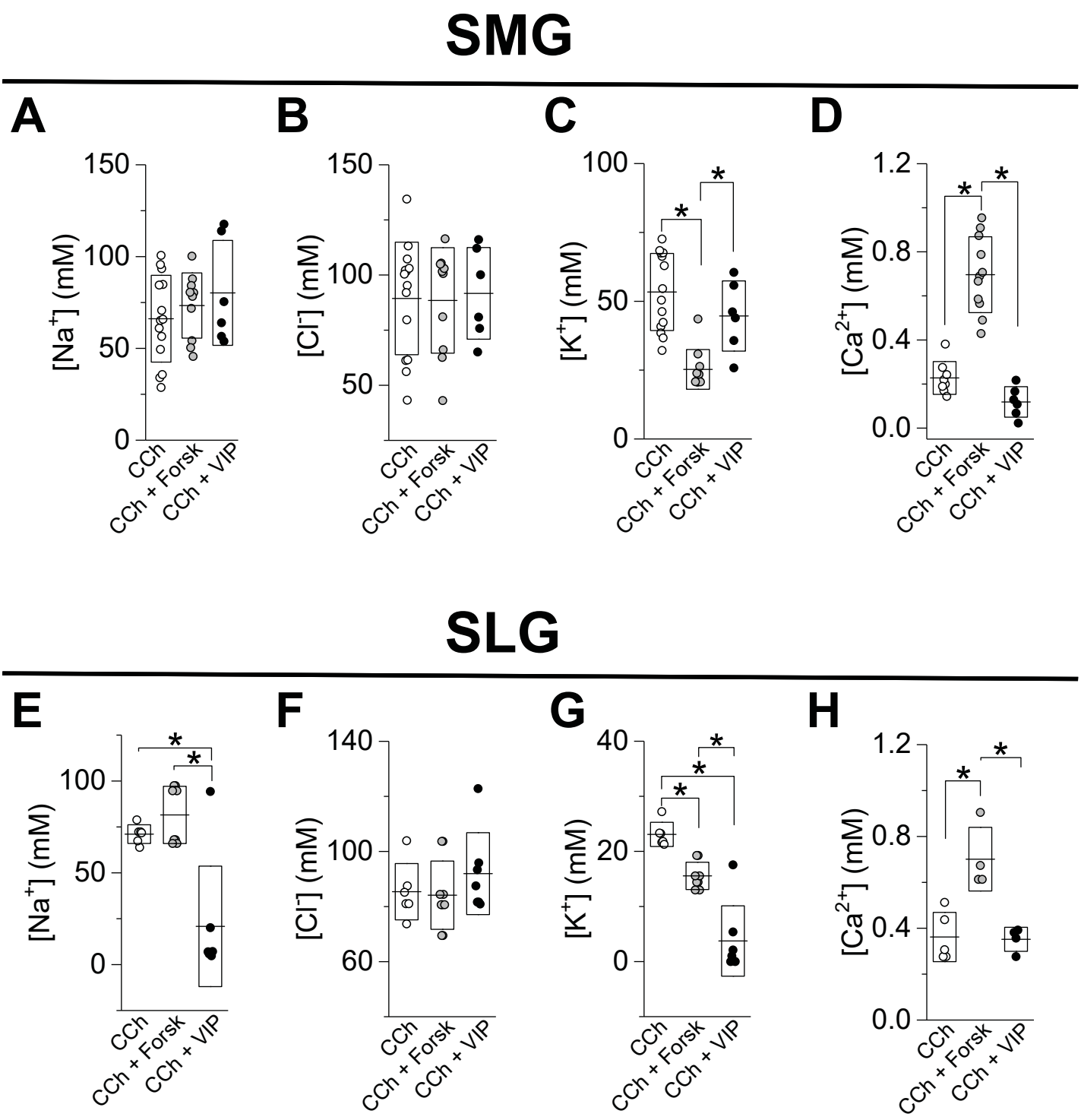


Figure 5

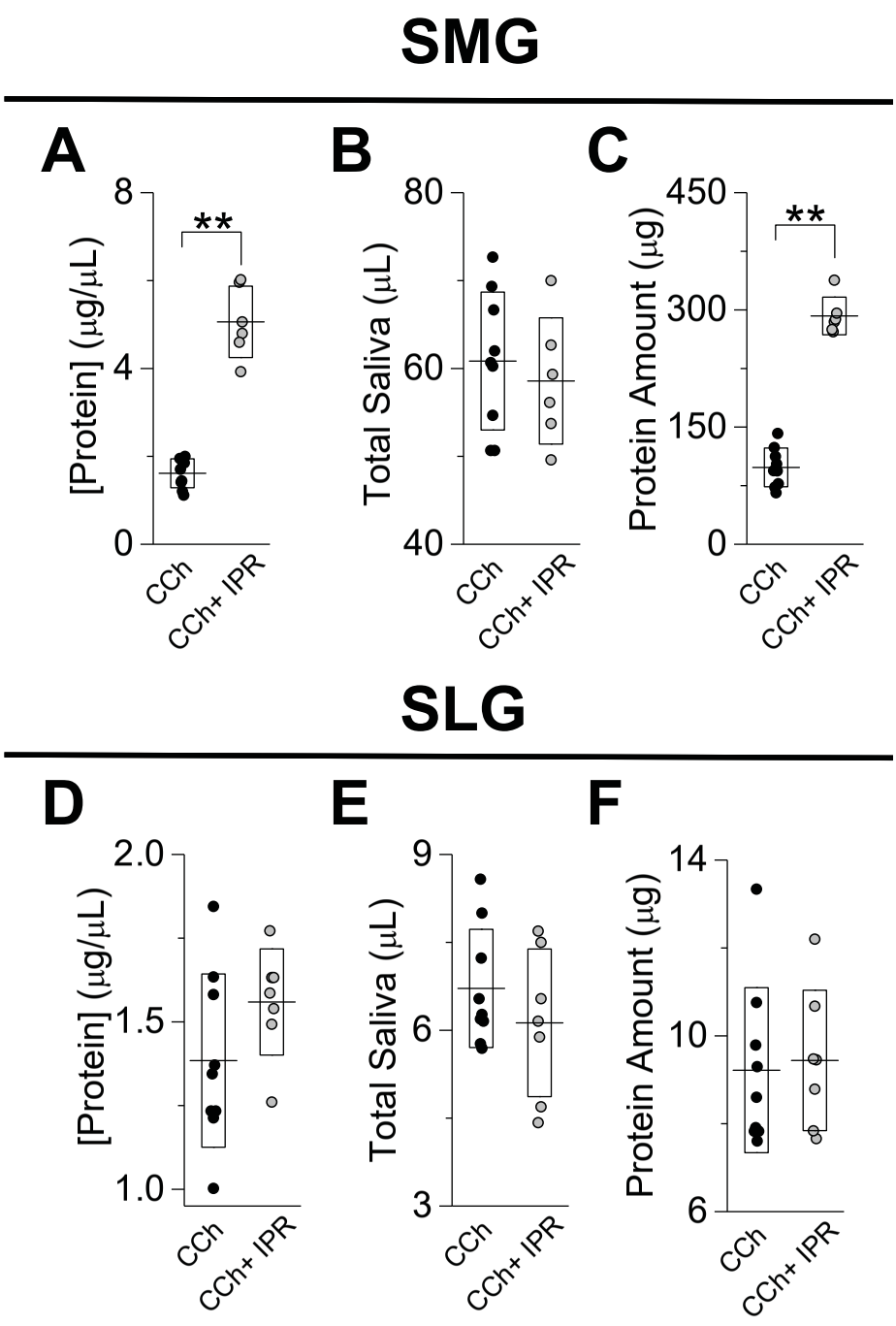
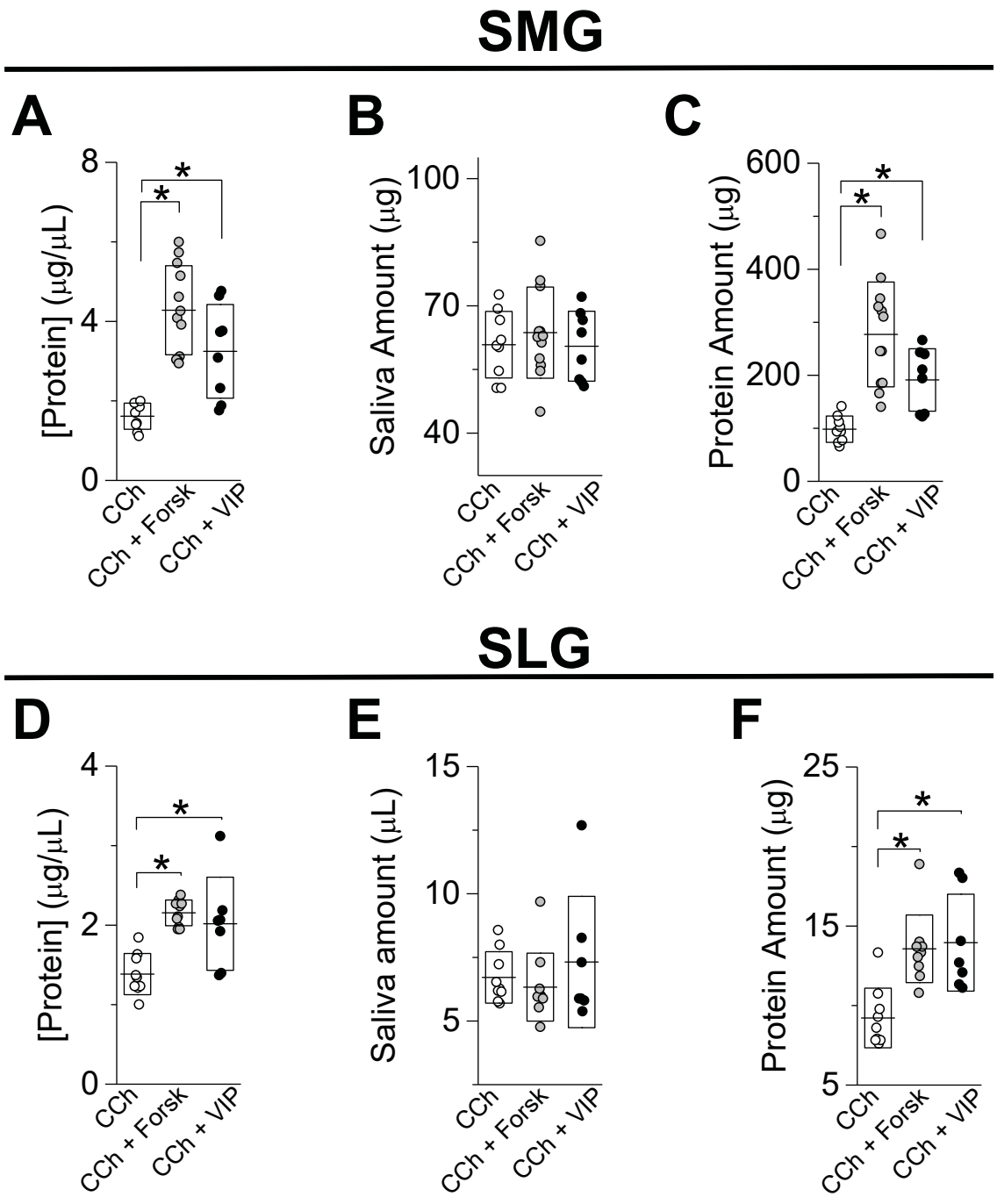

SMG
A
B
C
[Protein] (μg/μL)
8
4
0
**
CCh
CCh+ IPR
Total Saliva (μL)
80
60
40
Protein Amount (μg)
450
300
150
0
SLG
D
E
F
2.0
1.5
1.0
9
6
3
14
10
6

Figure 6


SMG
A
[Protein] (μg/μL)
8
4
0
B
Saliva Amount (μg)
100
70
40
C
Protein Amount (μg)
600
400
200
0
CCh
CCh + Forsk
CCh + VIP
SLG
D
[Protein] (μg/μL)
4
2
0
E
Saliva amount (μL)
15
10
5
F
Protein Amount (μg)
25
15
5
*