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The cAMP-phosphodiesterase 4 (PDE4) controls β-adrenoceptor- and CFTR-dependent saliva secretion in mice.

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Abstract

Saliva, while often taken for granted, is indispensable for oral health and overall well-being, as inferred from the significant impairments suffered by patients with salivary gland dysfunction. Here, we show that treatment with several structurally-distinct PAN-PDE4 inhibitors, but not a PDE3 inhibitor, induces saliva secretion in mice, indicating it is a class-effect of PDE4 inhibitors. In anesthetized mice, while neuronal regulations are suppressed, PDE4 inhibition potentiates a β-adrenoceptor-induced salivation, that is ablated by the β-blocker Propranolol and is absent in homozygous ΔF508-CFTR mice lacking functional CFTR. These data suggest that PDE4 acts within salivary glands to gate saliva secretion that is contingent upon the cAMP/PKA-dependent activation of CFTR. Indeed, PDE4 contributes the majority of total cAMP-hydrolytic capacity in submandibular-, sublingual-, and parotid glands, the three major salivary glands of the mouse. In awake mice, PDE4 inhibitor-induced salivation is reduced by CFTR deficiency or β-blockers, but also by the muscarinic blocker Atropine, suggesting an additional, central/neuronal mechanism of PDE4 inhibitor action. The PDE4 family comprises four subtypes, PDE4A-D. Ablation of PDE4D, but not PDE4A-C, produced a minor effect on saliva secretion, implying that while PDE4D may play a predominant role, PDE4 inhibitor-induced salivation results from the concurrent inactivation of multiple (at least two) PDE4 subtypes. Taken together, our data reveal a critical role for PDE4/PDE4D in controlling CFTR function in an *in vivo* model and in inducing salivation, hinting at a therapeutic potential of PDE4 inhibition for cystic fibrosis and conditions associated with xerostomia.

Keywords

PDE4; β-adrenergic signaling; salivation; cAMP; CFTR

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Author contributions:

IVA and AB performed the salivation assays; IVA, AB, LA, DS, and WR generated the animals, maintained the mouse colonies, and performed the biochemical assays; WR designed the experiments and analyzed the data; WR wrote a first draft and all authors edited and approved the manuscript.

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INTRODUCTION

It is now well appreciated that in humans and animals alike, saliva is indispensable for oral health, and in extension for the organisms' overall health[1–3]. While largely comprised of water, saliva contains critical minerals and electrolytes (such as sodium, potassium, calcium, chloride), buffers (bicarbonate), a plethora of proteins including digestive enzymes (e.g. amylase), various antimicrobials (including cystatins, lysozyme, agglutinins, and secretory immunoglobulins/IgA), as well as an abundance of glycoproteins, of which mucins (e.g. Muc5B) are the most eminent. Together, they facilitate critical tasks including the lubrication and moistening of oral mucosal surfaces, the maintenance of microbial homeostasis and innate immune defense, they assist in food digestion, bolus formation, swallowing, and taste, and play key roles in the mineralization of teeth and wound healing[2–4]. Humans produce between 0.5 and 1 L of saliva each day and its importance is plainly demonstrated by the significant discomfort and impairments suffered by patients with salivary gland hypofunction, which include xerostomia, the feeling of dry mouth, a loss of taste sensation, difficulty chewing, digesting and swallowing food leading to malnutrition/weight loss, an increased incidence of oral infections, sialosis, enamel hypomineralization and dental caries[1–3, 5]. Salivary gland hypo/dys-function is caused by various medical conditions or interventions including ageing, radiation therapy (e.g. for head and neck cancers), autoimmune diseases (such as Sjögren's syndrome), infection/inflammation affecting the salivary glands, diabetes, or by a wide range of xerogenic medications (including anticholinergics, anti-histamines, some antidepressants/antipsychotics, or antihypertensives)[5]. Current treatment options are limited and include parasympathomimetics, particularly the $M_{1/3}$-muscarinic-acetylcholine receptor agonists Pilocarpine and Cevimeline, as well as symptomatic (mouth washes or gels) or homeopathic treatments.

Saliva is produced by three main glands, the submandibular gland (SMG), the sublingual gland (SLG), and the parotid gland (PG), as well as a multitude of smaller glands[1], which are regulated by both the sympathetic and the parasympathetic autonomic nervous system and produce secretions with distinct compositions[6]. An intracellular increase in calcium (e.g. in response to M_3AChR or α_1-adrenoceptor activation) induces production of large volumes of watery saliva that is low in protein content. Conversely, an increase in the second messenger cAMP (e.g. in response to β-adrenergic stimulation) induces secretion of saliva with high protein content[3, 5].

The cellular concentration of cAMP is defined by the interplay between G_s- and G_i-coupled receptor signaling that determines the rate of cAMP synthesis by adenylyl cyclases, and in equal measure by the rate of cAMP degradation catalyzed by cyclic nucleotide phosphodiesterases (PDEs). In humans and most mammalian model species, including mouse and rat, PDEs are encoded by 21 genes, which in turn are grouped into 11 PDE families by sequence homology[7, 8]. The PDE4 family is the largest of the PDE families, comprising four PDE4 genes or subtypes, PDE4A, PDE4B, PDE4C, and PDE4D[8]. Each PDE4 gene in turn is expressed as numerous protein variants, that are generated *via* use of alternate promoters and transcription start sites, or by alternative splicing. These variants are distinguished by their cell- and tissue-specific expression patterns, unique post-translational

regulation, and their recruitment into distinct macromolecular signaling complexes and subcellular compartments[9]. As a result, individual PDE4 subtypes and protein variants exert unique cellular and physiologic functions. PDE4s are also widely expressed, so that one or more PDE4 variants are found in almost every cell of the body, and combined, they often contribute a substantial amount of total cellular cAMP hydrolytic capacity. Given their wide distribution, it is not surprising that PAN-selective PDE4 inhibition produces an array of potentially therapeutic benefits, including broad-spectrum anti-inflammatory properties, improvement in cognition and memory, metabolic- or cardiovascular effects[7, 10–12]. While exploring potential anti-inflammatory benefits of PDE4 inhibition in a model of bacterial lung infection, we noticed an obvious, excessive salivation in mice pretreated with PDE4 inhibitors when we subsequently handled the animals during intratracheal infection, and have further explored this observation.

EXPERIMENTAL

Drugs

Piclamilast (RP73401; 3-(Cyclopentyloxy)-N-(3,5-dichloropyridin-4-yl)-4-methoxybenzamide), Rolipram (4-(3-cyclopentyloxy-4-methoxyphenyl)pyrrolidin-2-one), Roflumilast (3-(cyclopropylmethoxy)-N-(3,5-dichloropyridin-4-yl)-4-(difluoromethoxy)benzamide), Cilostamide (N-cyclohexyl-N-methyl-4-[(2-oxo-1H-quinolin-6-yl)oxy]butanamide, and Atropine were from Cayman Chemical (Ann Arbor, MI), Isoprenaline, Pilocarpine, and Propranolol from Millipore Sigma (St. Louis, MO, USA), and RS25344 (1-(3-nitrophenyl)-3-(pyridin-4-ylmethyl)pyrido[2,3-d]pyrimidine-2,4-dione) was obtained from Santa Cruz Biotech (Santa Cruz, CA). All drugs were initially dissolved in DMSO, subsequently diluted into phosphate-buffered saline (PBS), pH 7.4, containing final concentrations of 5% DMSO and 5% Cremophor EL (Millipore Sigma, St. Louis, MO) and were applied by intraperitoneal (i.p.) injection (100 μl per 20 g body weight).

Animals

Wild-type C57BL/6 mice for experimentation were generated in-house using breeders obtained from Charles River Laboratories (Wilmington, MA). Mice deficient in PDE4A[13], PDE4B[14] and PDE4D[15] were generated by Drs. S.-L. Catherine Jin and Marco Conti (Stanford University, CA; also see[16]) and kindly distributed *via* the Mutant Mouse Resource and Research Centers (MMRRC, http://www.mmrrc.org, PDE4A stock ID# 034793-UCD, PDE4B stock ID# 034682-UCD, PDE4D stock ID# 034588-UCD) of the University of California at Davis. PDE4C knockout mice ($Pde4c^{tm1.1(KOMP)Wtsi/J}$) were generated by the National Institutes of Health (NIH) Knockout Mouse Program (KOMP; www.komp.org) and kindly distributed *via* the KOMP repository at the University of California at Davis. Please find a description of the PDE4C knockout mouse here[17]; additional details are available on the website of the Mutant Mouse Regional Resource Centers (MMRRC; http://www.mmrrc.org; Stock number 049025-UCD). Mice carrying the ΔF508-CFTR ($Cftr^{tm1Kth}$) mutation, that is common among cystic fibrosis patients, were generated by Dr. Kirk R. Thomas (University of Utah) and kindly distributed *via* The Jackson Laboratory (stock 002515 | ΔF; Bar Harbor, ME, USA). To alleviate their established phenotype of lethality resulting from bowel obstructions, the ΔF508-CFTR colony was maintained on laxative

(50% Golytely; Braintree Laboratories, Braintree, MA, USA) in the drinking water. All mice were maintained on a C57BL/6 background and group housed four mice per cage with *ad libitum* access to food and water in a temperature-controlled (22–23°C) vivarium with a 12-h light/dark cycle. Adult mice ≥10 weeks of age and ≥18 g of body weight were used for experimentation by equally and randomly dividing cage littermates into experimental groups. Unless indicated otherwise, experiments were performed using male mice. Experimenters were blinded to the identity of the injected drugs until data acquisition and analyses were completed. All experiments and procedures were conducted in accordance with the guidelines described in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA) and were approved by the University of South Alabama Institutional Animal Care and Use Committee. For euthanasia, animals were injected i.p. with EUTHASOL® Euthanasia Solution (Patterson Veterinary, Greeley, CO, USA) followed by cervical dislocation.

Scoring salivation in awake mice

An experimenter, blinded to drug treatments or genotypes, scored the mice three times at 10, 20, and 30 min after drug injection as either “normal/not salivating = 0” or “abnormal/increased salivation=1”. For each animal, the number/sum of positive “1” scores out of the three performed is reported.

Measurement of saliva secretion rate

Saliva secretion was measured as reported previously with minor modifications[18]. In short, after induction of anesthesia using Ketamine/Xylazine (80 and 10 mg/kg in PBS; i.p.), mice were placed on their sides, their mouths were wiped out with tissue, and narrow, pre-weighed strips of filter paper (4 mm × 20 mm) were placed 7 mm deep into their downward-facing cheek to absorb saliva (see Fig. 3A). The filter papers are replaced every 10 min for a total of 60 min and saliva production is calculated as the increase in weight of the paper strips before and after placement in the mouth of the mice. Two different protocols were applied to evaluate the effect of PDE4 inhibitor treatment on saliva production in anesthetized mice. In the short protocol (see scheme in Fig. 3B), PDE4 inhibitors or solvent control are injected (i.p.), and measurement of saliva production is initiated as soon as the mice lose consciousness (3–5 min) after Ketamine/Xylazine administration. In the longer protocol (see Fig. 3C), test drugs (e.g. PDE4 inhibitor, Isoprenaline, Pilocarpine) are injected 15 min after administration of Ketamine/Xylazine. For several data sets, both time courses of cumulative saliva production as well as the total amount of saliva produced in 60 min is reported. Saliva production after treatment with high-dose Pilocarpine (1 mg/kg) is so substantial, that filter papers are soaked past the 7 mm depth within 2–5 min. Thus, for Pilocarpine only, filter papers were replaced once or twice within a given 10 min time period as needed. If animals started to recover from anesthesia during the 60 min time course of the experiment (as detected by independent limb or head movements), additional doses of 25% of the original volume of Ketamine/Xylazine (80 and 10 mg/kg in PBS; i.p.) were administered to maintain anesthesia. PDE inhibitors were generally administered at a dose of 1 mg/kg (i.p.), which has been shown to produce ≥50% of maximal efficacy on a variety of acute phenotypes of PDE4 inhibition in mice[17, 19, 20], and thus likely reflects ≥50% target engagement.

Measurement of cAMP-PDE activity in salivary glands

Salivary glands were extracted from mice, flash-frozen in liquid nitrogen and stored at −80°C until processing. Tissues were then homogenized in buffer containing 20 mM HEPES (pH 7.4), 1 mM EDTA, 0.2 mM EGTA, 150 mM NaCl, 20% sucrose, HaltTM Protease & Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford, IL) and 1% Triton X-100 using a Dounce glass homogenizer. After a 30-min rotation at 4°C, cell debris was pelleted with a 10-min centrifugation at 20,000 g at 4°C, and soluble extracts were then subjected to cAMP-PDE activity assays following a protocol described previously[21] with minor modifications. In brief, samples were assayed in a reaction mixture of 200 μl containing 40 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1.34 mM β-mercaptoethanol, 1 μM cAMP, and 0.1 μCi [^{3}H]cAMP (Perkin Elmer, Waltham, MA) for 10 min at 37°C followed by heat inactivation in a boiling water bath for 1 min. The PDE reaction product 5′-AMP was then hydrolyzed by incubation of the assay mixture with 50 μg *Crotalus atrox* snake venom (Sigma-Aldrich, St. Louis, MO) for 20 min at 37°C and the resulting adenosine was afterwards separated by anion exchange chromatography on 1 ml of AG1-X8 resin (Bio-Rad Laboratories, Hercules, CA) and quantitated by scintillation counting. PDE4 activity was defined as the fraction of total cAMP-PDE activity inhibited by 10 μM of the archetypal PDE4 inhibitor Rolipram compared to solvent control (final concentration of 1% DMSO in the assay reaction).

Data and Statistical Analysis

All data are expressed as the mean ± SEM and n numbers indicate the number of individual animals assessed and are represented by individual dots in the scatter plots. The GraphPad Prism 8.3 software (GraphPad Software Inc, San Diego, CA, USA) was used to perform statistical analyses. Mann-Whitney test with 95% confidence interval was used to compare two treatment groups and Kruskal-Wallis followed by Dunn's *post hoc* test was used to determine differences between more than two treatment groups. Time courses were analyzed using two-way ANOVA with Bonferroni's post hoc test. Statistical differences are indicated as # (not significant; $p>0.05$), * ($p<0.05$), ** ($p<0.01$), and *** ($p<0.001$).

RESULTS

Treatment with PAN-PDE4 inhibitors induces salivation in awake/conscious mice.

While performing intratracheal infections, we noted that mice pretreated with PDE4 inhibitors exhibited an unusual, elevated salivation (see representative images in Fig. 1A/B). The effect was replicated by several, structurally-distinct PAN-PDE4 inhibitors including Rolipram, Roflumilast, Piclamilast/RP73401, and RS25344 (all at 1 mg/kg; i.p.), as scored by an experimenter blinded to drug treatments at 10, 20, and 30 min after drug injection (Fig. 1C). Conversely, treatment with the PDE3-inhibitor Cilostamide had no effect (Fig. 1C). Together, these data suggest that increased salivation is a class-effect of PAN-selective PDE4 inhibition in mice. It is well established that activation of M_3-muscarinic acetylcholine receptors (M_3-AChR) or β-adrenoceptors may induce salivation *via* central effects and/or by acting directly on salivary gland cells. Indeed, treatment with the M_3-mAChR agonist Pilocarpine induced visible salivation in the mice that was blunted by treatment with the muscarinic acetylcholine receptor (mAChR) antagonist Atropine, whereas

the β-adrenoceptor agonist Isoprenaline induced salivation that was sensitive to treatment with the β-blocker Propranolol (Fig. 1D). Intriguingly, salivation induced by treatment with two structurally-distinct PDE4 inhibitors, Rolipram and RS25344 (see formulas in Fig. 1C), was ablated by blockade of either β-adrenoceptor signaling or mAChR signaling (Fig. 1E), suggesting that PDE4 inhibitor-induced salivation requires both receptor pathways.

In mice and other mammals, saliva is produced by three main glands, the submandibular gland (SMG), the sublingual gland (SLG), and the parotid gland (PG), as well as a multitude of smaller glands[1]. To assess the relative expression of PDE4 in these tissues, we measured cAMP-PDE activity in detergent extracts prepared from these glands in the presence or absence of the PDE4 inhibitor Rolipram (10 μM) *in vitro*. As shown in Fig. 2, PDE4 activity, defined as the fraction of cAMP-PDE activity inhibited by the archetypal PDE4 inhibitor Rolipram, contributes a major portion of total cAMP-PDE activity in each of these glands. This indicates that PDE4 inhibitor-induced salivation may result, at least in part, from direct action of PDE4 inhibitors in the gland cells, rather than exclusively from indirect effects, such as from PDE4 inhibition in sensory- or central nervous system components of salivary regulation.

In anesthetized mice, PDE4 inhibition potentiates saliva production induced by β-adrenoceptor-, but not muscarinic receptor stimulation.

We next aimed to quantify glandular saliva production in response to PDE4 inhibitor treatment. To this end, mice were anesthetized using Ketamine/Xylazine and saliva production was assessed by the weight increase of thin filter paper strips that were placed in the mouths of mice for 10 min at a time (see representative image in Fig. 3A). If PDE4 inhibitors were injected as soon as the animal was unconscious (3–5 min after Ketamine/Xylazine; see timeline in Fig. 3B), highly variable rates of saliva production were observed (Fig. 3D and two left bars of Fig. 3E). In some mice, PDE4 inhibition hardly induced any saliva production over solvent controls, whereas in others, a substantial saliva production was induced. Conversely, if PDE4 inhibitor administration was delayed until 15 min after induction of anesthesia (see timeline in Fig. 3C), responses were more consistent, but PDE4 inhibition produced only a miniscule increase in saliva production over solvent controls (two right bars in Fig. 3E). Our interpretation of this finding is that inhibition of neuronal activity during deep anesthesia ablates a neurotransmitter signal that is required for, and that is amplified by PDE4 inhibition in awake mice. Given that blockade of β-adrenoceptor- or mAChR-signaling ablated PDE4 inhibitor-induced salivation in awake mice, we thus further explored these two pathways.

Treatment with the β-agonist Isoprenaline (Iso) dose-dependently induced salivation in deeply anesthetized mice that plateaued at ~4 mg/g/h (Fig. 4A and Supplementary Fig. 1A). In the presence of a low dose of Isoprenaline (0.01 mg/kg), which by itself did not induce significant salivation, co-treatment with the PDE4 inhibitor Rolipram produced a substantial potentiation of saliva production in a dose-dependent manner (Fig. 4B and Supplementary Fig. 1B). Potentiation of β-agonist-dependent salivation in mice was replicated by distinct PDE4 inhibitors, including Roflumilast, Piclamilast, or RS25344, confirming it as a class effect of PAN-PDE4 inhibitors (Fig. 4C). Moreover, β-agonist-dependent salivation in

anesthetized mice was ablated by treatment with the β-blocker Propranolol, whereas blockade of mAChR signaling with Atropine had no effect (Fig. 4D and Supplementary Figs. 1C/D). Treatment with the M_3-mAChR agonist Pilocarpine produced substantial saliva production in Ketamine/Xylazine-anesthetized mice, that was ablated by Atropine (Fig. 4E and Supplementary Fig. 1E). However, contrary to β-adrenergic stimulation, salivation induced by a low/submaximal dose of Pilocarpine was not further enhanced by co-treatment with the PDE4 inhibitor Rolipram (Fig. 4F). Taken together, these data suggest that under conditions of neuronal depression induced by anesthesia, direct inhibition of PDE4 in the salivary glands does not produce significant salivation by itself (Fig. 3E), but potently enhances salivation induced by minimal activation of receptors that lead to increased cAMP production (e.g. β-adrenoceptors) (Fig. 4C). Conversely, salivation induced by mACh receptors that couple to G_q and intracellular calcium release (e.g. M_3-AChR) is not potentiated by PDE4 inhibition in anesthetized animals (Fig. 4F).

Prior reports have suggested some sex-differences in the amount of saliva produced in mice[22]. While male mice were primarily used throughout this study, we have repeated critical findings using female C57BL/6 mice and observed a similar pattern of spontaneous salivation after PDE4 inhibitor treatment in awake mice (Supplementary Fig. 2A), and a substantial potentiation of salivation in the presence of low-dose Isoprenaline in anesthetized mice (Supplementary Fig. 2B/C).

PDE4 inhibitor-induced salivation is CFTR-dependent.

Prior reports indicated that β-adrenoceptor-dependent salivation in mice is dependent upon, and proportional to cystic fibrosis transmembrane conductance regulator (CFTR) function[18, 22]. Thus, the cAMP/PKA-mediated activation of CFTR current likely gates β-adrenoceptor-induced salivation. To assess whether PDE4 inhibitor-induced salivation is dependent upon functional CFTR, and may reflect a cAMP/PKA-mediated activation of the channel, we explored salivation in mice homozygous for the common cystic fibrosis mutation ΔF508-CFTR, which do not express functional CFTR. As shown in Fig. 5A, PDE4-inhibitor induced salivation observed in awake wild-type mice was significantly reduced in homozygous ΔF508-CFTR mice. In addition, anesthetized homozygous ΔF508-CFTR mice did not produce any measurable saliva levels in response to β-adrenergic stimulation with Isoprenaline, nor to co-treatment with Isoprenaline and the PDE4 inhibitor Rolipram, compared to heterozygous mice (Fig. 5B and Supplementary Fig. 3). Conversely, treatment with the M_3-agonist Pilocarpine induced comparable levels of saliva production in both heterozygous and homozygous ΔF508-CFTR mice (Fig. 5B and Supplementary Fig. 3C). These data indicate that potentiation of the cAMP/PKA-mediated activation of CFTR is one mechanism whereby PDE4 inhibition induces salivation.

PDE4 inhibitor-induced salivation results from the concurrent inactivation of multiple PDE4 subtypes.

The PDE4 family comprises four subtypes, PDE4A, PDE4B, PDE4C, and PDE4D, and each have been shown to play unique roles in the body. To discern whether one of the four PDE4 subtypes exerts a predominant role in the regulation of salivation, we assessed salivation in systemic/global PDE4-KO mice for each of the four PDE4 subtypes. Visual scoring of the

mice revealed that none of the four PDE4-KO mouse lines replicated the abnormal salivation induced by treatment with PAN-PDE4 inhibitors (no positive salivation scores in n=8 for each line). Measurement of saliva production in anesthetized mice produced a similar pattern. In the presence of low-dose Isoprenaline, none of the four PDE4KO mouse lines replicated the substantial amount of saliva produced by injection with a PDE4 inhibitor (Rolipram; 1 mg/kg), nor did ablation of any PDE4 subtype protect the animals from salivation induced by treatment with the PDE4 inhibitor Rolipram (Fig. 6A). That none of the PDE4KO mouse lines replicates the amount of saliva produced by injection of a PAN-PDE4 inhibitor, nor is protected from the effect of PDE4 inhibitor, suggests that PDE4 inhibitor-induced salivation results from the concurrent inhibition of multiple (at least two) PDE4 subtypes. Saliva production in PDE4D-KO mice, while clearly minor compared to the effect of PAN-PDE4 inhibitor treatment, trended to be higher compared to wild-type controls in mice treated with 0.01 mg/kg Iso, and was significantly increased over wild-type controls in mice treated with the higher dose of 0.04 mg/kg Iso (Fig. 6B). These data suggest that PDE4D, while not the sole PDE4 subtype involved, is likely the primary, or at least one of the PDE4 subtypes involved in the direct regulation of saliva secretion in the salivary glands of mice.

DISCUSSION

Inducing salivation is a class effect of PAN-PDE4 inhibitors in mice.

We report here that inhibition of PDE4, but not inhibition of PDE3, stimulates elevated saliva production in mice, as detected by observational scoring (Fig. 1A–C) or by measuring saliva secretion rates in anesthetized mice (Fig. 4C). Salivation is dose-dependently induced by the archetypal PDE4 inhibitor Rolipram (Fig. 4B) as well as several other, structurally-distinct PAN-PDE4 inhibitors, including Roflumilast, Piclamilast or RS25344 (all 1 mg/kg i.p.; Figs. 1C and 4C), suggesting that salivation is a class-effect of PDE4 inhibition in mice. While not mechanistically explored, salivation has been noted previously during toxicity studies of PDE4 inhibitors in rats[23], dogs[24], as well as monkeys[25, 26], suggesting the effect may be conserved among mammalian species. Thus, if it extends to humans, our findings may hint at a therapeutic potential of PDE4 inhibition for conditions associated with salivary gland hypofunction and xerostomia.

The PDE4 family comprises four subtypes, PDE4A to PDE4D, that each have been shown to exert distinct and non-overlapping physiological and pathophysiological roles[7, 16]. We show here that selective ablation of any of the four individual PDE4 subtypes in mice does not replicate the substantial salivation observed by PAN-PDE4 inhibitor treatment, suggesting that inactivation of several (at least two) distinct PDE4 subtypes is required for maximal stimulation of salivation (Fig. 6A). While significantly less efficacious than PAN-PDE4 inhibition, genetic deletion of PDE4D in mice does significantly increase saliva production compared to wild-type littermates (Fig. 6A/B), suggesting that PDE4D is one of the PDE4 subtypes that affect salivation; perhaps even the most critical one.

Individual PDE4 subtypes are distinguished by a multitude of unique post-translation regulations as well as the differential recruitment in distinct subcellular compartments and macromolecular signaling complexes. As a result, individual PDE4 subtypes mediate

distinct functions even if expressed in the same cell. Thus, PDE4 inhibition may induce salivation by inhibiting several (or the same) PDE4 subtype(s) located in distinct cell types, and inactivation producing salivation *via* physiologic additivity or synergism. But salivation may also be produced by inactivation of several PDE4 subtypes in the same cell, thereby leading to additive/synergistic effects in elevating intracellular cAMP/PKA signaling in salivary gland cells.

Given that the expression of several PDE4 isoforms has been shown to be cAMP responsive[27–29], it is theoretically possible that the actual effect/impact of ablating one of the four PDE4 subtypes on salivary secretion (see Fig. 6) may be partly obscured by compensatory changes in expression of other PDE4 isoforms. We consider this less likely given the large body of data suggesting that PDEs are functionally not interchangeable, and because such compensatory changes in PDE4 expression were not observed in other primary cells or tissues of PDE4-KO mice[16]. Nevertheless, the possibility of compensatory changes in PDE isoform expression or activity in the salivary glands of the different PDE4-KO mouse lines remains to be explored in future studies.

Development of subtype-selective PDE4 inhibitors is a promising approach to retain the many therapeutically beneficial effects of the PAN-PDE4 inhibitors available to date (e.g. anti-inflammatory, memory/cognition-enhancing) while alleviating their common side effects, mainly nausea and emesis. Thus, PDE4D should be the primary target for future development of subtype-selective PDE4 inhibitors to induce salivation.

Given a lack of highly subtype-selective PDE4 inhibitors, it remains unclear which PDE4 subtypes mediate the adverse effects of PAN-PDE4 inhibitors in humans. A prior report revealed that genetic ablation of PDE4D shortens the duration of Ketamine/Xylazine-induced anesthesia in mice[30], thus replicating the effects of PAN-PDE4 inhibitors in this paradigm, which has been proposed as a correlate of nausea and emesis in species that are anatomically unable to vomit (e.g. mice/rats). If this model were an accurate predictor of the role of PDE4D in mediating the adverse effect of PAN-PDE4 inhibitors, it would be challenging to derive any therapeutic benefits from the inhibition of PDE4D, including the mitigation of hyposalivation. However, while the Ketamine/Xylazine-anesthesia test is a reliable measure of α_2-adrenoreceptor antagonism, it does have limitations as a predictor of emetic potential as reviewed recently[31]. Moreover, at least one study has shown that inhibitors with some selectivity for PDE4D exhibited reduced vomiting compared to PAN-PDE4 inhibitors in several species[32]. Finally, using gastric retention as an alternative correlate of nausea and emesis in mice, we recently suggested that it may be the concurrent inhibition of multiple PDE4 subtypes that is responsible for the adverse effects of PAN-PDE4 inhibitors[17]. Thus, in our opinion, the association of individual PDE4 subtypes with emesis and/or nausea remains inconclusive.

Distinguishing central and direct effects of PDE4 inhibition on salivation.

While inhibition of PDE4 promotes salivation in both awake (Fig. 1) and anesthetized mice (Fig. 4), there are some differences in the molecular mechanisms involved. First, in awake mice, treatment with PDE4 inhibitors induced substantial salivation by itself (Fig. 1A–C), whereas in deeply anesthetized mice, salivation induced by PDE4 inhibition *per se* is minor

(see Fig. 3E) and inducing substantial levels of PDE4 inhibitor-induced salivation requires priming with low doses of a β-agonist (Fig. 4B/C). Second, PDE4 inhibitor-induced salivation in awake mice is dependent upon both muscarinic- and β-adrenergic signaling, given it is blocked by either Atropine or Propranolol (Fig. 1E), whereas PDE4 inhibition potentiates β-adrenoceptor-dependent salivation in anesthetized mice, but has no effect on M_3-dependent salivation (Fig. 4D). Given that anesthesia principally involves the inhibition of neuronal activity, it is tempting to speculate that PDE4 inhibitor-induced salivation in anesthetized mice results directly from PDE4 inhibition in salivary glands and not from effects on neuronal regulation of salivation (see scheme in Fig. 7). The fact that PDE4 contributes the major portion of total cAMP-hydrolytic capacity in all three major salivary glands (Fig. 2) supports this notion. Indeed, a prior study has shown that inhibition of PDE4 promoted the release of amylase from parotid acinar cells in culture[33], which is consistent with the notion that β-adrenoceptor- and cAMP-dependent saliva secretions are rich in protein[1, 2]. Conversely, we propose that in awake mice, PDE4 inhibition may induce salivation *via* additional, central/neuronal regulations, in line with the high expression of PDE4 in brain[34] and the fact that PDE4 inhibition has been shown to affect other autonomic nervous system regulations including control of body temperature or gastrointestinal motility[17, 20]. In an intriguing parallel, although the most widely used drug to induce salivation, Pilocarpine, can induce salivation by acting directly on the salivary glands (see Fig. 4E and Fig. 5B), the drug may yet mediate its pronounced effect on salivation largely *via* central mechanisms. This is supported by the observation that intracerebroventricular (i.c.v) injection of Pilocarpine induces salivation in rats[35, 36], and that *vice versa*, lesions in the medial preoptic area and the lateral hypothalamus, or i.c.v.-injection of muscarinic blocker Atropine, impair salivation in response to peripheral Pilocarpine[36–39]. Curiously, central α_2-adrenoceptor agonism has been shown to inhibit the central actions of Pilocarpine on salivation[40–42]. Given that PAN-PDE4 inhibitors act as potent, physiologic α_2-adrenoceptor blockers in mammals[30, 43, 44], it is possible that in awake mice, PDE4 inhibition mediates salivation by releasing a central, α_2-adrenoceptor-dependent inhibition of muscarinic signaling, which may explain why Atropine is effective in ablating PDE4 inhibitor-induced salivation in awake mice (Fig. 1E).

While we show that inhibition of PDE4 in anesthetized mice does not potentiate salivation induced by muscarinic receptor activation with low-dose pilocarpine (Fig. 4F), this does not principally exclude a potential role for PDE4 in regulating salivation induced by other secretagogues that act *via* activation of G_q-coupled receptors and intracellular calcium signaling, given the multitude of cAMP-signaling events known to regulate receptor- and/or ion channel functions. Furthermore, and in light of the observation that muscarinic stimulation has been shown to increase cGMP signaling in rabbit parotid acinar cells[45], the role of PDE families other than PDE3 and PDE4 (Fig. 1C) as well as the role of cGMP signaling in the regulation of saliva secretion remain to be explored.

PDE4, CFTR, and Cystic Fibrosis.

β-agonist-induced saliva production in mice has been shown to be gated by CFTR in the salivary glands and be dependent upon both the expression level and the cAMP/PKA-mediated activation of the channel[18, 22] (Fig. 7). The rate of β-adrenoceptor-induced saliva

secretion has thus been proposed as a suitable *in vivo* correlate of CFTR function in mice, given that mice do not sweat, and the classical sweat-chloride test that is used to assess CFTR function in humans, cannot be applied. On this basis, our study is also the first report that inhibition of PDE4, but not inhibition of PDE3, stimulates CFTR function in an *in vivo* model (Fig. 5). The critical role of PDE4 in regulating CFTR function in salivary glands aligns with prior reports identifying PDE4 as the predominant PDE family regulating CFTR function in airway, intestinal, and renal epithelial cells[46–50]. Activation of PDE4 using small-molecule allosteric activators has thus been proposed as a therapeutic approach to alleviate the cAMP/PKA-mediated hyperactivation of CFTR and its associated cyst formation in models of autosomal dominant polycystic kidney disease (ADPKD)[50]. Conversely, inhibition of PDE4, which is widely expressed throughout the cells of the bronchi and lung parenchyma[51], has been shown to activate CFTR in non-CF as well as ΔF508-CFTR human bronchial airway epithelial cells in culture[46], thus confirming PDE4 as a promising target to potentiate the effects of CFTR correctors and potentiators to restore CFTR function in CF patients. In this context, it is worth noting that several prior reports suggested a predominant role for the PDE4 subtype PDE4D in controlling CFTR activity in both airway and intestinal epithelial cells[46, 47, 49, 52]. In line with our finding of a pre-eminent, but not exclusive role of PDE4D in controlling β-adrenoceptor- and CFTR-dependent salivation (Figs. 5 and 7), these data suggest that PDE4D may serve a conserved role in controlling CFTR function across various cells and tissues throughout the body, and that targeting PDE4D may thus serve to alleviate CFTR hypofunction.

Targeting PDE4 in settings of salivary gland hypofunction.

There remains a critical need for effective treatments for salivary gland hypofunction, particularly for patients presenting with severe symptoms such as in Sjögren's syndrome. While the parasympathomimetics Pilocarpine and Cevimeline are widely prescribed, their use can be limited by significant side effects resulting directly from their muscarinic agonism, and they certainly cannot be prescribed if xerostomia is the result of anti-cholinergic medications to begin with[1, 2]. Thus, inhibition of PDE4/PDE4D may represent a novel therapeutic approach for xerostomia, particularly since in awake mice, inhibition of PDE4 in the brain (Fig. 1E) and inhibition of PDE4 in salivary glands (Fig. 3) appear to synergize in the induction of salivation (Fig. 7). In addition, while not measured here, the composition of saliva produced by muscarinic activation or PDE4 inhibition is expected to be distinct, and the effects of both treatments may thus be complementary. Muscarinic activation (e.g. with Pilocarpine) is well-known to produce large volumes of watery saliva with low protein content, thus principally ameliorating hydration of the oral mucosa. Conversely, saliva produced upon β-adrenergic stimulation (and thus likely also upon PDE4/PDE4D inhibition) is high in protein content and may be better suited to ameliorate deficits in food digestion or innate defense that are associated with salivary gland hypofunction. Indeed, a prior report has shown that PDE4 inhibition potentiates the β-adrenoceptor-dependent release of amylase from parotid glands *in vitro*[33]. Moreover, underlining the significance of saliva produced upon β-adrenergic-, cAMP- and CFTR-dependent salivation, an increased incidence of dental caries has been reported in ΔF508-CFTR cystic fibrosis mice, which lack functional CFTR[53]. Finally, although the causes of salivary gland dysfunction are varied, there is often an inflammatory component. This is apparent in

Sjögren's syndrome, an autoimmune disease, but also in salivary gland dysfunction caused by infections, diabetes or ageing. Targeting PDE4, particularly the PDE4 subtypes PDE4B and PDE4D[54–57], is well established to exert broad-spectrum anti-inflammatory properties and to dampen both innate and adaptive immune responses. Therefore, in addition to stimulating salivary secretions, thus alleviating the symptoms of salivary gland hypofunction, PDE4 may also exert therapeutic benefits by alleviating the inflammatory responses that cause salivary gland dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

Atr	Atropine
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CNS	central nervous system
DMSO	Dimethyl sulfoxide
i.p.	intraperitoneal
Iso	Isoprenaline
KO	Knockout
PANS	parasympathetic autonomic nervous system
PBS	Phosphate-buffered saline
PDE	cyclic nucleotide phosphodiesterase
PDE4	cAMP phosphodiesterase 4
Pilo	Pilocarpine
Prop	Propranolol
Roli	Rolipram
SANS	Sympathetic autonomic nervous system

WT	Wild-type

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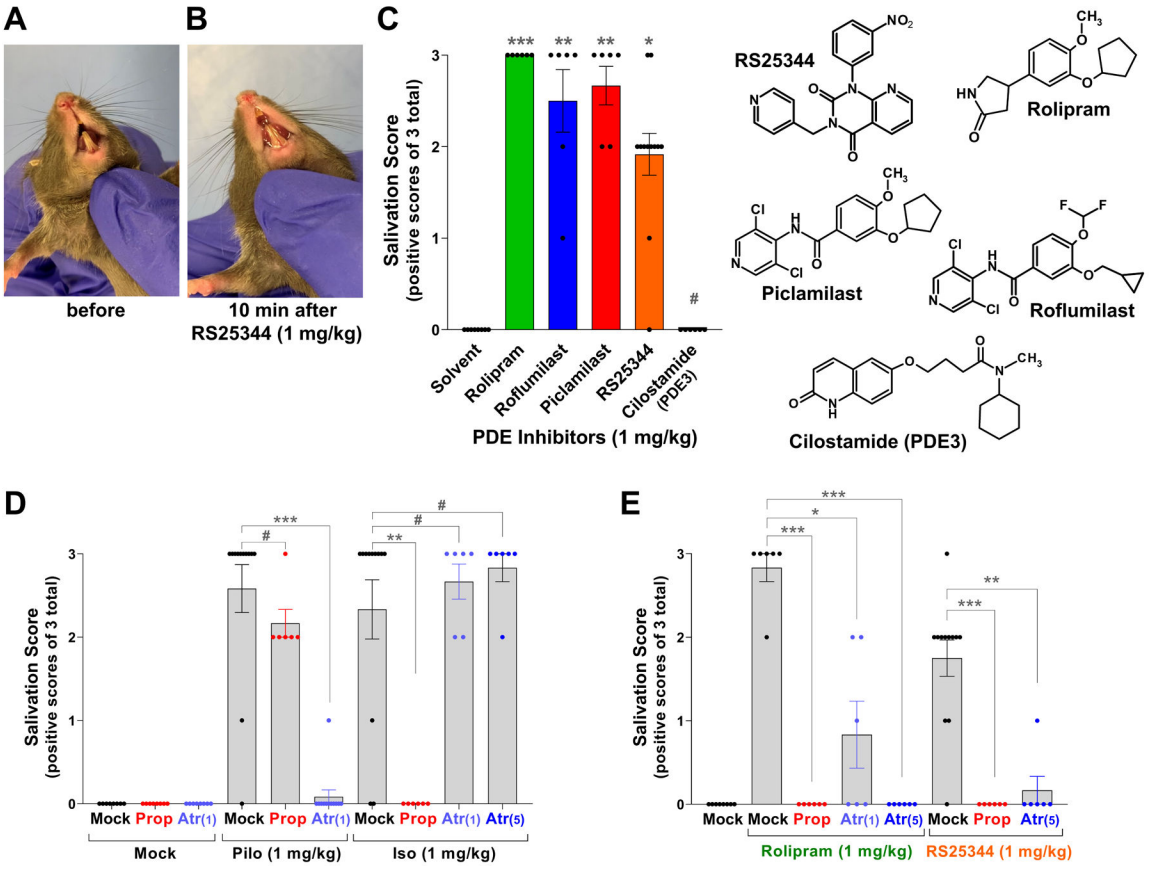

Figure. 1. Salivation is a class effect of PAN-selective PDE4 inhibitors in mice.

Male C57BL/6 mice were injected intraperitoneally (i.p.) with structurally distinct PDE inhibitors, the M_3 muscarinic receptor agonist Pilocarpine (Pilo, 1 mg/kg), the β-adrenoceptor agonist Isoprenaline (Iso, 1 mg/kg) or solvent controls (Mock). The animals were scored at 10, 20, and 30 min after drug injection by an experimenter blinded to the treatments as either "1" elevated/abnormal salivation, or "0" no elevated/abnormal salivation and the sum of the three scores obtained at different times is reported for each mouse. (**A/B**) Representative images of the same mouse shortly before drug injection showing no abnormal salivation (**A**) and at 10 min after treatment with the PDE4 inhibitor RS25344 (1 mg/kg) (**B**) exhibiting excessive drug-induced salivation. (**C**) Mice were injected with the PAN-PDE4 inhibitors Rolipram, Roflumilast, Piclamilast/RP73401, or RS25344 (all at 1 mg/kg, i.p.), the PDE3-selective inhibitor Cilostamide (1 mg/kg), or solvent (Mock) control and salivation was scored three times at 10, 20, and 30 min after drug injection. Treatment with any of the PDE4 inhibitors produced salivation, whereas inhibition of PDE3 did not, revealing salivation as a class-effect of PAN-PDE4 inhibitors. The chemical structures of the PDE inhibitors tested are shown for comparison. (**D/E**) 30 min after pre-treatment with the mAchR blocker Atropine at a dose of 1 mg/kg (Atr(1)) or 5 mg/kg (Atr(5)), the β-adrenoceptor blocker Propranolol (Prop, 5 mg/kg, i.p.) or solvent control (Mock), mice were injected with the M_3 muscarinic receptor (M_3R) agonist Pilocarpine (Pilo, 1 mg/kg) or the β-adrenoceptor agonist Isoprenaline (Iso, 1 mg/kg) (**D**), or the PDE4 inhibitors Rolipram (1 mg/kg) or RS25344 (1 mg/kg) (**E**), and salivation was scored at 10, 20, and 30 min after

drug injection. As expected, salivation induced by M_3R agonism (Pilocarpine) was selectively ablated by a muscarinic blocker, Atropine, but not a βAR blocker, Propranolol. In the same vein, salivation induced by an βAR agonist (Isoprenaline) was selectively ablated by the β-blocker Propranolol, but unaffected by the muscarinic blocker Atropine. Conversely, salivation induced by PDE4 inhibitors Rolipram or RS25344 was ablated by treatment with either Propranolol or Atropine, suggesting that both β-adrenergic and muscarinic signaling converge to mediate PDE4 inhibitor-induced salivation. Data represent the mean ± SEM. In scatter plots, each dot represents a different animal. Statistical significance was determined using Kruskal-Wallis and Dunn's post hoc tests and is indicated as # (not significant; $p>0.05$), * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

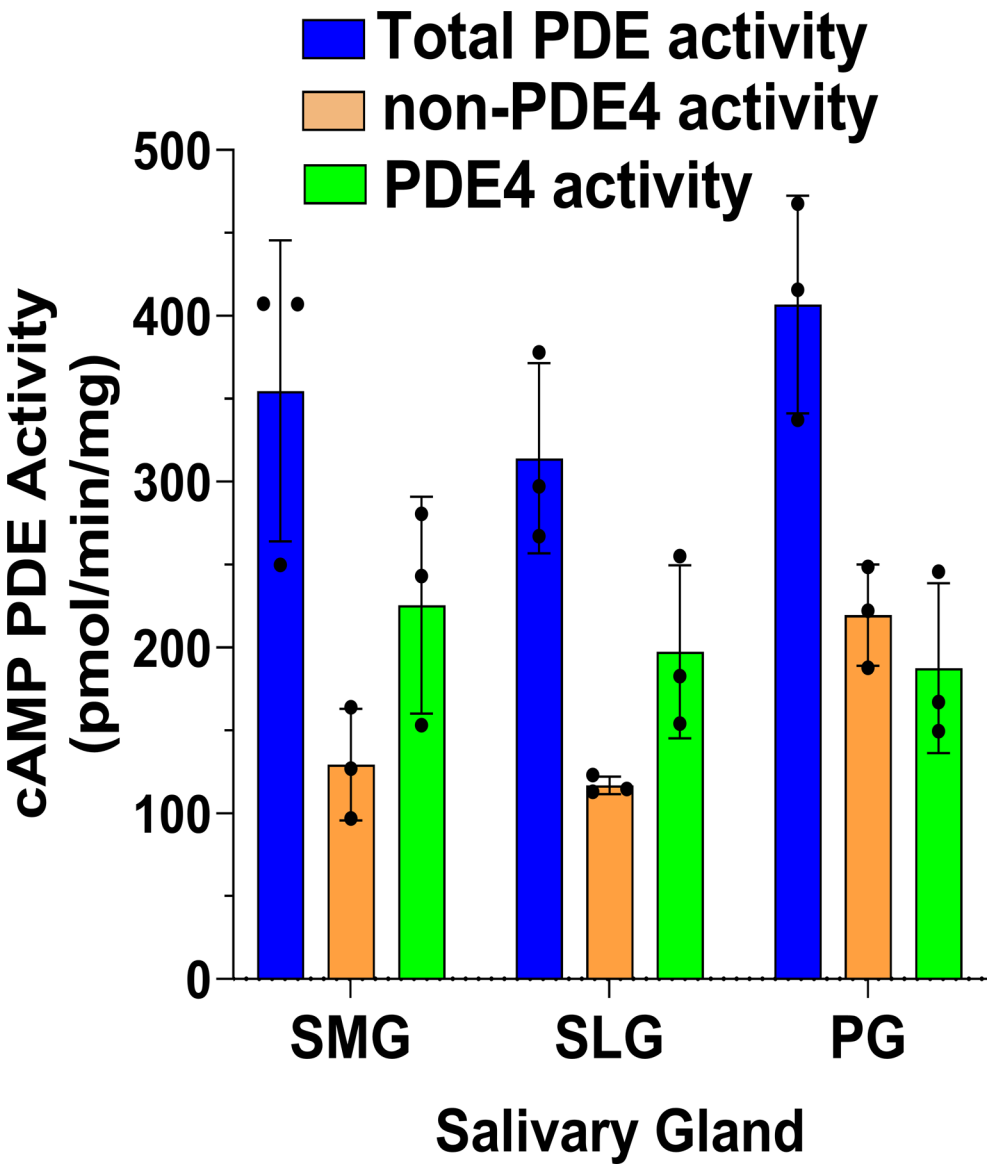

Figure 2. PDE4 contributes the majority of cAMP-PDE activity in mouse salivary glands.
Detergent extracts prepared from submandibular (SMG), sublingual (SLG), and parotid (PG) salivary glands were subjected to *in vitro* cAMP-PDE activity assays in the presence or absence of the PDE4 inhibitor Rolipram (10 μM). Total cAMP-PDE activity is defined as the rate of cAMP hydrolysis measured in the absence of Rolipram, whereas PDE4 and non-PDE4 activity are defined as the fraction of total activity that is either inhibited or that is insensitive to inhibition by Rolipram, respectively. All data represent the mean ± SEM. In the scatter plot, each dot represents a gland isolated from a different animal (n=3).

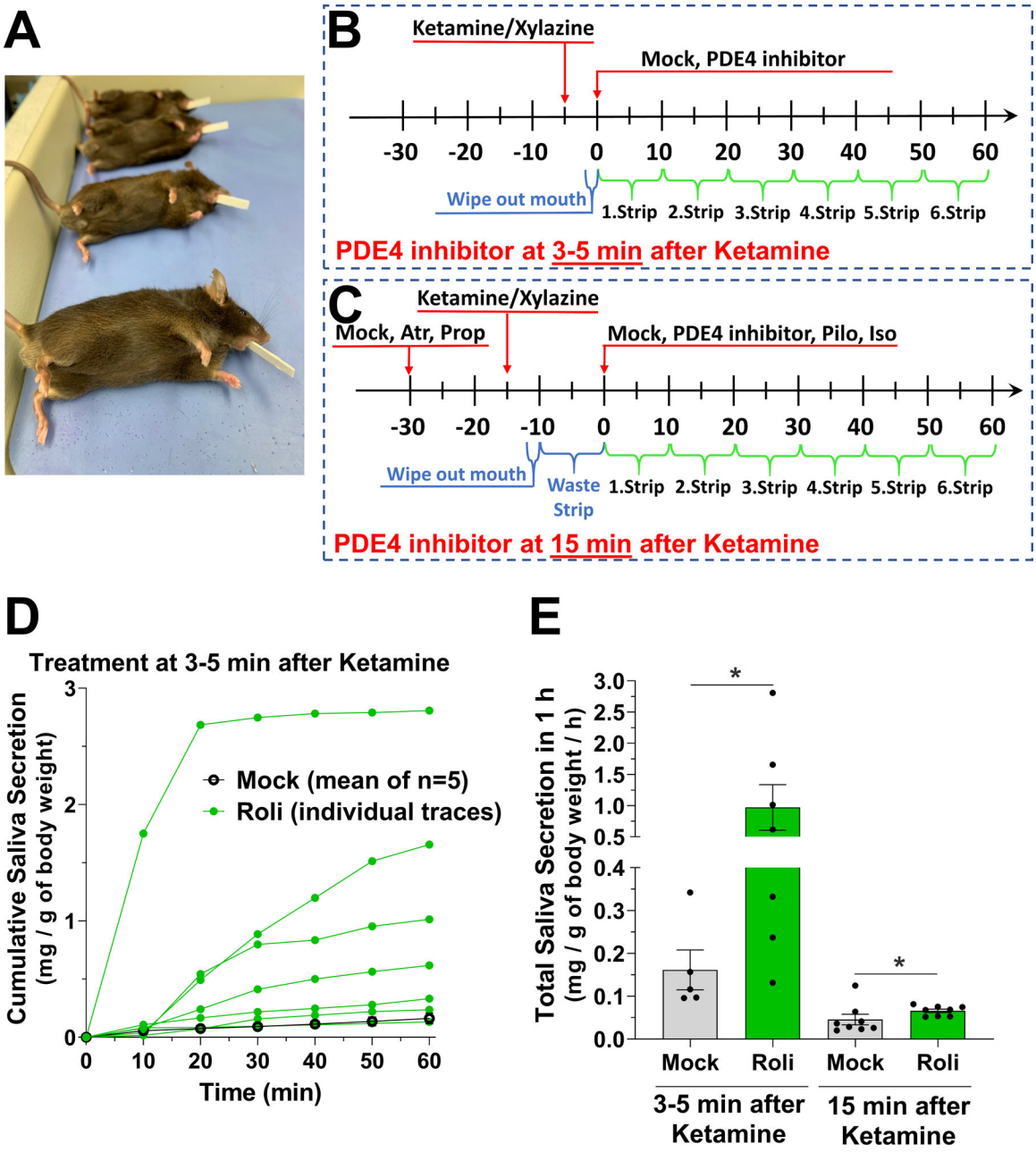

Figure 3. Measurement of saliva production in anesthetized mice.

(**A**) A representative image illustrating the approach to measure saliva production *via* increased weight of pre-weighed filter paper strips. (**B**/**C**) Schematic timelines for the experimental approach of measuring saliva production with PDE4 inhibitor injection following either 3–5 min (**B**) or 15 min (**C**) after induction of Ketamine/Xylazine anesthesia. In the shortened timeline (**B**), mice were put on their sides as soon as they became unconscious (3–5 min after Ketamine/Xylazine), their mouth was wiped out with a piece of tissue to remove baseline saliva, followed by injection of PDE4 inhibitor or solvent control and immediate placement of the first of 6 pre-weighed filter paper strips for measurement of saliva production. Using the longer timeline (**C**), mice were placed on their side at 5 min

after Ketamine/Xylazine, their mouth was wiped out and a filter strip was placed in their mouths for 10 min to absorb baseline saliva. Ten min later, this filter strip was discarded, PDE4 inhibitors or other drugs were injected, followed by placement of the first pre-weighed filter used for measurement of saliva production. (**D**) Mice treated with the PDE4 inhibitor Rolipram (1 mg/kg, i.p.) immediately after achieving anesthetic plane (3–5 min after Ketamine/Xylazine injection; see timeline in (**B**)) exhibit highly variable rates of saliva production over the subsequent hour with some mice producing substantial amounts of saliva, whereas other animals do not exhibit elevated saliva production compared to solvent controls (Mock). (**E**) PDE4 inhibitor-induced saliva production depends on the timing of drug injection. Shown is total saliva production in mice after i.p. injection of the PDE4 inhibitor Rolipram (Roli; 1 mg/kg) or solvent control (Mock) as soon as animals become unconscious after induction of Ketamine/Xylazine anesthesia (3–5 min; left two bars), or if the drugs were injected at 15 min after Ketamine/Xylazine anesthesia. Data represent the mean ± SEM. In scatter plots, each dot represents a different animal. Statistical significance was determined using Mann-Whitney test with 95% confidence interval and is indicated as * ($p<0.05$).

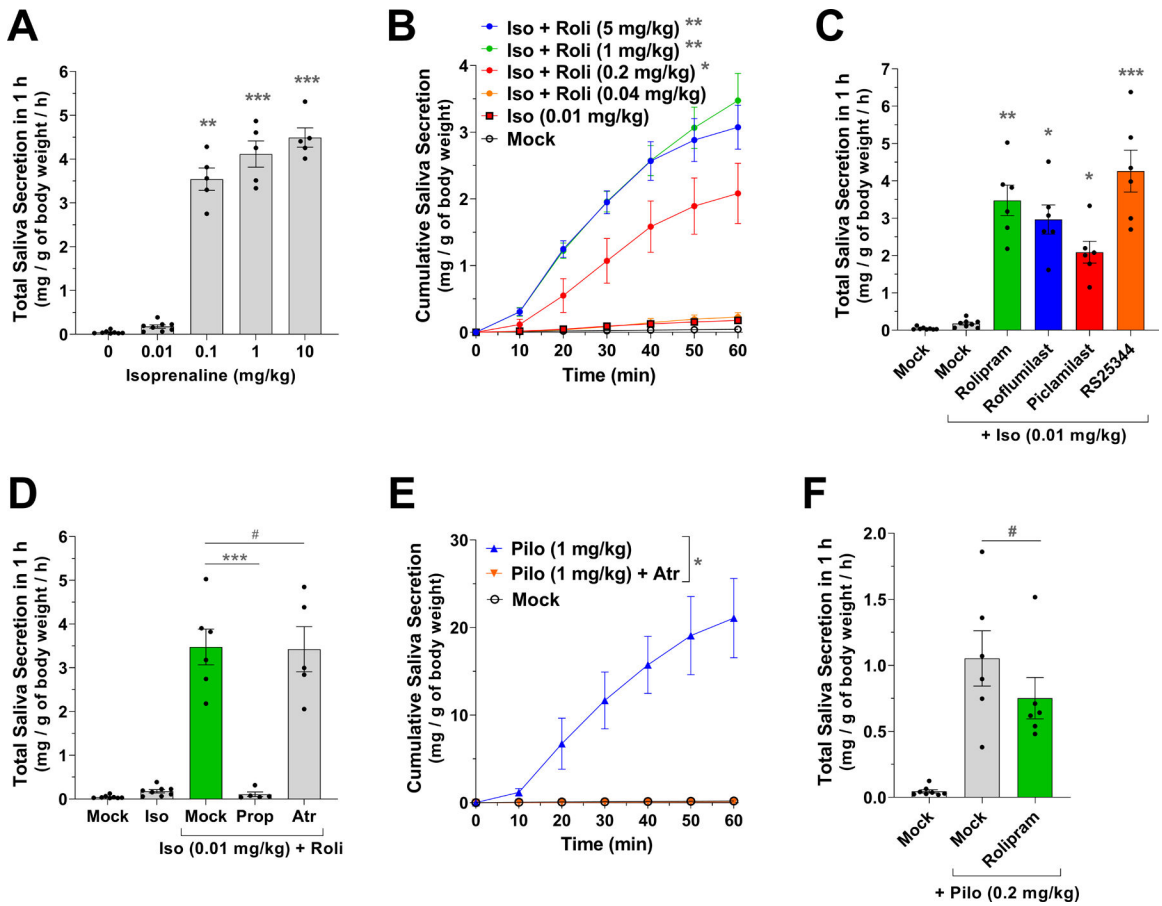

Figure 4. PDE4 inhibition potentiates β-adrenoceptor-dependent salivation in anesthetized mice.

Fifteen minutes after induction of Ketamine/Xylazine-anesthesia, mice were injected i.p. with the indicated drugs and saliva production was measured for the next 60 min. (**A**) Dose-dependent induction of salivation by the β-adrenoceptor agonist Isoprenaline (Iso). (**B**) In the presence of low-dose Isoprenaline (Iso; 0.01 mg/kg), the PDE4 inhibitor Rolipram (Roli; n=6) dose-dependently induces significant saliva production. (**C**) Potentiation of saliva production in the presence of low-dose Iso (0.01 mg/kg) is induced by PDE4 inhibitors Rolipram, Roflumilast, Piclamilast/RP73401, and RS25344 (all 1 mg/kg), suggesting it is a class-effect of PAN-PDE4 inhibitors. (**D**) Pretreatment with the β-blocker Propranolol (Prop; 5 mg/kg), but not the mAChR blocker Atropine (Atr, 5 mg/kg) prevents salivation induced by the PDE4 inhibitor Rolipram (Roli; 1 mg/kg) in the presence of low-dose Iso (0.01 mg/kg). (**E**) The M_3-AChR agonist Pilocarpine (Pilo; 1 mg/kg; n=5) induces substantial salivation that is ablated by pretreatment with the muscarinic antagonist Atropine (Atr; 5 mg/kg; $p<0.001$). (**F**) Co-treatment with the PDE4 inhibitor Rolipram does not enhance saliva production induced by low-dose Pilocarpine (Pilo; 0.2 mg/kg). All data represent the mean ± SEM. In scatter plots, each dot represents a different animal. For total saliva production shown in bar graphs, statistical significance was determined using Kruskal-Wallis and Dunn's post hoc tests; for time courses, two-way ANOVA and Bonferroni's post hoc tests were used. Statistical significance is indicated as # (not significant; $p>0.05$), * ($p<0.05$), ** ($p<0.01$), and *** ($p<0.001$).

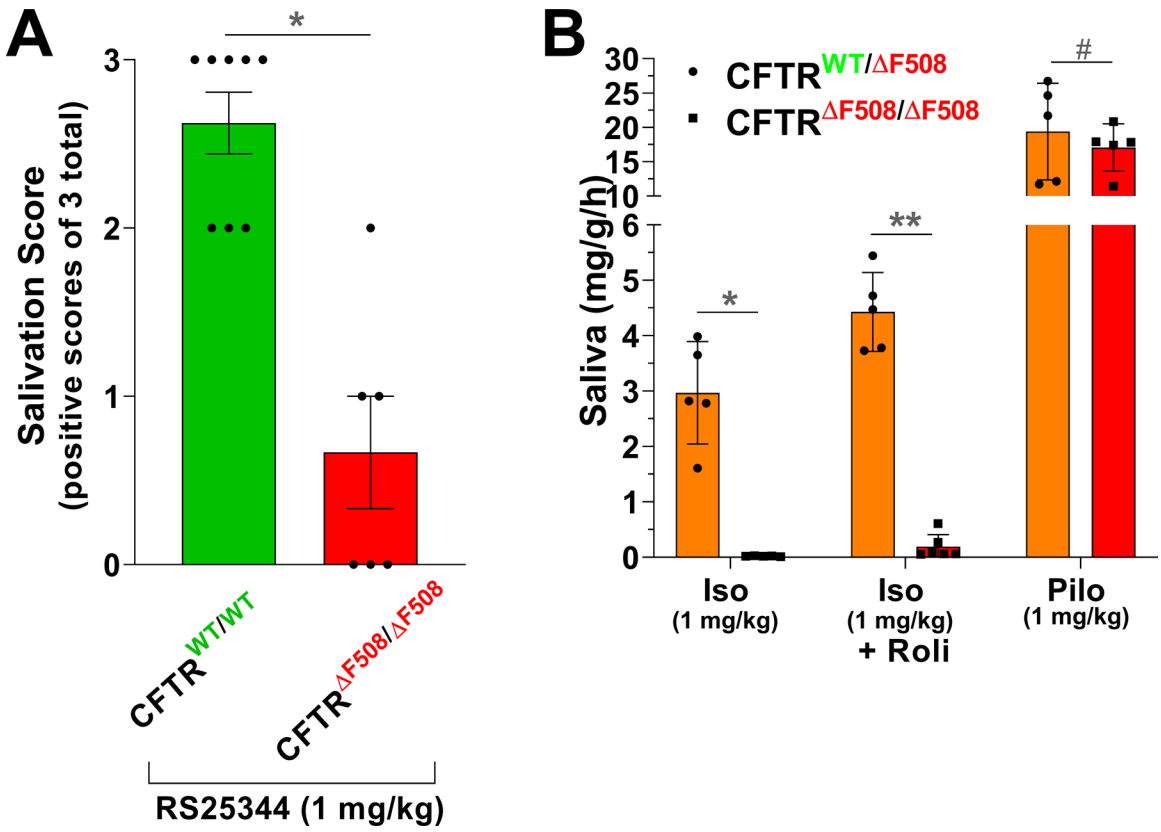

Figure 5. PDE4 inhibitor-induced salivation is CFTR-dependent.

(**A**) Homozygous ΔF508-CFTR mice (CFTR$^{\mathbf{\Delta F508/\Delta F508}}$), which lack functional CFTR, or wild-type controls (CFTR$^{\mathbf{WT/WT}}$) were injected with the PAN-PDE4 inhibitor RS25344 (1 mg/kg; i.p.) and animals were scored at 10, 20, and 30 min after drug injection for abnormal salivation by an experimenter blinded to the treatments. The sum of positive salivation scores for the three scorings performed is reported for each mouse. (**B**) Fifteen minutes after induction of Ketamine/Xylazine-anesthesia, mice were injected with the β-adrenoceptor agonist Isoprenaline (Iso; 1 mg/kg), the PDE4 inhibitor Rolipram (Roli; 1 mg/kg), or the muscarinic receptor agonist Pilocarpine (Pilo; 1 mg/kg) and saliva production was measured for the next 60 min. Saliva production in response to Iso in mice heterozygous for the ΔF508-CFTR mutation (CFTR$^{\mathbf{WT/\Delta F508}}$) was slightly reduced compared to wild-type mice (see Fig. 4A). Conversely, neither Iso by itself, nor combination treatment with Iso and Roli induced significant saliva production in homozygous ΔF508-CFTR mice (CFTR$^{\mathbf{\Delta F508/\Delta F508}}$). However, saliva production in response to the muscarinic agonist Pilocarpine (Pilo; 1 mg/kg) is preserved in homozygous ΔF508-CFTR mice. Data represent the mean ± SEM. In scatter plots, each dot represents a different animal. Statistical significance was determined using Mann-Whitney test with 95% confidence interval (**A**) or Kruskal-Wallis followed by Dunn's post hoc test (**B**) and is indicated as # (not significant; $p>0.05$), * ($p<0.05$), and ** ($p<0.01$).

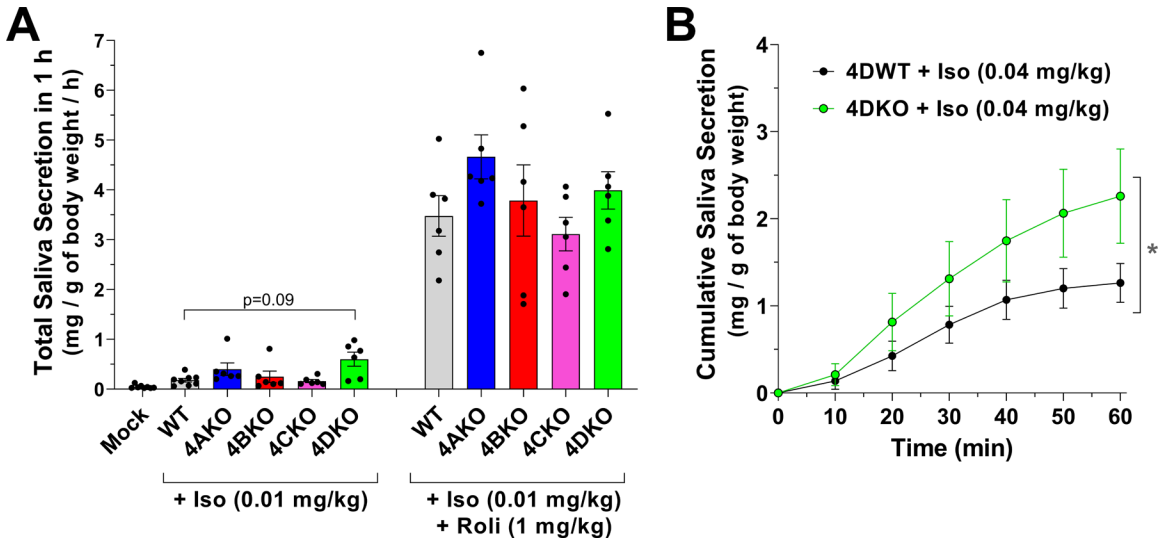

Figure 6. PAN- but not subtype-selective PDE4 inactivation induces salivation.

Fifteen minutes after induction of Ketamine/Xylazine-anesthesia, mice were injected with the PDE4 inhibitor Rolipram (1 mg/kg) and/or the β-adrenoceptor agonist Isoprenaline (Iso; 0.01 mg/kg in (**A**), 0.04 mg/kg in (**B**)) and saliva production was measured for the next 60 min. (**A**) Shown is the total amount of saliva produced within 60 min of drug injection in mice deficient in PDE4A (4AKO), PDE4B (4BKO), PDE4C (4CKO) or PDE4D (4DKO), or in wild-type (WT) controls. There were no significant differences ($p>0.05$) in saliva production between any PDE4-KO mice and wild-type controls after treatment with either Iso or after treatment with Iso+Roli. Conversely, treatment with Iso+Roli produced significantly more saliva than treatment with Iso alone ($p<0.001$) in any of the PDE4-KO lines or in wild-type controls. (**B**) Cumulative saliva secretion in PDE4D-KO mice and wild-type littermates in response to injection of 0.04 mg/kg Iso. All data represent the mean ± SEM. In scatter plots, each dot represents a different animal. For total saliva production shown in bar graphs, statistical significance was determined using Kruskal-Wallis and Dunn's post hoc tests; for time courses, two-way ANOVA and Bonferroni's post hoc tests were used. Statistical significance is indicated as * ($p<0.05$).

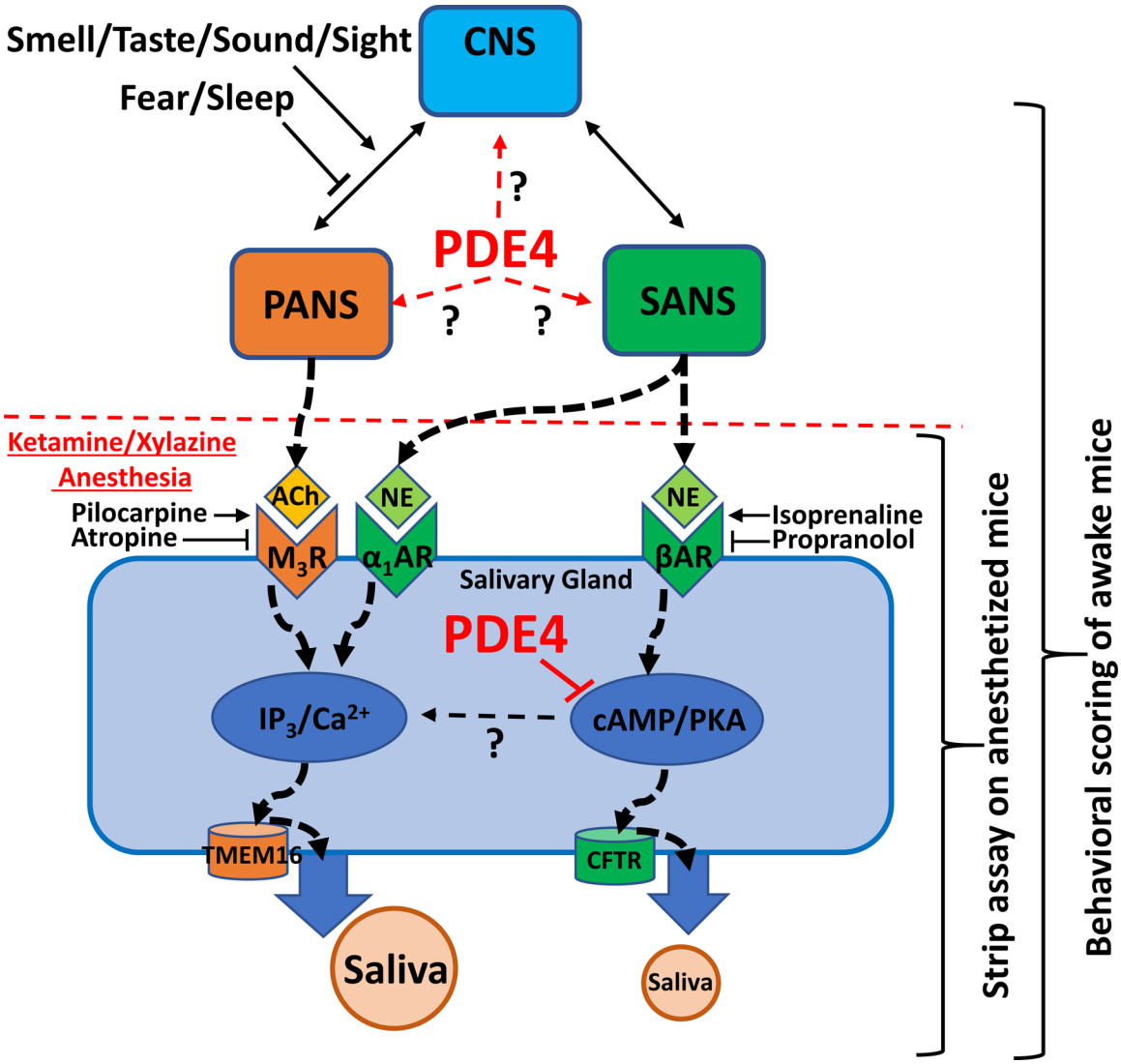

Figure 7: Scheme illustrating the role(s) of PDE4 in the regulation of salivation.

The scheme illustrates the known mechanisms of autonomic nervous system control and the intracellular signaling events that mediate saliva secretion, as well as the potential role(s) of PDE4 in inducing salivation. As shown within the blue rectangle representing the salivary glands, direct stimulation of glandular G_q-coupled receptors, such as M_3-muscarinic acetylcholine receptors (M_3R) or α_1-adrenergic receptors (α_1AR), and the subsequent rise in intracellular Ca^{2+} mediates the release of high volumes of watery saliva that is dependent upon activation of the calcium-activated chloride channel TMEM16A[58, 59]. In addition, stimulation of intracellular cAMP signaling, such as upon activation of β-adrenergic receptors (βAR), promotes saliva secretion *via* a pathway dependent upon the cAMP/PKA-activation of the anion channel CFTR[18, 22]. Under conditions of inhibited neuronal signaling due to Ketamine/Xylazine-induced anesthesia, only the events shown below the red striated line are detected. Under these conditions, inhibition of PDE4/PDE4D selectively potentiates a β-adrenoceptor-, cAMP-, and CFTR-dependent saliva secretion (Fig. 4B/C), which is completely ablated in homozygous ΔF508-CFTR mice (Fig. 5B), that lack functional CFTR,

or by treatment with the β-blocker Propranolol (Fig. 4D). On the other hand, in anesthetized mice, PDE4 inhibition does not affect salivation induced by the direct action of the M_3R agonist Pilocarpine on salivary glands. However, in awake/conscious mice, inhibition of PDE4 induces salivation *via* an additional, central/neuronal mechanism that remains to be defined and may involve the sympathetic autonomic nervous system (SANS), the parasympathetic autonomic nervous system (PANS), or the central nervous system (CNS), but is ablated by the muscarinic blocker Atropine (Fig. 1E), and only partially dependent upon CFTR (Fig. 5A).