

Cftr and ENaC ion channels mediate NaCl absorption in the mouse submandibular gland

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Cystic fibrosis is caused by mutations in *CFTR*, the cystic fibrosis transmembrane conductance regulator gene. Disruption of CFTR-mediated anion conductance results in defective fluid and electrolyte movement in the epithelial cells of organs such as the pancreas, airways and sweat glands, but the function of CFTR in salivary glands is unclear. Salivary gland acinar cells produce an isotonic, plasma-like fluid, which is subsequently modified by the ducts to produce a hypotonic, NaCl-depleted final saliva. In the present study we investigated whether submandibular salivary glands (SMGs) in $\Delta F508$ mice (*Cftr* ^{$\Delta F/\Delta F$}) display ion transport defects characteristic of cystic fibrosis in other tissues. Immunolocalization and whole-cell recordings demonstrated that Cftr and the epithelial Na⁺ (ENaC) channels are co-expressed in the apical membrane of submandibular duct cells, consistent with the significantly higher saliva [NaCl] observed *in vivo* in *Cftr* ^{$\Delta F/\Delta F$} mice. In contrast, Cftr and ENaC channels were not detected in acinar cells, nor was saliva production affected in *Cftr* ^{$\Delta F/\Delta F$} mice, implying that Cftr contributes little to the fluid secretion process in the mouse SMG. To identify the source of the NaCl absorption defect in *Cftr* ^{$\Delta F/\Delta F$} mice, saliva was collected from *ex vivo* perfused SMGs. *Cftr* ^{$\Delta F/\Delta F$} glands secreted saliva with significantly increased [NaCl]. Moreover, pharmacological inhibition of either Cftr or ENaC in the *ex vivo* SMGs mimicked the *Cftr* ^{$\Delta F/\Delta F$} phenotype. In summary, our results demonstrate that NaCl absorption requires and is likely to be mediated by functionally dependent Cftr and ENaC channels localized to the apical membranes of mouse salivary gland duct cells.

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Abbreviations CCh, carbachol; CF, cystic fibrosis; CFTR (Cftr), human (murine) cystic fibrosis transmembrane conductance regulator; ΔF , phenylalanine 508 deletion mutation; IPR, isoproterenol; IBMX, 3-isobutyl-1-methylxanthine; SMG, submandibular gland.

Introduction

The first stage of the digestion process takes place in the oral cavity, where saliva is secreted in response to ingested food. Saliva is a combination of the fluid secreted by three major pairs of glands (parotid, submandibular and sublingual) and numerous minor salivary glands. Salivary gland acinar cells are classified according to the type of secretion they produce: parotid glands are composed of serous acini which secrete a watery saliva rich in amylase, an enzyme for digesting starches; the sublingual glands are primarily composed of mucous cells that secrete a viscous mucin-rich fluid that lubricates the ingested food and protects the oral cavity from mechanical, chemical

and bacterial insults; and the submandibular glands are composed of a mixture of serous and mucous acinar cells.

The significance of saliva in the upper gastrointestinal tract can be inferred from individuals displaying hyposalivation defects, causing difficulties in tasting, chewing and swallowing, and increased incidence and severity of infections leading to dental caries, periodontal disease, and oral and oesophageal ulcers. Salivary gland dysfunction is most frequently observed as a side-effect of medications and irradiation therapy, as well as diseases such as Sjögren's syndrome and cystic fibrosis.

Cystic fibrosis (CF) is the most common genetic disease in Caucasians, occurring in approximately 1 out of 3200 live births (Quinton, 1999). Identification of the gene

associated with this condition (*CFTR*) has led to the characterization of the functional properties of the *CFTR* protein (Riordan *et al.* 1989). Of the more than 1000 mutations associated with CF, deletion of phenylalanine 508 ($\Delta F508$) is the most common, occurring in about 70% of cases (Kerem *et al.* 1989). *CFTR* codes for a cAMP-activated anion channel highly expressed in epithelial tissues, including salivary glands.

Studies of the effects of CF on human salivary gland function have produced conflicting results. For example, it has been reported that either CF has no effect on flow (Blomfield *et al.* 1973, 1976) or it reduces salivation (Davies *et al.* 1990, 1991). Moreover, little effect of CF has been generally noted on the concentration of Na^+ , K^+ , Cl^- or protein in submandibular gland (SMG) saliva, but defective Cl^- reabsorption and HCO_3^- secretion have been described in SMG ductal saliva from some CF patients (Blomfield *et al.* 1973; Davies *et al.* 1990, 1991). The reason for the large variability in the reported effects of CF on salivary gland function is unknown. Most of these studies were performed prior to the advent of methods for determining the nature of the mutation, which relates to the severity of disease.

To gain further insight into the effects of CF on salivary gland function, we have utilized the mouse CF model system which reproduces many of the defects observed in the human disease (Zeihner *et al.* 1995). Specifically, we examined whether mice homozygous for the $\Delta F508$ mutation (*Cftr* $^{\Delta F/\Delta F}$) express defects characteristic of CF related to fluid secretion or NaCl reabsorption. The stimulated saliva flow was normal in *Cftr* $^{\Delta F/\Delta F}$ mice whereas the NaCl content of saliva was significantly elevated in mice lacking *Cftr*, verifying that this channel is a major regulator of NaCl reabsorption by SMG duct cells. The mouse CF model may therefore provide a valuable system for examining the physiological significance and the regulation of ion absorptive mechanisms.

Methods

General methods and materials

Cftr $^{\Delta F/\Delta F}$ mice were generated by introduction of a deletion corresponding to the human $\Delta F508$ allele via gene targeting (Zeihner *et al.* 1995). Targeted disruptions of the murine *Nhe2* and *Nhe3* genes were performed previously (Schultheis *et al.* 1998; Bell *et al.* 1999). All animals were bred and housed in micro-isolator cages with *ad libitum* access to laboratory chow and water. Water used for *Cftr* $^{+/+}$ and *Cftr* $^{\Delta F/\Delta F}$ mice was supplemented with GoLYTELY (Braintree Laboratories Inc., Braintree, MA, USA), an oral osmotic laxative used to increase the survival of *Cftr* $^{\Delta F/\Delta F}$ mice (Clarke *et al.* 1996). Experiments were performed at the University of Rochester using protocols approved by the University Committee on

Animal Resources and in compliance with the policies and regulations described by Drummond (2009). Sex- and age-matched adult mice between 2 and 6 months old were used. Reagents were purchased from Sigma-Aldrich unless otherwise indicated.

Tissue processing and immunohistochemistry

Mouse submandibular gland fixation and immunostaining procedures were performed as described elsewhere (Romanenko *et al.* 2008). The rabbit anti-*CFTR* polyclonal antibody was from Cell Signaling Technology, Inc. (Danvers, MA, USA; cat. no. 2269) and an anti-mouse α -ENaC polyclonal antibody was generated by injecting rabbits with a peptide directed at amino acids 46–68 of the mouse α -ENaC sequence (LGKGDKREEQALGPEPSEPRQPTC). This region was selected based on the rat α -ENaC epitope employed previously (Masilamani *et al.* 1999) and differs from the rat sequence in two amino acids. Rabbit serum was affinity purified using a kit, following the manufacturer's instructions (Sulfolink Immobilization Kit, Pierce Biotechnology Inc., Rockford, IL, USA). Dilutions were used for both antibodies (1:100). The specificity of the anti- α -ENaC antibody was tested using CHO cells expressing the murine α , β and/or γ subunits of ENaC (online Supplemental Material, Fig. S1B).

Measurement of fluid secretion *in vivo*

Mice were anaesthetized by intraperitoneal injection of chloral hydrate (400 mg (kg body wt) $^{-1}$) prior to saliva collection as described (Romanenko *et al.* 2008). The level of anaesthesia was monitored by assessing the animals responsive to toe pinch. *In vivo* fluid secretion data were normalized by body weight (μl (kg body wt) $^{-1}$). Immediately after collection of saliva, animals were killed by carbon dioxide inhalation, and the diaphragm and aorta were severed to ensure that the animals were dead.

Ex vivo submandibular gland perfusion

Ex vivo SMG perfusion was performed as previously reported (Romanenko *et al.* 2007). Secretion was stimulated by addition of a combination of muscarinic (0.3 μM carbachol, CCh) and β -adrenergic (5 μM isoproterenol, IPR) agonists to the perfusion solution. Where indicated, ENaC (amiloride) and *Cftr* (*CFTR*(INH)-172) channel blockers were added to the perfusion solution. Salivary gland acini are leaky epithelia, and consequently relatively large compounds like amiloride and *CFTR*(INH)-172 have access to the luminal surface (Romanenko *et al.* 2008).

Analysis of saliva composition

Saliva pH was measured immediately after collection with a pH-sensitive electrode (Thermo Scientific, Beverly, MA, USA). Saliva samples were then stored at -86°C until further analysis. Na^+ , K^+ and Cl^- concentrations were analysed as previously described (Romanenko *et al.* 2008).

Electrophysiological recordings

Whole-cell recordings were performed on isolated SMG acinar and granular duct cells as described elsewhere (Romanenko *et al.* 2007, 2008).

Solutions used for Cftr-mediated Cl^- currents recordings were as follows: high Cl^- external solution (in mM): 150 NaCl, 1 CaCl_2 , 1 MgCl_2 , 10 Hepes, 20 sucrose, pH 7.4; low Cl^- external solution (in mM): 10 NaCl, 140 sodium gluconate, 1 CaCl_2 , 1 MgCl_2 , 10 Hepes, 20 sucrose, pH 7.4; the internal pipette solution (in mM): 120 CsCl, 10 TEACl, 1 MgCl_2 , 0.5 EGTA, 1 ATP, 10 Hepes, pH 7.4. Cftr-mediated Cl^- currents were elicited by addition to the perfusate of a cAMP-increasing cocktail (10 μM forskolin + 100 μM 3-isobutyl-1-methylxanthine (IBMX)). CdCl_2 at 300 μM and 10 μM amiloride were added to external solutions to prevent ClC-2 -mediated Cl^- currents and ENaC-mediated Na^+ currents, respectively (Dinudom *et al.* 1993; Romanenko *et al.* 2008). Solutions used to record ENaC-mediated Na^+ currents were as follows: external solution (in mM): 135 sodium glutamate, 5 potassium glutamate, 2 CaCl_2 , 2 MgCl_2 , 10 Hepes, 20 sucrose, pH 7.2; the internal pipette solution (in mM): 145 potassium glutamate, 5 Na_4EGTA , 3 CaCl_2 , 10 Hepes, pH 7.2.

Biotinylation procedure

In brief, dispersed submandibular cells from *Cftr*^{+/+} and *Cftr* ^{$\Delta F/\Delta F$} mice were rinsed twice with ice-cold phosphate-buffered saline (PBS; pH 8.0) prior to the addition of 80 μl of 10 mM Sulfo NHS-SS-Biotin (Pierce) stock solution per ml of PBS as previously described (Gonzalez-Begne *et al.* 2007).

Western blot analysis

Western blot experiments from whole-cell lysates and affinity enriched biotinylated plasma membrane fractions were performed as previously described (Gonzalez-Begne *et al.* 2007; Romanenko *et al.* 2008). Antibodies used were anti- β and anti- γ rabbit polyclonal antibodies (Ergonul *et al.* 2006) or an anti-mouse α -ENaC rabbit polyclonal antibody. Dilutions used for α -, β - and γ -ENaC antibodies were 1 : 100, 1 : 1000 and 1 : 250, respectively.

Statistical analysis

Results are presented as the mean \pm S.E.M. Statistical significance was determined using Student's *t* test or ANOVA, followed by Bonferroni's test for multiple comparisons using Origin 7.0 Software (OriginLab Corp., Northampton, MA, USA). *P* values of less than 0.05 were considered statistically significant. All experiments were performed using three or more separate preparations.

Results

Cftr is functionally expressed in the apical pole of SMG duct cells

Activation of an apical anion channel is the rate limiting step for fluid secretion by the acinar secretory cells of most exocrine glands, including salivary glands. The Cftr anion channel has been postulated to fulfil this secretory role in the SMG (Zeng *et al.* 1997b). We performed immunolocalization studies to verify the expression and localization of Cftr channels in the mouse SMG. Figure 1A and B show that Cftr-dependent immunostaining in male and female glands, respectively, was associated with granular and striated duct cells, but little if any staining was detected in acinar cells. The Cftr-associated immunostaining pattern in the SMG was similar to the distribution of Cftr previously described in mouse SMG (Zeihner *et al.* 1995). At the subcellular level, most of the immunostaining was confined to the apical pole of ducts cells,

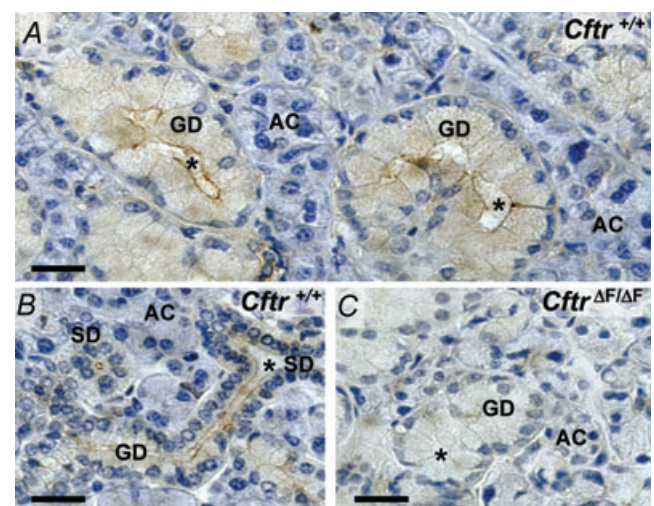


Figure 1. Immunohistochemical localization of Cftr channel protein in mouse SMG

A and B, immunoperoxidase labelling for Cftr in submandibular glands from male (A) and female (B) *Cftr*^{+/+} mice. Sections show staining of striated (SD) and granular ducts (GD) with no apparent immunolabelling in acinar cells (AC). C, immunostaining is not present in SMG from $\Delta F508$ (*Cftr* ^{$\Delta F/\Delta F$}) mice. Asterisks show luminal spaces. Bars = 50 μm .

consistent with the apical expression of Cfr channels in other epithelial tissues (Zeiber *et al.* 1995; Bothe *et al.* 2008). Essentially no specific staining was found in SMG tissue from *Cfr* ^{$\Delta F/\Delta F$} mice (Fig. 1C), in agreement with the lack of Cfr protein in the *Cfr* ^{$\Delta F/\Delta F$} SMG (Zeiber *et al.* 1995).

To verify if the staining pattern correlates with functional Cfr channels, single acinar and duct cells were isolated and tested for Cfr-mediated Cl⁻ currents using the whole-cell configuration of the patch clamp technique. Figure 2A and B shows that an ohmic conductance was elicited upon addition of a cAMP-increasing cocktail to the perfusate (10 μ M Forskolin + 100 μ M IBMX) in SMG duct cells isolated from *Cfr*^{+/+} mice. This conductance was carried by Cl⁻, since its

replacement by gluconate in the perfusate shifted the reversal potential to more positive voltages: reversal potential was -3.8 ± 1 mV at [Cl⁻]_o = 154 mM and $+54.1 \pm 14.7$ mV at [Cl⁻]_o = 14 mM (paired experiments, *n* = 3). The equilibrium potentials for Cl⁻ (*E*_{Cl}) under these conditions at room temperature were -3.9 mV and $+56.1$ mV, respectively. Moreover, the cAMP-activated Cl⁻ current was completely abolished by 10 μ M CFTR(INH)-172, a selective CFTR channel blocker (Ma *et al.* 2002). cAMP did not activate a Cl⁻ current in granular duct cells from *Cfr* ^{$\Delta F/\Delta F$} mice (Fig. 2C), confirming that *Cfr* encodes the cAMP-activated anion channel in mouse SMG duct cells. We also failed to detect a cAMP-activated Cl⁻ current in SMG acinar cells (Fig. 2D).

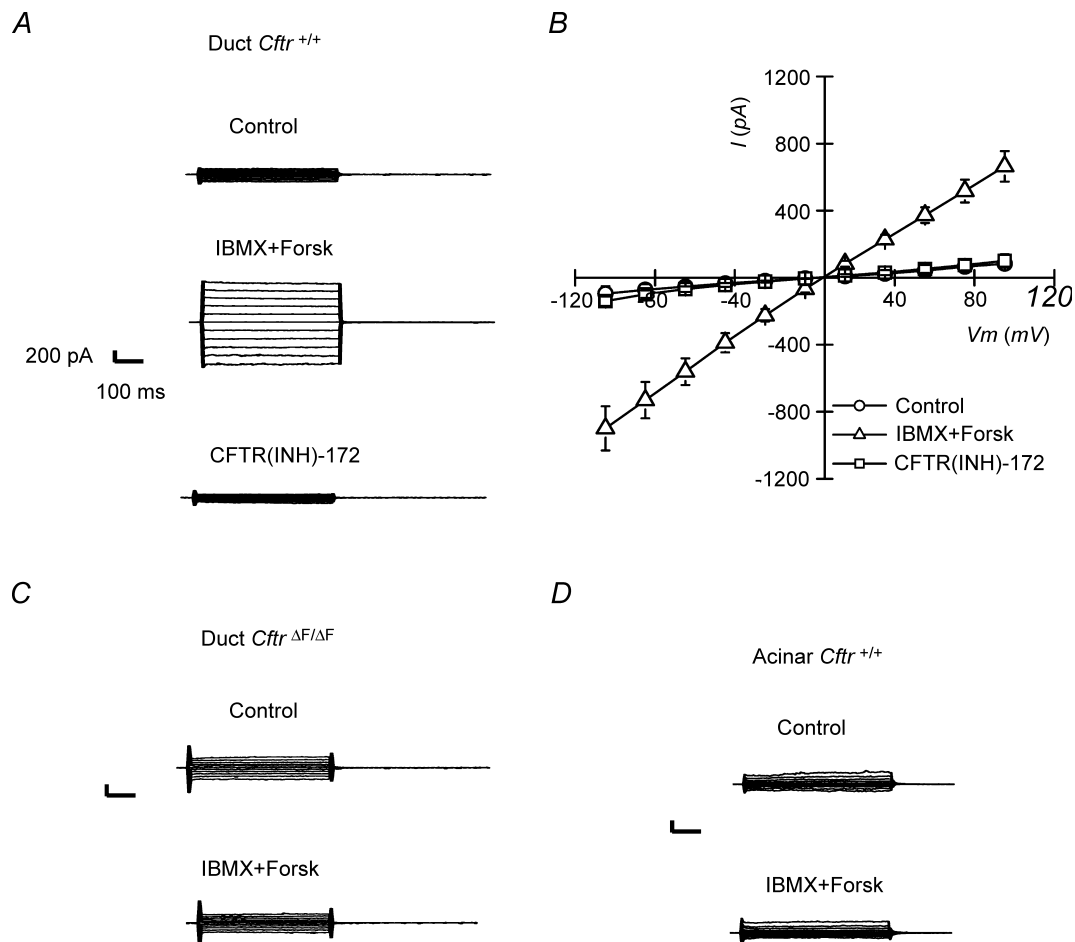


Figure 2. Cfr-mediated Cl⁻ currents in granular duct cells

Whole-cell Cl⁻ currents were recorded as described in Methods. *A*, cAMP-activated Cl⁻ currents elicited in response to square voltage steps in *Cfr*^{+/+} granular duct cells (from -100 to $+100$ mV, 20 mV steps). CFTR(INH)-172 at 10 μ M fully blocked Cfr-mediated currents. Scale: current 200 pA and time 100 ms. *B*, current-voltage relations from data like those shown in panel *A* (*n* = 5). *C*, cAMP-activated Cl⁻ currents were not present in granular duct cells isolated from $\Delta F508$ (*Cfr* ^{$\Delta F/\Delta F$}) mice (*n* = 4). *D*, submandibular acinar cells isolated from *Cfr*^{+/+} mice also failed to display cAMP-activated Cl⁻ currents (*n* = 4).

Defective NaCl absorption in *Cftr*^{ΔF/ΔF} SMG

Most of whole saliva (~90%) is secreted by two of the three major salivary glands, the submandibular and parotid glands. To directly evaluate whether Cftr is involved in acinar fluid secretion and/or ductal function in the SMG, the *in vivo* saliva flow rates and ionic compositions were determined in littermate *Cftr*^{+/+} and *Cftr*^{ΔF/ΔF} mice (Snouwaert *et al.* 1992; Zeiher *et al.* 1995). Figure 3A and B shows that the flow rate and total volume of saliva generated during stimulation with the broad-range cholinergic secretagogue pilocarpine were modestly decreased in *Cftr*^{ΔF/ΔF} mice, suggesting that Cftr contributes little to fluid secretion in this organ under these conditions. To investigate the role of Cftr in ductal modification of saliva, we determined the ion composition of pilocarpine-stimulated SMG saliva (Fig. 3C). The [Cl⁻] and [Na⁺] were significantly increased, whereas the [K⁺] was not affected, in the *Cftr*^{ΔF/ΔF} mouse CF model.

We previously found that *in vivo* saliva production in response to systemic administration of pilocarpine may not faithfully reflect perturbations of the saliva production machinery (Romanenko *et al.* 2007). Consequently, we also used an *ex vivo*, perfused SMG preparation to eliminate the circulating factors and neural inputs that complicate the interpretation of *in vivo* experiments. A combination of muscarinic (0.3 μM CCh) and β-adrenergic (5 μM IPR) stimulation was used to enhance ductal function (Romanenko *et al.* 2008). Supplemental Table 1 shows that Na⁺ and Cl⁻ reabsorption was strongly stimulated in the SMG upon β-adrenergic stimulation, while K⁺ secretion was not significantly different, mimicking the *in vivo* effects of pilocarpine on salivary gland function (compare to Fig. 3C). The salivary flow rates and secretion kinetics were essentially identical in SMG from wild-type and *Cftr*^{ΔF/ΔF} mice (Fig. 4A). In contrast, both the [Cl⁻] and [Na⁺] were significantly increased more than 2-fold in *Cftr*^{ΔF/ΔF} saliva, whereas the [K⁺] was unchanged (Fig. 4B). It should be noted that the [Cl⁻] in saliva is less than the sum of the [K⁺] and [Na⁺], suggesting that other anions must be secreted to maintain electroneutrality. HCO₃⁻, which permeates the Cftr anion channel and is a major regulator of saliva pH, is the second most abundant physiological anion after Cl⁻ in biological fluids, including saliva (Sommer *et al.* 1975). Consequently, we measured the saliva pH in littermate wild-type (pH 8.18 ± 0.04, *n* = 13) and *Cftr*^{ΔF/ΔF} (pH 7.63 ± 0.06, *n* = 8; *P* < 0.01, *t* test) mice. This decrease in saliva pH suggests that the [HCO₃⁻] is likely to have decreased in parallel with the [Cl⁻] increase in *Cftr*^{ΔF/ΔF} mice.

The above functional data demonstrate that Cl⁻ reabsorption is dependent on Cftr expression, but the molecular mechanism involved in this process remains unclear. The simplest interpretation of the above results

is that the apical Cftr channel acts as the Cl⁻ influx pathway in SMG duct cells. However, it has been postulated that CFTR modulates the activity and expression of several ion channels and ion transporters (Kunzelmann, 2001). To test whether Cftr channels are essential for Cl⁻

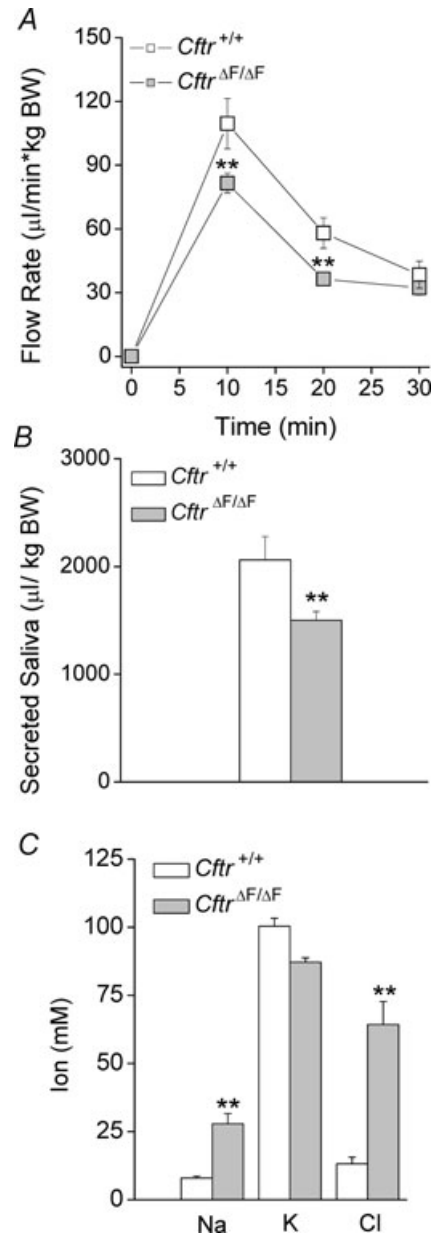


Figure 3. *In vivo* effects of the *Cftr* ΔF508 mutation on fluid secretion and ion composition of SMG saliva

Secretion was stimulated by intraperitoneal injection of pilocarpine HCl (10 mg (kg body weight)⁻¹). *In vivo* fluid secretion data were normalized by body weight (μl (kg body weight)⁻¹). A, saliva flow in response to stimulation was slightly affected in ΔF508 (*Cftr*^{ΔF/ΔF}) mice. B, total submandibular saliva secreted. C, Na⁺ and Cl⁻ saliva concentrations were significantly increased in the ΔF508 CF mouse model. Data are given as the mean ± s.e.m. ***P* < 0.05, *t* test; *n* = 16 for each genotype.

reabsorption, *ex vivo* pharmacological experiments were performed. Treatment with the CFTR-specific blocker CFTR(INH)-172 mimicked the *Cftr*^{ΔF/ΔF} phenotype, i.e. fluid secretion was not affected (Fig. 5A) but the saliva [NaCl] was increased (Fig. 5B), suggesting that functional *Cftr* protein is required to mediate Cl⁻ reabsorption in the mouse SMG.

The role of ENaC in SMG NaCl reabsorption

Both genetic (Fig. 4B) and pharmacological (Fig. 5B) disruption of *Cftr* activity increased the saliva Na⁺ concentration. One explanation of these results is that Na⁺ influx is dependent upon Cl⁻ reabsorption in the SMG.

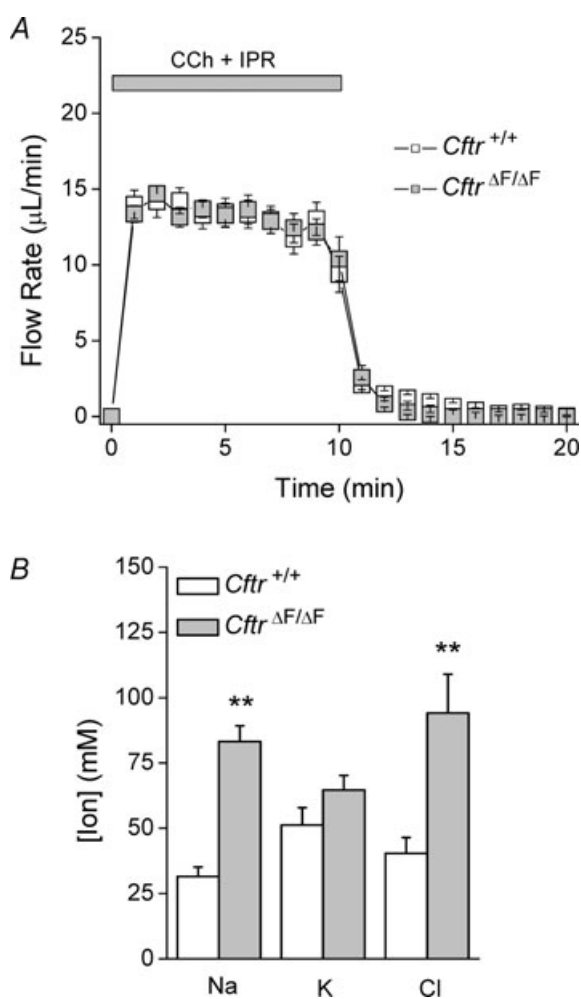


Figure 4. *Ex vivo* effects of the *Cftr* ΔF508 mutation on fluid secretion and ion composition of SMG saliva

Ex vivo submandibular glands were used to determine the flow rate and ion composition of saliva in *Cftr*^{+/+} and ΔF508 (*Cftr*^{ΔF/ΔF}) mice. Carbachol (CCh, 0.3 μM) and isoproterenol (IPR, 5 μM) were used to stimulate secretion. Flow rate is expressed as μL min⁻¹. A, saliva flow in response to stimulation (*Cftr*^{+/+}, open squares, *n* = 13; *Cftr*^{ΔF/ΔF}, grey squares, *n* = 8). B, ion composition of saliva collected in panel A. Data are given as the mean ± the s.e.m. ***P* < 0.05, *t* test.

Several coupled NaCl absorption mechanisms have been described in absorptive epithelia (Kunzelmann & Mall, 2002; Baum, 2008), all of which require a Na⁺-influx pathway in the apical membrane. Consistent with this model, Na⁺-transporting proteins have been detected in SMG, including Nhe2 and Nhe3 Na⁺/H⁺ exchangers and the ENaC Na⁺ channel (Dinudom *et al.* 1993; Lee *et al.* 1998). Table 1 shows a summary of the *ex vivo* experiments performed with *Nhe2*^{-/-} and *Nhe3*^{-/-} glands to test whether these Na⁺ ion transporters mediate Na⁺ reabsorption. NaCl reabsorption was not impaired by null mutations in either Na⁺/H⁺ exchanger (nor was K⁺ secretion), similar to previous *in vivo* results in the mouse parotid gland (Park *et al.* 2001).

Considering that NaCl reabsorption was normal in *Nhe2*^{-/-} and *Nhe3*^{-/-} mice, we hypothesized that the epithelial Na⁺ channel ENaC is likely to be involved in this process. Taking advantage of ENaC's sensitivity to low concentrations of amiloride (Kellenberger *et al.* 2003), *ex vivo* experiments revealed that neither the kinetics nor the total volume of secreted saliva was affected by amiloride treatment (Fig. 5C). This result rules out potential side-effects on other amiloride-sensitive proteins involved in fluid secretion, in particular the Na⁺/H⁺ exchanger Nhe1 (Park *et al.* 2001). Conversely, ion composition analysis demonstrated that amiloride treatment impaired Na⁺ reabsorption nearly 3-fold in the SMG (Fig. 5D). Qualitatively similar to *Cftr* inhibition (Figs 3, 4 and 5), this manoeuvre also impaired Cl⁻ reabsorption, providing additional evidence that Na⁺ and Cl⁻ transport are functionally linked in this NaCl absorptive tissue.

Functional ENaC channels are expressed in the apical membrane of SMG duct cells

Based on the amiloride sensitivity of NaCl reabsorption, ENaC appears to be directly linked to Na⁺ absorption, the molecular counterpart to the *Cftr*-mediated Cl⁻ influx in the SMG. This model requires that ENaC be localized to the apical membrane of SMG duct cells. Consequently, immunolocalization studies were performed on mouse SMG. The specificity of a rabbit polyclonal antibody generated against the N-terminal segment of α-ENaC was tested on CHO cells expressing the murine α, β and/or γ subunits of ENaC (Supplementary Fig. S1A). Similar to the staining pattern for *Cftr* (Fig. 1), Fig. 6A and B shows strong α-ENaC-associated immunostaining at the apical poles of granular and striated SMG duct cells, but no staining was detected in acinar cells. To verify that the α-ENaC staining pattern correlates with functional ENaC channels, SMG granular duct cells were isolated and assessed for an amiloride-sensitive, ENaC-mediated Na⁺ current using the whole-cell configuration of the patch

clamp technique. A robust, time-independent Na^+ current was elicited in response to different voltage steps in isolated granular duct cells (Fig. 6C and D). This Na^+ conductance relied on ENaC activity because it was essentially abolished by $10 \mu\text{M}$ amiloride treatment ($80.2 \pm 4.4\%$ inhibition at -60 mV , $n = 8$). The current–voltage relation of the amiloride-sensitive current revealed that the reversal potential was more than $+40 \text{ mV}$, consistent with a Na^+ conductance ($E_{\text{Na}} = +47.7 \text{ mV}$ at room temperature). Together, these results provide further evidence for the involvement of ENaC channels in the functional coupling of NaCl absorption in submandibular duct epithelium.

α -ENaC protein level and activity are reduced in *Cftr* ^{$\Delta F/\Delta F$} SMG

The above results demonstrate that absorption of Na^+ and Cl^- is dependent on both ENaC and Cftr channels

Table 1. Nhe2 and Nhe3 Na^+/H^+ exchangers are not involved in NaCl reabsorption

(mM)	Wild-type	<i>Nhe2</i> ^{-/-}	<i>Nhe3</i> ^{-/-}
Na^+	$31.5 \pm 3.6(13)$	$31.1 \pm 9.5(5)$	$25.9 \pm 6.4(8)$
K^+	$51.2 \pm 6.7(13)$	$52.2 \pm 5.0(5)$	$64.3 \pm 7.5(8)$
Cl^-	$40.41 \pm 6.0(13)$	$51.28 \pm 4.9(5)$	$42.9 \pm 7.0(8)$

Ion composition analysis of *ex vivo* submandibular saliva from wild-type, *Nhe2*^{-/-} and *Nhe3*^{-/-} mice stimulated with carbachol (CCh, $0.3 \mu\text{M}$) and isoproterenol (IPR, $5 \mu\text{M}$). Wild-type mice values were taken from Supplemental Table 1 ($0.3 \mu\text{M}$ CCh + $5 \mu\text{M}$ IPR). Data are given as the mean \pm s.e.m. and the number of experiments is given in parentheses. Note that there were no significant differences between groups, ANOVA.

in the duct epithelium of the mouse SMG. Because Na^+ reabsorption was impaired in the *Cftr* ^{$\Delta F/\Delta F$} SMG, we predicted that functional disruption of Cftr might also affect ENaC activity in this epithelium. To test

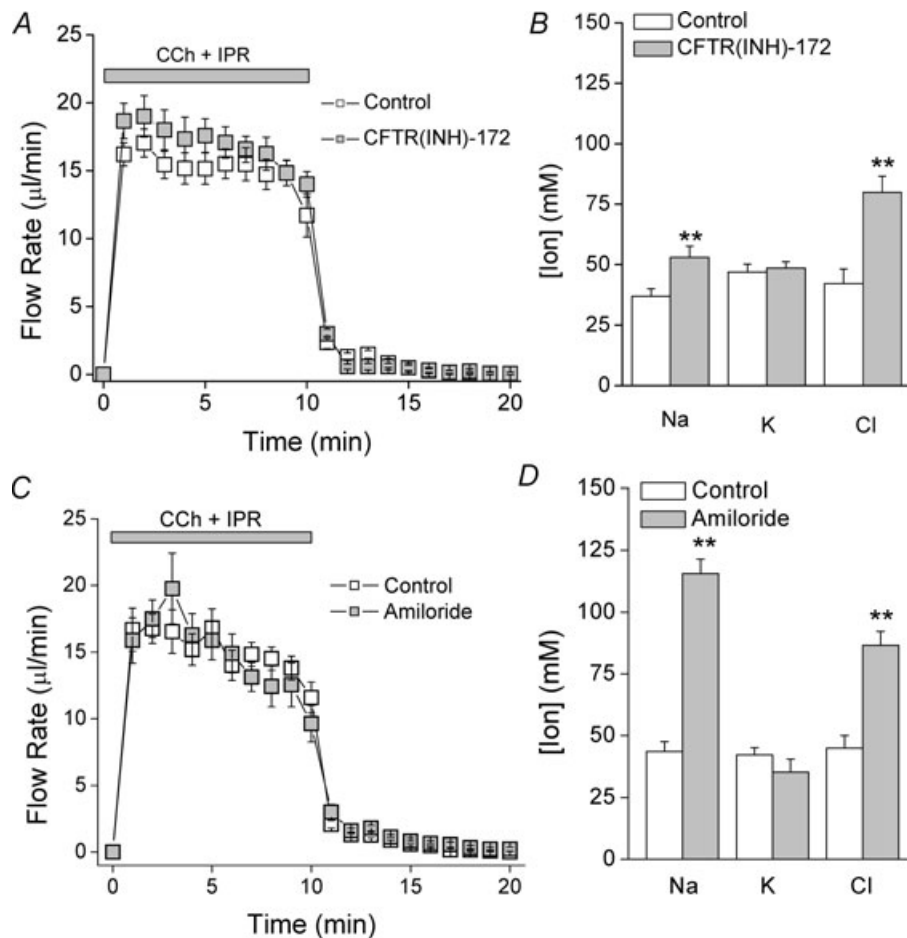


Figure 5. Pharmacological blockade of Cftr and ENaC ion channels mimics the *Cftr* ^{$\Delta F/\Delta F$} phenotype

Wild-type *ex vivo* submandibular glands (Control, *Cftr*^{+/+}) were perfused with $3 \mu\text{M}$ CFTR(INH)-172 or $10 \mu\text{M}$ amiloride for 30 min prior to and during stimulation. Carbachol (CCh, $0.3 \mu\text{M}$) and isoproterenol (IPR, $5 \mu\text{M}$) were used to stimulate secretion. *A*, saliva flow in response to stimulation (Control, open squares, $n = 8$; CFTR(INH)-172 perfused glands, grey squares, $n = 8$). *B*, ion composition of saliva collected in panel *A*. *C*, saliva flow in response to stimulation (Control, open squares, $n = 15$; amiloride-perfused glands, grey squares, $n = 12$). *D*, ion composition of saliva collected in panel *C*. Data are given as the mean \pm the s.e.m. ** $P < 0.05$, *t* test.

this hypothesis, whole-cell recordings were performed on isolated SMG granular duct cells from *Cftr*^{+/+} and *Cftr*^{ΔF/ΔF} mice and the ENaC-mediated Na⁺ current magnitudes compared. Indeed, the results from these experiments (Fig. 7A) show that the ENaC-mediated Na⁺ current was severely decreased in *Cftr*^{ΔF/ΔF} SMG duct cells (*Cftr*^{ΔF/ΔF} = -4.9 ± 1.5 pA pF⁻¹ vs. *Cftr*^{+/+} = -15.3 ± 1.5 pA pF⁻¹ at -60 mV; $P < 0.001$, *t* test).

The decrease in ENaC-mediated Na⁺ currents in *Cftr*^{ΔF/ΔF} SMG duct cells could be due to either changes in channel properties or a decrease in the

expression of ENaC at the plasma membrane. To monitor the plasma membrane expression of α-ENaC, western blots were performed on biotinylated proteins isolated by streptavidin affinity chromatography. Figure 7B shows that expression of the α-ENaC subunit in the plasma membrane was dramatically reduced in *Cftr*^{ΔF/ΔF} SMG. Western blots of whole-cell lysates also found that the total cellular level of α-ENaC protein was considerably decreased in *Cftr*^{ΔF/ΔF} SMG (left panel, Fig. 7C), demonstrating that the decrease in α-ENaC expression was not restricted to the plasma membrane. This effect was limited to the α-ENaC subunit because no apparent changes were observed in the total cellular expression of β- or γ-ENaC (middle and right panels of Fig. 7C, respectively) using previously characterized antibodies (Ergonul *et al.* 2006).

Discussion

The fluid secreted by salivary gland acinar cells is an isotonic NaCl-rich fluid. This NaCl-rich fluid is modified during its passage through the ductal epithelium, where much of the NaCl is reabsorbed, and because the ducts are relatively impermeable to water, NaCl reabsorption results in the production of a hypotonic saliva. Apical Cl⁻ channels are key components of both the fluid secretion and NaCl reabsorption mechanisms in exocrine salivary glands, while fluid secretion by acinar cells also requires the apical water channel Aqp5 (Ma *et al.* 1999; Krane *et al.* 2001). Although results are conflicting, some clinical studies suggest that CFTR may be important for fluid secretion and NaCl reabsorption in salivary glands (Blomfield *et al.* 1973; Davies *et al.* 1990, 1991), but the role of CFTR in these processes has not been experimentally demonstrated. The present study provides evidence that NaCl absorption requires and is likely to be mediated by functionally interdependent Cftr and ENaC channels localized to the apical membranes of mouse SMG duct cells.

We found that mouse SMG tissue displayed strong Cftr immunostaining in the apical membrane of duct cells, with no apparent Cftr-specific staining in acinar cells, as previously noted (Zeiber *et al.* 1995). Consistent with our immunolocalization studies, we also failed to detect Cftr-mediated Cl⁻ currents in whole-cell recordings of SMG acinar cells, and the total volume of *ex vivo*-stimulated SMG fluid was not affected by disruption of Cftr. These *ex vivo* results are in agreement with the lack of cAMP-activated Cl⁻ current and Cftr protein in acinar cells. Conversely, it has previously been reported that the secretion of *in vivo* whole saliva stimulated with a β-adrenergic agonist was reduced in *Cftr*^{-/-} mice (Best & Quinton, 2005) and that Cftr channels are expressed in the apical membranes of both rat and mouse submandibular

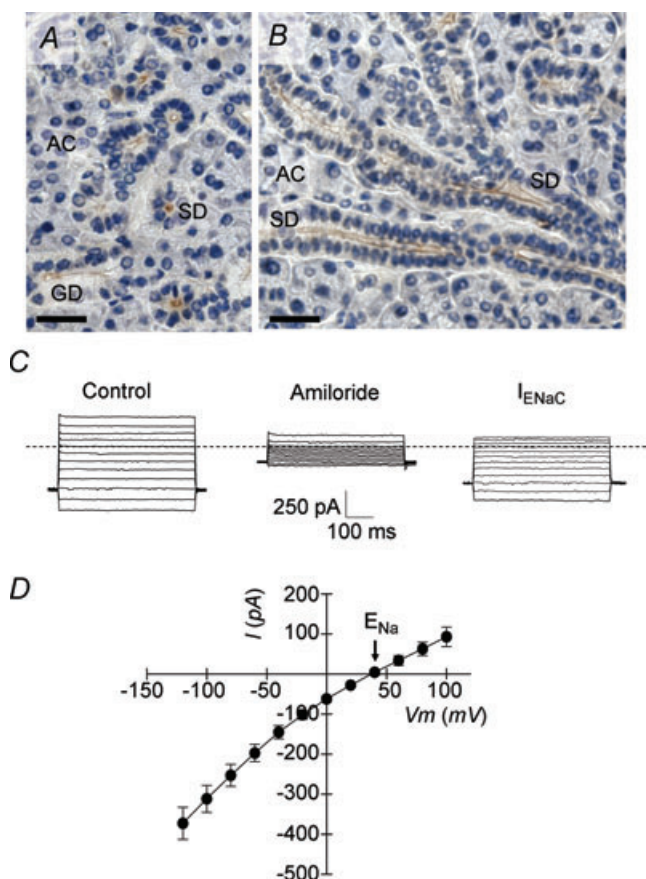


Figure 6. Immunohistochemical and functional evidence for α-ENaC expression in mouse SMG

Immunoperoxidase labelling of α-ENaC in wild-type submandibular glands (Control, *Cftr*^{+/+}). Transverse (A) and longitudinal (B) sections showing the staining of striated (SD) and granular ducts (GD) with no apparent immunolabelling in acinar cells (AC). Bars = 50 μm. C, whole-cell Na⁺ currents were recorded as described in Methods. Na⁺ currents elicited in response square voltage steps in wild-type (Control, *Cftr*^{+/+}) granular duct cells (from -120 to $+100$ mV, 20 mV steps). Amiloride at 10 μM blocked ENaC-mediated currents. Scale: current 250 pA and time 100 ms, respectively. Dashed line indicates zero current level. D, ENaC-mediated current–voltage relation was obtained by subtracting whole-cell currents after amiloride treatment from those recorded before amiloride addition ($n = 8$). Data are given as the mean ± S.E.M.

acinar and duct cells (Zeng *et al.* 1997b; Ishibashi *et al.* 2008). We do not know the basis for these apparent differences, but it could be related to several factors including differences in stimulation protocols, whole saliva *vs.* gland-specific saliva collection methods, type of Cftr mutation, and/or mouse background. Alternatively, we detected a modest decrease in the secretion of *in vivo* whole saliva (Fig. 3) that was not detected in the *ex vivo*-stimulated SMG fluid in *Cftr*^{ΔF/ΔF} mice. We believe that the differences between the *in vivo* and *ex vivo* model

systems are likely to be due to absorptive defects in the intestinal tract of CF mice (Clarke *et al.* 1996), or circulating factors and/or neural inputs that complicate the interpretation of *in vivo* experiments, rather than an effect on the ion transport machinery itself (Romanenko *et al.* 2007).

In contrast to acinar cells, granular duct cells displayed large cAMP-activated Cl⁻ currents. The currents in granular duct cells were sensitive to the CFTR-specific blocker CFTR(INH)-172 and were absent in duct cells

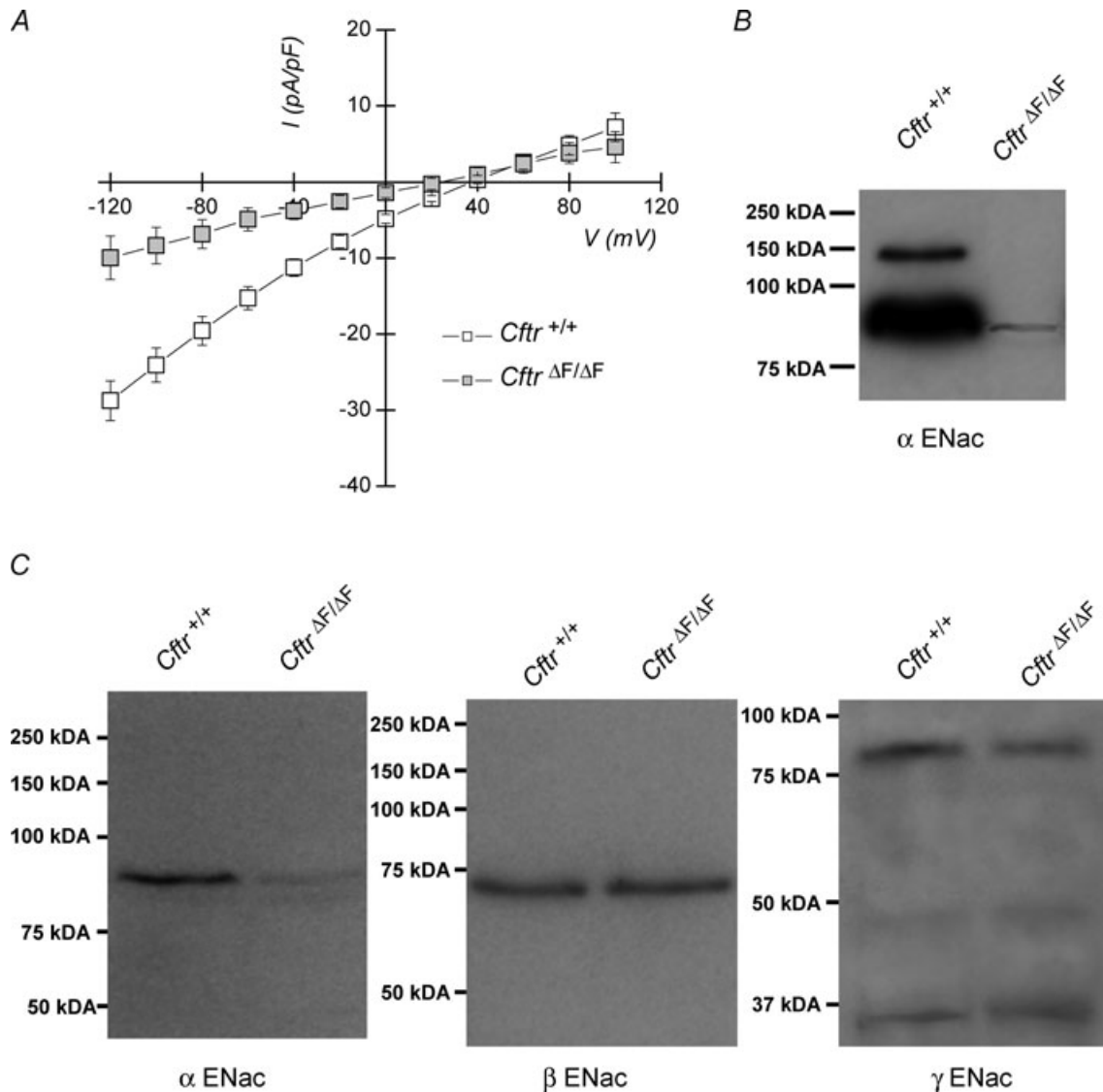


Figure 7. α-ENaC currents and protein level are reduced in *Cftr*^{ΔF/ΔF} SMG

A, whole-cell Na⁺ currents were recorded as described in Methods. ENaC-mediated current densities *versus* voltage plots in *Cftr*^{+/+} (open squares) and ΔF508 (*Cftr*^{ΔF/ΔF}; grey squares) SMG duct cells. Current densities were obtained by dividing the current magnitude by the cell capacitance and expressing as pA pF⁻¹ ($n = 8$ for *Cftr*^{+/+} and $n = 9$ for *Cftr*^{ΔF/ΔF}). Data are given as the mean ± s.e.m. ****** $P < 0.05$, t test. Data for *Cftr*^{+/+} mice are the same as those shown in Fig. 6C. B, α-ENaC immunoreactivity in submandibular plasma membrane-enriched proteins from *Cftr*^{+/+} and *Cftr*^{ΔF/ΔF} mice. C, α-, β- and γ-ENaC immunoreactivities in submandibular whole-cell lysates from *Cftr*^{+/+} and *Cftr*^{ΔF/ΔF} mice. α-, β- and γ-ENaC immunoreactive bands ran at molecular weights of approximately 90, 73 and 80 kDa, respectively, similar to the expected sizes of these proteins in mice.

isolated from *Cftr*^{ΔF/ΔF} mice, confirming that *Cftr* encodes the cAMP-activated anion channel in this cell type. The *Cftr*-mediated Cl⁻ current in SMG duct cells was similar to the cAMP-activated Cl⁻ current previously described in mouse granular duct cells (Dinudom *et al.* 1995). Furthermore, both *in vivo* and *ex vivo* experiments revealed that the [Cl⁻] of SMG saliva was dramatically increased in *Cftr*^{ΔF/ΔF} mice, suggesting that *Cftr* anion channels are involved in Cl⁻ reabsorption. Because the *ex vivo* volume of fluid was essentially unchanged in *Cftr*^{ΔF/ΔF} mice, the increase in [Cl⁻] was not due to changes in saliva flow rate. The most straight forward interpretation of the increase in the [Cl⁻] in *Cftr*^{ΔF/ΔF} mice is that the *Cftr* anion channel is involved in Cl⁻ reabsorption. However, *Cftr* modulates the activity and expression of other ion channels and transporters involved in NaCl reabsorption (Kunzelmann, 2001). Consequently, disruption of *Cftr* expression may not directly test whether *Cftr* mediates the Cl⁻ influx in mouse submandibular duct cells. To examine this possibility, we used a pharmacological approach to avoid possible effects of *Cftr* gene disruption on the expression of other ion-transporting proteins. Pharmacological blockade of *Cftr* with CFTR(INH)-172 in the SMG of wild-type mice mimicked the *Cftr*^{ΔF/ΔF} phenotype, i.e. fluid secretion was not affected whereas Cl⁻ reabsorption was impaired. Taken together, the results of these pharmacological and genetic experiments suggest that *Cftr* is directly involved in Cl⁻ reabsorption. It should be noted that in both *in vivo* and *ex vivo* experiments, the saliva [Cl⁻] in *Cftr*^{ΔF/ΔF} mice was less than predicted if *Cftr* is the only Cl⁻ reabsorption pathway. This suggests that either residual ΔF508 channels are present or that SMG duct cells express other Cl⁻ absorption pathways.

The Na⁺ absorption mechanism in mouse SMG is robust, reabsorbing much of the Na⁺ secreted by acinar cells (typically from 145 mM to less than 30 mM). Nhe2 and Nhe3 Na⁺/H⁺ exchangers, which are expressed in the apical membrane of salivary gland duct cells (Park *et al.* 2001), may contribute to Na⁺ absorption in other epithelial tissues (Schultheis *et al.* 1998; Guan *et al.* 2006). Consequently, we hypothesized that Nhe2 and/or Nhe3 might be involved in SMG Na⁺ reabsorption, either directly as Na⁺ influx pathways or by regulating intracellular pH, which may regulate *Cftr* and/or ENaC activity (Reddy *et al.* 2008). However, *ex vivo* submandibular gland experiments performed on *Nhe2*^{-/-} and *Nhe3*^{-/-} mice revealed that neither Na⁺/H⁺ exchanger was required for Na⁺ reabsorption, consistent with previous *in vivo* results in the mouse parotid gland (Park *et al.* 2001). On the other hand, Na⁺ reabsorption was severely impaired by 10 μM amiloride. Amiloride is a relatively specific inhibitor of ENaC at this low concentration, suggesting that ENaC is involved in submandibular Na⁺ reabsorption. In agreement with this observation, immunolocalization

and patch clamp experiments showed that ENaC channels are expressed in the apical membrane of submandibular duct cells. The ENaC-mediated Na⁺ current in duct cells was similar to the amiloride-sensitive Na⁺ current previously described in mouse granular duct cells (Dinudom *et al.* 1993).

SMG Na⁺ reabsorption was also dependent on *Cftr* expression and/or activity, i.e. like the [Cl⁻], the Na⁺ concentration increased in the saliva from *Cftr*^{ΔF/ΔF} mice as well as in wild-type glands treated with the CFTR channel blocker CFTR(INH)-172. Similarly, reabsorption of both Na⁺ and Cl⁻ was dramatically reduced upon amiloride inhibition of ENaC. These results suggest that Na⁺ and Cl⁻ influx across the apical membrane of SMG duct cells is mediated by ENaC and *Cftr* channels, respectively, and their coordinated activity drives the NaCl reabsorption process in this gland. In agreement with this NaCl reabsorption model, our immunolocalization and electrophysiology studies provide direct evidence that ENaC and *Cftr* channels are co-expressed in the same SMG duct cells. Such a functional interaction between ENaC and *Cftr*, and the resulting coupled NaCl uptake in SMG duct cells, closely resembles the functional interplay found between these two channels in sweat gland duct epithelium (Reddy *et al.* 1999; Reddy & Quinton, 2003).

The simplest interpretation of our data is that ENaC and *Cftr* coupling is mediated by electrochemical driving forces. The low intracellular Cl⁻ concentration in SMG ductal cells provides a favourable gradient for Cl⁻ entry upon channel opening (Zeng *et al.* 1997a). Furthermore, Na⁺ entry through ENaC would promote apical membrane depolarization, thus increasing the driving force for Cl⁻ influx via *Cftr* channels. Conversely, it can be postulated that ENaC Na⁺ channel activity is dependent on *Cftr*-induced hyperpolarization. Although electrochemical forces are likely to drive much of the functional interaction between ENaC and *Cftr* in submandibular duct cells, we cannot rule out a direct interaction as previously suggested (Berdiev *et al.* 2007). It is important to note that *Cftr* channels are gated by an increase in cAMP levels and salivary ducts are under sympathetic, β-adrenergic control (Denniss *et al.* 1978). Thus, it is plausible to speculate that the functional interplay between ENaC and *Cftr* channel activity may require β-adrenergic stimulation, that is through *Cftr* activation, as previously shown in sweat gland ducts (Reddy *et al.* 1999; Reddy & Quinton, 2003). Consistent with this hypothesis, we found that β-adrenergic stimulation enhanced not only Cl⁻ reabsorption, but Na⁺ reabsorption as well (Supplement Table 1).

The duct cells from *Cftr*^{ΔF/ΔF} mice also displayed a decreased ENaC-mediated Na⁺ current. In contrast to the pharmacological experiments where ENaC and *Cftr* protein expression was unchanged, the level of α-ENaC protein was severely reduced in the *Cftr*^{ΔF/ΔF} SMG,

but not expression of the β - or γ -ENaC subunits. It can be concluded that the reduced Na^+ reabsorption in the *Cftr* ^{$\Delta F/\Delta F$} mice is at least partially due to reduced expression of α -ENaC rather than a reduced driving force or a direct effect on the gating and/or sorting of ENaC channels upon *Cftr* disruption. Similarly, ENaC activity is dramatically reduced in human CF duct cells or in non-CF duct cells where CFTR activity had been blocked (Reddy *et al.* 1999; Reddy & Quinton, 2003). The decrease in ENaC activity in duct cells from both salivary and sweat gland cells in CF contrasts with the mechanism observed in CF lungs, where an increased ENaC-dependent Na^+ conductance is responsible for the Na^+ hyper-absorption, which is postulated to cause the airway pathology in CF disease (Stutts *et al.* 1995; Kunzelmann, 2001; Riordan, 2008). Consistent with this CF model of airway disease, over-expression of α -ENaC mimicked the CF Na^+ hyper-absorption phenotype (Mall *et al.* 2004). The different mechanisms through which ENaC activity is regulated by *Cftr* expression in exocrine salivary and sweat glands *versus* airway epithelium have important implications for our understanding of the pathophysiology of CF. One possibility is that *Cftr* expression somehow modulates the targeting to the plasma membrane or the functional activity of ENaC in airways (Stutts *et al.* 1995; Kunzelmann, 2001; Riordan, 2008). In contrast, *Cftr* is apparently necessary for α -ENaC gene expression in submandibular gland ducts.

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Author contributions

M.A.C., T.N. and J.E.M. designed the research; M.A.C., T.N., M.G.B. and J.M.C. performed the experiments; M.A.C. and T.N. analysed the data; S.M.W. generated and contributed the anti- α -ENaC rabbit polyclonal antibody. L.L.C. supplied mice for the breeder colonies. All authors wrote and approved the paper. The experiments were performed at the University of Rochester, NY, USA.

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