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RESEARCH PAPER

Protein phosphatase 1 coordinates CFTR-dependent airway epithelial HCO_3^- secretion by reciprocal regulation of apical and basolateral membrane Cl^--HCO_3^- exchangers

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BACKGROUND AND PURPOSE

Our recent studies on human airway serous-like Calu-3 cells showed that cAMP agonists stimulated a HCO_3^- rich secretion containing up to 80 mM HCO_3^-. This alkaline secretion relied on a coordinated switch in the activity of distinct Cl^--HCO_3^- anion exchangers (AE) located at different regions of the cell. At the apical membrane, cAMP agonists activated the electroneutral AE pendrin (SLC26A4), together with cystic fibrosis transmembrane conductance regulator (CFTR), while at the basolateral membrane the agonists inhibited AE2 (SLC4A2). However, the underlying mechanism(s) that orchestrates this cAMP-dependent switch in AE activity has not been elucidated.

EXPERIMENTAL APPROACH

Apical and basolateral Cl^--HCO_3^- exchange was assessed by measuring Cl^--dependent changes in intracellular pH (pH_i).

KEY RESULTS

We show that protein phosphatase 1 (PP1), together with CFTR, play central roles in this reciprocal regulation of AE activity. Activation of pendrin by cAMP agonists, but not inhibition of the basolateral exchanger, was protein kinase A-dependent. Knocking down CFTR expression, or blocking its activity with GlyH-101, led to incomplete inhibition of the basolateral AE by cAMP, supporting a role for CFTR in this process. Addition of the PP1/2A inhibitor, okadaic acid, but not the PP2A specific inhibitor fostreicin, mimicked the effect of cAMP stimulation. Furthermore, okadaic acid-treated Calu-3 monolayers produced a more alkaline fluid than untreated cells, which was comparable with that produced by cAMP stimulation.

CONCLUSIONS AND IMPLICATIONS

These results identify PP1 as a novel regulator of AE activity which, in concert with CFTR, coordinates events at both apical and basolateral membranes, crucial for efficient HCO_3^- secretion from Calu-3 cells.

Abbreviations

$0Cl^-$, absence of Cl^-; ADO, adenosine; AE, anion exchanger; ASL, airway surface liquid; CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; H_2-DIDS, 4,4′-diisothiocyano-1,2-diphenylethane-2,2′-disulfonate; KD, knockdown; KRB, HCO_3^--buffered Krebs solution; NBCe1B, Na^+-HCO_3^- cotransporter e1B; NKCC1, Na^+-K^+-$2Cl^-$ cotransporter; OA, okadaic acid; pH_i, intracellular pH; PKA, protein kinase A; PP, protein phosphatase; SMG, submucosal gland; VIP, vasoactive intestinal peptide

Introduction

The volume and composition of the airway surface liquid (ASL) is critical to the maintenance of ciliary function, mucus rheology and ultimately, to lung defence against inhaled noxious agents (Boucher, 2007). ASL volume and composition are in turn regulated by the ion transport activity of the surface epithelium as well by the underlying submucosal glands (SMGs) (Ballard and Spadafora, 2007). Failure in these transport processes, as in the inherited disease cystic fibrosis (CF), impairs mucus hydration, reduces mucociliary clearance and predisposes the lungs to bacterial infection and disease (Boucher, 2007). Because SMGs are thought to produce the majority of upper ASL in healthy individuals (Reid, 1960), elucidating the mechanism of ion and fluid transport in these glands is very important for understanding lung function in health and disease. Recent *ex vivo* studies from isolated SMGs have shown that fluid secretion is primarily driven by the active secretion of both HCO_3^- and Cl^- in human, sheep, ferret and pig airways (Joo *et al.*, 2001; 2002; Inglis *et al.*, 2003; Ballard and Inglis, 2004). Furthermore, studies from transgenic CF mice and pigs have shown that cAMP-dependent anion secretion from isolated glands is defective due to lack of functional CF transmembrane conductance regulator (CFTR), highlighting an essential role for this channel in anion and fluid transport (Ianowski *et al.*, 2008; Joo *et al.*, 2010; Cho *et al.*, 2011b). Consistent with the idea that CFTR is vital for HCO_3^- secretion, SMGs isolated from CF patients have been shown to secrete a more acidic fluid than non-CF glands (Song *et al.*, 2006). Indeed, recent *in vivo* and *in vitro* studies from normal and CF pigs have provided convincing evidence that ASL pH is vital for innate defence in the lungs (Pezzulo *et al.*, 2012).

Until recently, it has been thought that the exit pathway for HCO_3^- in human airways was solely through CFTR (Smith and Welsh, 1992; Illek *et al.*, 1997; Devor *et al.*, 1999; Krouse *et al.*, 2004), but our recent studies using the human serous-like cell line, Calu-3, has challenged this paradigm (Garnett *et al.*, 2011). In this study, we established that CFTR predominately controlled the rate of liquid secretion, via an increase in electrogenic Cl^- efflux, while the anion exchanger SLC26A4 (pendrin) mediated the majority of HCO_3^- exit across the apical membrane via coupled exchange of Cl^- for HCO_3^-. Our work therefore identified a critical role for pendrin in controlling the composition of the luminal fluid in human airway secretory cells.

We have also shown that Calu-3 cells possess a basolateral AE, which is likely to be SLC4A2 (AE2) based on previous RT-PCR, immunofluorescence and knockdown (KD) studies (Loffing *et al.*, 2000; Huang *et al.*, 2012; Shan *et al.*, 2012). In order for adequate HCO_3^- secretion to occur under cAMP-stimulated conditions, this basolateral Cl^--HCO_3^- exchanger must be inhibited to avoid 'short-circuiting' HCO_3^- transport across the apical membrane, which we have previously reported (Garnett *et al.*, 2011). However, the underlying cellular mechanism responsible for this inhibition in Calu-3 cells, or in other HCO_3^- secreting epithelia where a similar inhibition occurs (Ishiguro *et al.*, 2002), is unknown. In the present study, we provide the first evidence that protein phosphatase (PP) 1 coordinates both the activation of CFTR/pendrin at the apical membrane as well as the inhibition of the basolateral AE by cAMP agonists. We also demonstrate that CFTR is involved in basolateral anion exchange inhibition, but not in PP1 regulation *per se*. Therefore, PP1 appears to be a key molecule in orchestrating transepithelial HCO_3^- and fluid secretion from human serous-like airway cells.

Methods

Calu-3 cell culture

The human adenocarcinoma-derived cell line, Calu-3 [passages 20–50 (Shen *et al.*, 1994)], was grown in Eagle's minimal essential medium plus 10% fetal calf serum, 2 mM L-glutamine, 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 1% non-essential amino acids (Sigma-Aldrich, Gillingham, UK) and incubated in humidified air containing 5% CO_2 at 37°C. CFTR KD Calu-3 cells (MacVinish *et al.*, 2007) were cultured in the same media supplemented with Geneticin (400 μg mL^{-1}; G418; Sigma-Aldrich). Note that CFTR KD experiments were performed at the same time as those presented previously (Garnett *et al.*, 2011), in which we showed ~75% KD of CFTR protein expression by Western blot.

Calu-3 cells were seeded onto clear Costar Transwell® inserts (0.45 μm pore size; Corning, Amsterdam, The Netherlands) at 250 000 cells cm^{-2}, to form a confluent monolayer with a transepithelial resistance of 700–900 ohms cm^{-2} (measured using an epithelial voltohmmeter; World Precision Instruments, Hitchin, UK) after approximately 5 days of growth, which remained stable up to 14 days post-seeding. Experiments were carried out 7–14 days postseeding.

Measurement of intracellular pH (pH_i)

Cells were loaded with the pH-sensitive fluorescent dye 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (10 μM) for 60 min at 37°C in a HEPES-buffered salt solution, which consisted of 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Na-HEPES, and 10 mM D-glucose set to pH 7.4. Transwells were placed in a perfusion chamber, mounted onto an inverted microscope stage (Nikon), and perfused with a HCO_3^--buffered Krebs solution (KRB), which

consisted of 115 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM D-glucose and adjusted to pH 7.4 by bubbling with a 95% O_2, 5% CO_2 mixture at 37°C. Apical and basolateral bath volumes were 0.5 and 1 mL and were perfused at a rate of 3 and 6 mL min^{-1}, respectively. pH_i was measured from 15 to 20 cells, using a Life Sciences Microfluorimeter System (Life Sciences Resources, Cambridge, UK). Ratio values were calibrated to pH_i using the high K^+-nigericin method (10 μM), as described previously (Garnett *et al.*, 2011). Mean changes in pH_i were estimated by calculating the average pH_i over 60 s (120 data points). The initial rate of pH_i change ($\Delta pH_i/\Delta t$) was calculated by linear regression fitted to a minimum of 40 data points. Note that in some figures, pH_i responses are presented as a percentage of control response, due to variability in the absolute magnitude of pH changes in response to Cl^- removal between cultures. For Cl^--free KRB, NaCl was substituted with sodium gluconate, with 6 mM calcium gluconate replacing 1 mM $CaCl_2$ to compensate for the Ca^{2+} buffering capacity of gluconate, and 5 mM KCl was replaced with 2.5 mM K_2SO_4. For Na^+-free KRB, 115 mM NMDG-Cl, replaced NaCl and 25 choline-HCO_3 replaced $NaHCO_3$. Atropine (10 μM) was included to block muscarinic receptors. For anion-substitution experiments, Cl^- was replaced with the appropriate anion (Garnett *et al.*, 2011). All general chemicals were purchased from Sigma-Aldrich except for forskolin (Tocris, Bristol, UK), GlyH-101 (Calbiochem, Nottingham, UK), okadaic acid (Calbiochem) and 8CPT-2Me-cAMP (Tocris).

Transepithelial liquid secretion rates and pH measurements

The rate and pH of liquid secreted from Calu-3 monolayers was measured over a 24 h period as previously described (Garnett *et al.*, 2011).

Statistical analysis

Results are presented as mean ± SE, where *n* indicates the number of experiments. Statistical analysis was performed using either a paired Student's *t*-test or one-way ANOVA with Bonferroni's *post hoc* test. *P* values of <0.05 were considered statistically significant.

Results

Profile of the basolateral Cl^--HCO_3^- exchanger

We have previously reported (Garnett *et al.*, 2011) that polarized cultures of Calu-3 cells produce an alkalinization in their pH_i in response to the removal of extracellular basolateral Cl^- in the absence of cAMP stimulants. This change in pH_i is consistent with the presence of a basolateral Cl^--HCO_3^- exchanger, such that Cl^- removal reverses the AE and drives HCO_3^- into the cell. The response of Calu-3 cells to basolateral Cl^- withdrawal is illustrated in Figure 1A. Overall, $0Cl^-$

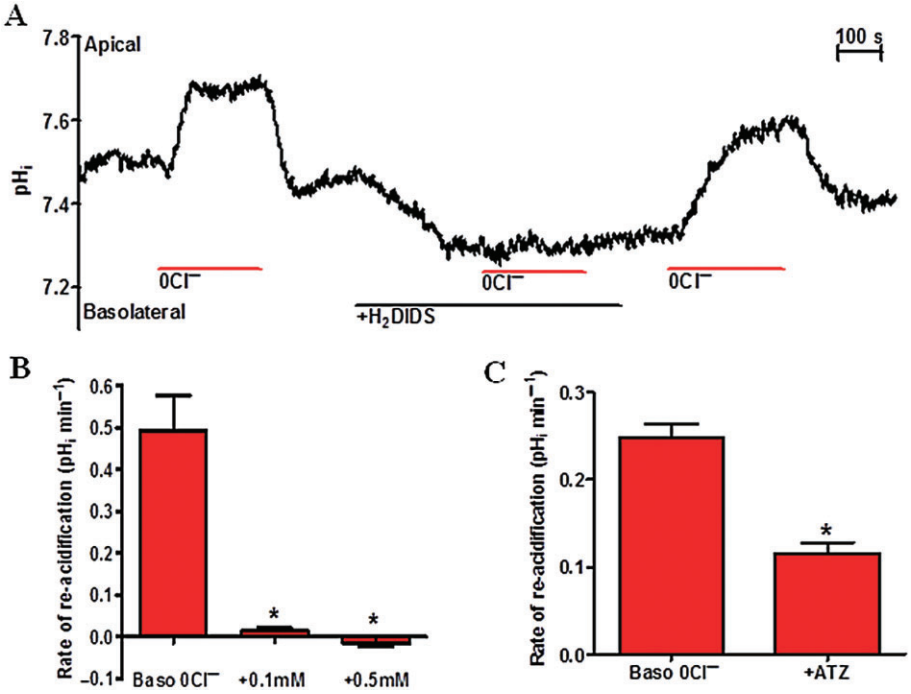


Figure 1

Pharmacological profile of basolateral Cl^--dependent changes in pH_i in Calu-3 cells. A: Representative trace illustrating the effect of basolateral H_2-DIDS (500 μM; indicated by black bar below trace) on changes in pH_i following the removal of basolateral Cl^- (indicated by red bars below trace). B: The effect of basolateral H_2-DIDS (100 and 500 μM) on the mean rate of re-acidification in pH_i upon re-addition of basolateral Cl^- ($n = 4$; paired observations. *$P < 0.05$ compared with Baso $0Cl^-$). C: The effect of the carbonic anhydrase inhibitor acetazolamide (ATZ; 100 μM) on the mean rate of re-acidification in pH_i upon re-addition of Cl^- ($n = 5$; *$P < 0.001$ compared with Baso $0Cl^-$). *Baso $0Cl^-$* denotes the removal of Cl^- (replaced with gluconate) from the basolateral solution.

(substituted with gluconate) produced an alkalinization in pH_i of 0.45 ± 0.02 pH units ($n = 4$). Restoring Cl^- to the basolateral perfusate caused pH_i to recover at a rate of 0.49 ± 0.08 pH units min^{-1} ($n = 4$; Figure 1B). To investigate the properties of this putative AE, the effect of the generic anion transport inhibitor 4,4′-diisothiocyano-1,2-diphenylethane-2,2′-disulfonate (H_2-DIDS) was tested. Figure 1A & B shows that both 0.1 mM and 0.5 mM H_2-DIDS completely abolished the pH_i response to Cl^- removal ($P < 0.05$, paired t-test; $n = 4$; Figure 1A & B).

The HCO_3^--dependence of the basolateral AE was tested by removing HCO_3^- and buffering solution pH to 7.4 using HEPES (see Materials and Methods). In HCO_3^--free conditions, the removal of basolateral Cl^- caused a small acidification in pH_i of 0.07 ± 0.04 pH units ($P < 0.05$; $n = 4$). These results suggest that there is little or no OH^- transport by the basolateral exchanger. Previous studies have shown that SLC4A2 (AE2) is sensitive to acetazolamide because of its association with the cytoplasmic form of carbonic anhydrase (CA) II (Vince and Reithmeier, 2000). Figure 1C shows that 100 μM acetazolamide reduced the rate of re-acidification in response to the re-addition of basolateral Cl^- by 46.5 ± 10.5% ($P < 0.01$; $n = 5$). In the absence of HCO_3^- production by CA, intracellular HCO_3^- levels are likely maintained by uptake through the Na^+-HCO_3^- cotransporter e1B (NBCe1B), thus sustaining basolateral Cl^--HCO_3^- exchange. Overall, the results in Figure 1 provide clear evidence for a DIDS-sensitive Cl^--HCO_3^- anion exchanger on the basolateral membrane of Calu-3 cells, which is consistent with a previous report showing SLC4A2 expression on the basolateral, but not apical, membrane of Calu-3 cells by immunofluorescence (Loffing *et al.*, 2000).

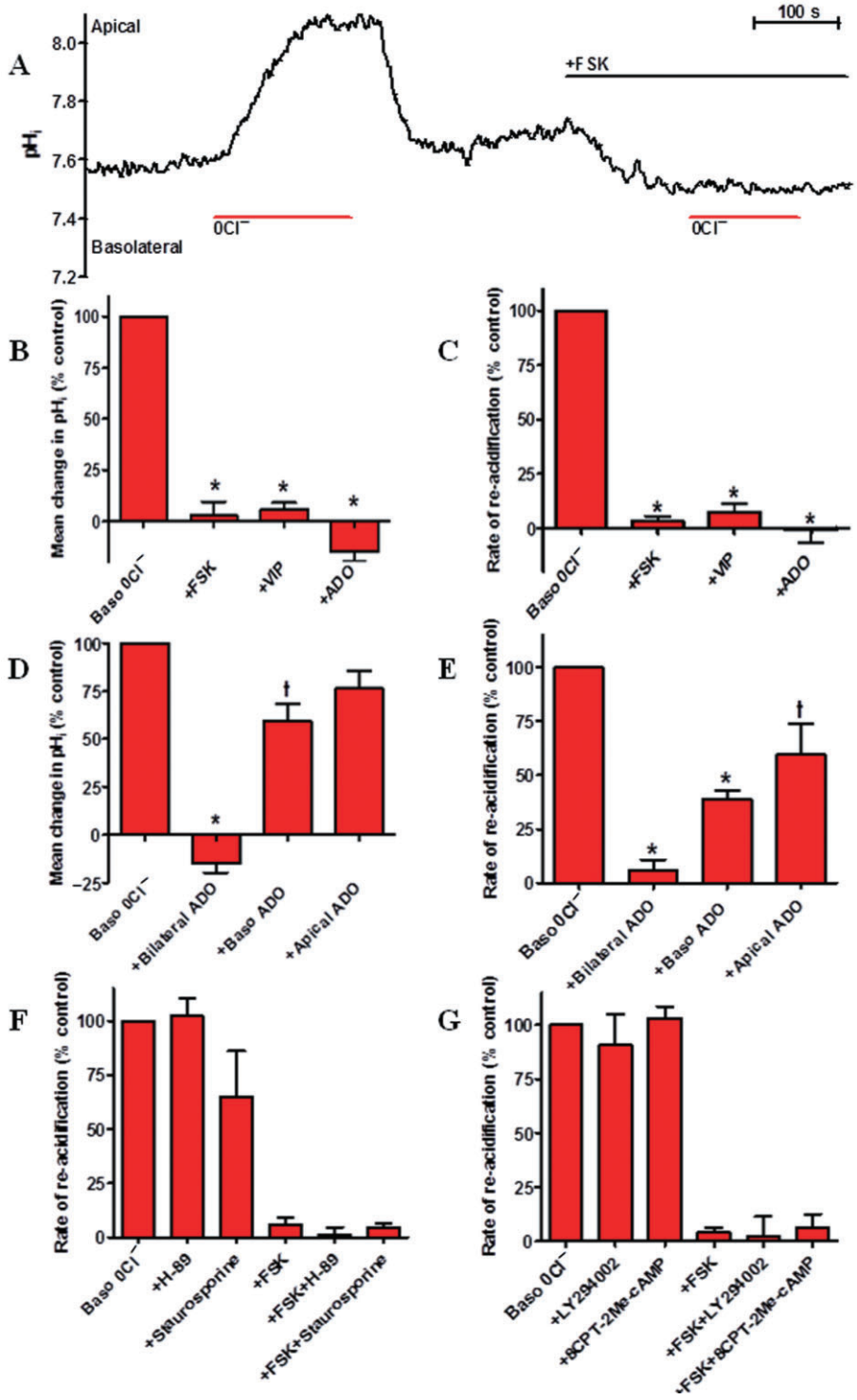
Inhibition of the basolateral Cl^--HCO_3^- exchanger by cAMP agonists

Basolateral AE activity was completely abolished following the addition of the cAMP agonist forskolin (5 μM, applied to apical solution; Figure 2A), but the inactive analogue, dideoxyforskolin, had no effect (mean alkalinization of 0.35 ± 0.05 pH_i units in response to basolateral Cl^- removal; $P > 0.05$ compared with control response; $n = 3$). Note that forskolin addition caused a characteristic slow, but significant, acidification in pH_i (Figure 2A) due to stimulation of HCO_3^- efflux from Calu-3 cells as previously reported (Garnett *et al.*, 2011). A similar inhibition of basolateral AE activity also occurred in cells stimulated by vasoactive intestinal peptide (VIP; 150 nM) in the basolateral perfusate, or by adenosine (ADO; 10 μM) when added simultaneously to both the apical and basolateral solutions (Figure 2B & C). Both these agonists are known to be linked to cAMP production in Calu-3 cells (Cobb *et al.*, 2002; Dérand *et al.*, 2004). Note that all three secretagogues have also been previously shown to fully activate the apical AE, pendrin, in these cells (Garnett *et al.*, 2011).

Although application of ADO to both apical and basolateral membranes completely inhibited basolateral anion exchange, when ADO was applied to the basolateral membrane alone, basolateral AE activity was reduced, but no longer eliminated (Figure 2D & E). In this case, ADO reduced the mean alkalinization produced by basolateral Cl^- removal by 40.5 ± 9.2% and the rate of re-acidification by 61.2 ± 4.1% ($P < 0.01$; $n = 3$). In marked contrast, application of ADO to the apical membrane only did not significantly inhibit the alkalinization produced by basolateral Cl^- removal ($P > 0.05$; $n = 3$; Figure 2D), despite full activation of apical pendrin under these conditions (mean alkalinization of 0.50 ± 0.10 pH_i units produced by apical Cl^- removal; $P > 0.05$ compared with responses in the presence of bilateral ADO or forskolin; $n = 3$; experiments ran in parallel). These results show that activation of the apical AE alone is not sufficient to inhibit basolateral AE activity, and suggest that local changes in cAMP concentration at both membranes are required to produce a 'switch' in AE activity. From the ADO experiments there appeared to be a partial correlation between agonist-induced acidification in pH_i, and the amount of inhibition of the basolateral AE. Bilateral ADO produced a mean acidification of 0.23 ± 0.01 pH_i units and also the greatest inhibition of the basolateral AE (rate % inhibition = 94.2 ± 4.9%; $P < 0.001$; $n = 3$; Figure 2E). In contrast, the addition of ADO to either the basolateral or apical membranes alone failed to produce a significant acidification in pH_i ($P > 0.05$; $n = 3$) and also failed to completely inhibit the basolateral AE ($P < 0.01$ compared with rate % inhibition in presence of bilateral ADO; $n = 3$; Figure 2E), unlike bilateral ADO.

Potential downstream targets of cAMP were examined to elucidate the mechanisms involved in the inhibition of the basolateral AE in Calu-3 cells. Despite protein kinase A (PKA) being involved in stimulation of the apical Cl^--HCO_3^- exchanger in these cells (Garnett *et al.*, 2011), neither the PKA-inhibitor H-89 (50 μM; 60 min pretreatment in Krebs solution), nor the general protein kinase inhibitor staurosporine (1 μM; 60 min pretreatment in Krebs solution), showed any significant effect on basolateral AE activity under non-stimulated conditions, or on the forskolin-induced inhibition of the basolateral AE ($P > 0.05$; Figure 2F). Cyclic AMP is known to activate phosphatidylinositol 3-kinase (PI3-kinase), which stimulates Cl^- and bile acid secretion in biliary epithelial cells, via IP_3 production (Kagawa *et al.*, 2002). However, the PI3-kinase inhibitor, LY294002 (20 μM; 60 min pretreatment in Krebs solution), had no effect on the response to cAMP (Figure 2G). Another downstream target of cAMP is the guanine-nucleotide exchange protein directly activated by cAMP (Epac). We tested for Epac involvement by incubating the cells with the Epac agonist, 8CPT-2Me-cAMP [50 μM; 60 min pretreatment in Krebs solution (Enserink *et al.*, 2002)], but like LY294002, this had no effect on basolateral AE activity (Figure 2G).

To investigate the apparent temporal 'switch' in apical and basolateral AE activity by cAMP elevation in more detail, we measured the lag time for (i) inhibition and (ii) activation of the respective AEs (Figure 3A and B, respectively). For these experiments, cells were first exposed to a Cl^--free solution prior to stimulation by 5 μM forskolin. In the absence of basolateral Cl^- (Figure 3A), forskolin produced an acidification in pH_i after 17.0 ± 2.0 s ($n = 4$). In the absence of apical Cl^- (Figure 3B), forskolin caused a rapid alkalinization in pH_i after 37.5 ± 4.0 s (response 1; $n = 4$), at a rate and magnitude (ΔpH_i change) similar to removal of apical Cl^- *after* stimulation with forskolin (5 μM; response 2; $P > 0.05$; $n = 4$). Comparison of the two time-courses suggests that forskolin



inhibits the basolateral AE prior to stimulating apical anion exchange activity ($P < 0.05$; $n = 4$).

CFTR-dependent inhibition of basolateral Cl^--HCO_3^- exchange

To investigate whether CFTR is involved in the inhibition of the basolateral Cl^--HCO_3^- exchanger by cAMP agonists, experiments were conducted on CFTR KD Calu-3 cells (Figure 4A, light grey trace), in which CFTR protein expression and secretory function is reduced by ~75% (MacVinish *et al.*, 2007; Garnett *et al.*, 2011). The removal of basolateral Cl^- under non-stimulated conditions produced an alkalinization of 0.46 ± 0.01 pH_i units ($P > 0.05$ compared with WT Calu-3 cells; $n = 4$), which was entirely H_2-DIDS sensitive (mean alkalinization of 0.02 ± 0.01 pH_i units in response to basolateral $0Cl^-$; $P < 0.05$; $n = 3$). Significantly, however, the re-acidification rate was reduced in these CFTR KD cells by 31.9 ± 10.4%, compared with WT Calu-3 cells (Figure 4B).

Figure 2

Inhibition of basolateral Cl^--HCO_3^- exchanger by cAMP in Calu-3 cells. A: Representative trace illustrating the effect of apical forskolin (FSK) addition (5 μM; indicated by black bar above trace) on changes in pH_i following the removal of basolateral Cl^- (indicated by red bars below trace). B: The effect of apical FSK (5 μM), basolateral VIP (150 nM) and bilateral ADO (10 μM) on the percentage mean change in pH_i following the removal of basolateral Cl^-. pH_i responses in the presence of FSK, VIP and ADO compared with control basolateral $0Cl^-$ responses. Agonists ran in parallel in separate experiments ($n = 4$; $^*P < 0.001$ compared with Baso $0Cl^-$). C: The effect of apical forskolin, basolateral VIP and bilateral ADO on the percentage mean rate of re-acidification following re-addition of basolateral Cl^- ($n = 4$; $^*P < 0.001$ compared with Baso $0Cl^-$). D: The effect of bilateral, apical only and basolateral only ADO (10 μM) addition on the percentage mean change in pH_i produced by basolateral Cl^- removal. pH_i responses in the presence of bilateral ADO, basolateral ADO and apical ADO compared with control baso $0Cl^-$ responses ($n = 3$; each condition ran in parallel in separate experiments. $^*P < 0.001$ compared with Baso $0Cl^-$. $^\dagger P < 0.01$ compared with Baso $0Cl^-$). E: The effect of bilateral, basolateral only and apical only ADO (10 μM) addition on the percentage rate of re-acidification upon basolateral Cl^- re-addition ($n = 3$; $^*P < 0.001$ compared with Baso $0Cl^-$. $^\dagger P < 0.05$ compared with Baso $0Cl^-$). F: The effect of the PKA inhibitor H-89 (50 μM) and of the protein kinase inhibitor staurosporine (1 μM) on the percentage rate of re-acidification upon basolateral Cl^- re-addition in non-stimulated and forskolin-stimulated cells. pH_i responses in the presence of H-89 and staurosporine compared with control baso $0Cl^-$ responses (± FSK) ($n = 4$; H-89 and staurosporine experiments ran in parallel). G: The effect, of the PI3 kinase inhibitor LY294002 (20 μM) and Epac agonist, 8CPT-2Me-cAMP (50 μM) on the percentage mean rate of re-acidification upon basolateral Cl^- re-addition in non-stimulated and forskolin-stimulated cells. pH_i responses in the presence of LY294002 and 8CPT-2Me-cAMP (± FSK) compared with control baso $0Cl^-$ (± FSK) responses ($n = 4$; Control, LY294002 and 8CPT-2Me-cAMP ran in parallel).

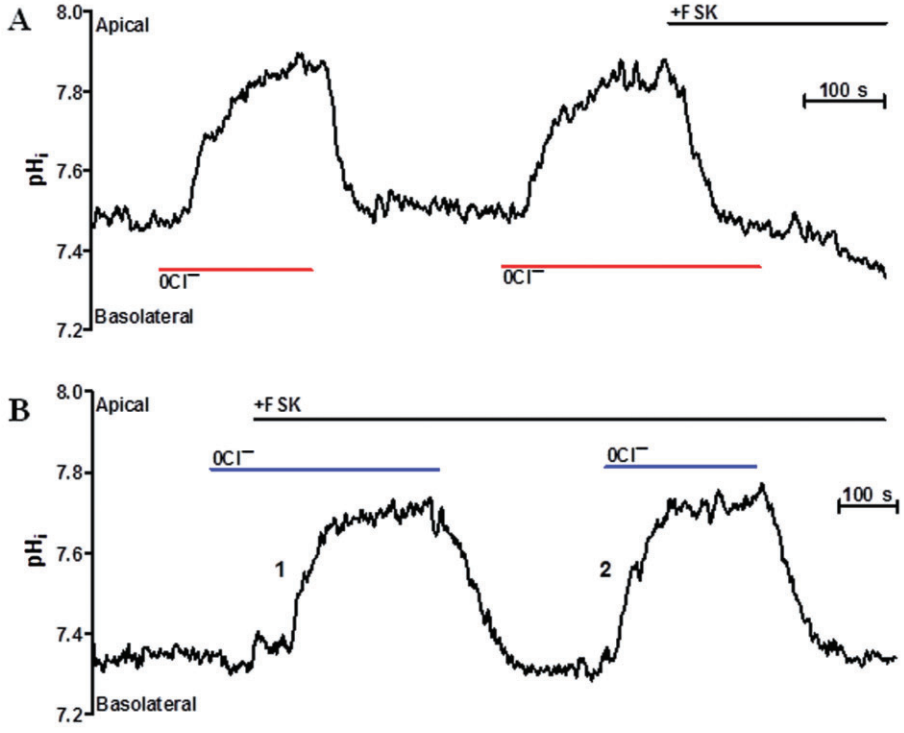


Figure 3

Forskolin-induced changes in Calu-3 cell pH_i in the absence of either basolateral or apical Cl^-. Time course studies for examining the effect of forskolin (5 μM) on pH_i when applied to cells exposed to (A) basolateral zero Cl^- and (B) apical zero Cl^- conditions. Panel B shows a comparison between the effect of forskolin (5 μM) added during apical zero Cl^- (response 1) or added prior to apical Cl^- removal (response 2). Note: bars above and below traces indicate changes to the apical and basolateral solutions, respectively.

Furthermore, forskolin was now unable to fully inhibit the basolateral AE (Figure 4A & C). To further investigate the role of CFTR in the inhibition of the basolateral AE by forskolin, the apical membrane of Calu-3 cells was exposed to the CFTR pore blocker GlyH-101 (Muanprasat *et al.*, 2004) prior to basolateral Cl^- removal (Figure 4D). Under non-stimulated conditions GlyH-101 had no effect on the mean alkalinization produced by the removal of basolateral Cl^- (Figure 4E), but the rate of re-acidification decreased by 56.5 ± 15.2% (Figure 4F). Similar to the results with CFTR KD cells, forskolin now failed to fully inhibit the basolateral exchanger in the presence of the CFTR blocker, with 56.1 ± 19.9% of the AE-induced pH_i change still present (Figure 4E). These results suggest that cAMP inhibition of basolateral Cl^--HCO_3^- exchange is mediated, in part, through a CFTR-dependent mechanism. Because we, and others, have previously shown

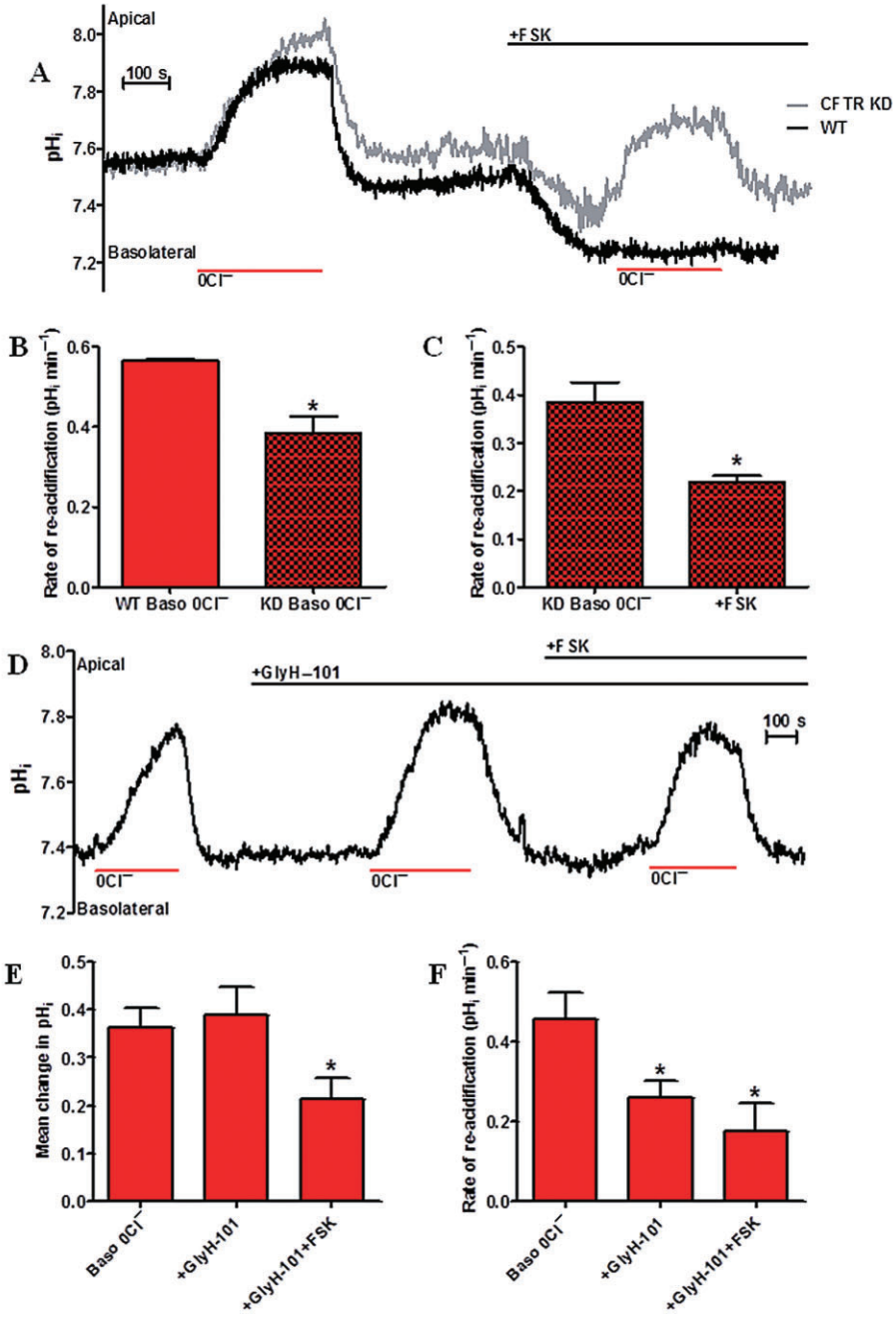


Figure 4

CFTR-dependent inhibition of the basolateral Cl^--HCO_3^- exchanger in normal and CFTR KD Calu-3 cells. A: Representative traces comparing the effects of forskolin (5 μM) on changes in pH_i following the removal of basolateral Cl^- in normal (WT) and CFTR KD Calu-3 cells. B: Comparison of the mean rate of re-acidification upon re-addition of basolateral Cl^-, between non-stimulated WT and CFTR KD Calu-3 cells ran in parallel ($n = 4$; $^*P < 0.05$ compared with Baso $0Cl^-$). C: The effect of forskolin (FSK; 5 μM) on the mean rate of re-acidification upon re-addition of Cl^-, in CFTR KD Calu-3 cells ($n = 4$; paired observations. $^*P < 0.05$ compared with Baso $0Cl^-$). D: Representative trace illustrating the effect of apical GlyH-101 (10 μM) on basolateral Cl^--dependent changes in pH_i in Calu-3 cells. E: The effect of the CFTR inhibitor GlyH-101 (10 μM) on mean alkalinization of pH_i following removal of basolateral Cl^-, in non-stimulated and forskolin-stimulated Calu-3 cells ($n = 4$; paired observations. $^*P < 0.05$ compared with Baso $0Cl^-$). F: The effect of the CFTR inhibitor GlyH-101 (10 μM) on the mean rate of re-acidification upon re-addition of Cl^-, in non-stimulated and forskolin-stimulated Calu-3 cells ($n = 4$; $^*P < 0.05$ compared with Baso $0Cl^-$).

that pendrin is not affected by this CFTR blocker (Garnett *et al.*, 2011; Stewart *et al.*, 2011), we can be confident that the reduction in basolateral AE activity by GlyH-101 is not via a change in pendrin activity. Furthermore, although GlyH-101 has recently been shown to block Cl^- transport by several other members of the SLC26 family, including A3, A6, A9 and A11 (Bertrand *et al.*, 2009; Stewart *et al.*, 2011), SLC26 mRNA expression and pharmacological data (Garnett *et al.*, 2011; Stewart *et al.*, 2011) argue that it is unlikely, but not excluded, that the activity of other SLC26 transporters in the apical membrane of Calu-3 cells accounts for the results we observe with GlyH-101.

Okadaic acid inhibits basolateral and stimulates apical Cl^--HCO_3^- exchange

Our results so far suggest that CFTR is involved in both the inhibition of the basolateral anion exchanger as well as the activation of the apical exchanger (pendrin) by cAMP agonists (Garnett *et al.*, 2011). To better understand how this reciprocal regulation of Cl^--HCO_3^- exchangers is coordinated, we investigated the role of PPs. To do this Calu-3 cells were pretreated with 100 nM okadaic acid (OA), a PP1/PP2A inhibitor (Haystead *et al.*, 1989), for 60 mins (pretreatment in Krebs solution). OA-treated Calu-3 cells had a more acidic pH_i compared with untreated cells (OA-treated cell $pH_i = 7.03 \pm 0.06$; untreated cell $pH_i = 7.43 \pm 0.03$; $P < 0.01$; $n = 15$). Surprisingly, OA exposure produced a switch in Cl^--HCO_3^- exchange activity that qualitatively mimicked the effect of cAMP agonists. In OA-treated cells the basolateral exchanger was now completely inactive, whilst apical Cl^- withdrawal produced a significant alkalinization, suggesting that OA treatment had also activated an apical Cl^--HCO_3^- exchanger (Figure 5A). Importantly, the alkalinization in pH_i following apical Cl^- removal in OA-treated cells could be further enhanced by forskolin (Figure 5A & B) to levels not significantly different to untreated cells exposed to forskolin (Figure 5B). OA treatment caused similar effects on the rate of re-acidification (Figure 5C). Because the profile of the OA-activated apical anion exchanger was similar to the forskolin-activated exchanger (Garnett *et al.*, 2011), being H_2-DIDS insensitive, Na^+-independent (Figure 5D) and only capable of transporting monovalent anions in exchange for HCO_3^- (Figure 5E), our data strongly suggest that this anion exchange was mediated by pendrin.

Increasing the concentration of OA from 100 to 500 nM did not further enhance apical Cl^--HCO_3^- exchange activity (under basal conditions), but lowering the concentration of OA to 10 nM failed to activate any anion exchange activity (Figure 6A). Similarly, the inhibition of the basolateral Cl^--HCO_3^- exchanger by OA was also only seen at the higher concentrations (100 and 500 nM, and not 10 nM; Figure 6B). Overall, this OA dose–response is consistent with the inhibition of PP1 rather than PP2A, according to the specificity of OA for the two PPs (PP2A IC_{50} ~0.5 nM; PP1 IC_{50} ~50 nM). These results also reveal that the stimulation of apical anion exchange activity by OA is unlikely to be due to a more acid pH_i, since cells treated with 10 nM OA had comparable pH_i (7.09 ± 0.25; $P > 0.05$; $n = 3$) but did not show an active apical anion exchanger (Figure 6A). In addition, although CFTR activity has been shown to be affected by pH_i (Reddy *et al.*, 1998; Chen *et al.*, 2009), this result also rules out a causal role for a pH_i-dependent change in CFTR activity as the mechanism underlying the activation of apical anion exchange by PPs.

To further investigate which PP was involved in the stimulation of the apical AE by OA, cells were pretreated to fostriecin, a potent PP2A inhibitor (PP2A IC_{50} 3.2 nM; PP1 IC_{50} 131 μM; Walsh *et al.*, 1997). 100 nM fostriecin (60 min pretreatment in Krebs solution), a concentration that should only inhibit PP2A, was unable to stimulate apical AE activity, in marked contrast to 100 nM OA. However, fostriecin pretreatment did modestly, but significantly, decrease forskolin-stimulated apical AE activity (Figure 6C). Fostriecin was also without effect on the basolateral AE ($P > 0.05$; $n = 4$; Figure 6D), strongly implicating PP1 in its inhibition. However, blocking PP2A did appear to restore some basolateral Cl^--HCO_3^- exchange activity under forskolin-stimulated conditions (Figure 6D). Taken together, these results suggest that PP1 inhibition is responsible for both the stimulation of apical AE activity as well as the reduction in basolateral AE activity, under resting conditions. However, PP2A appears to have some role in regulating both anion exchangers in the presence of forskolin.

PKA-dependence of OA-activated apical Cl^--HCO_3^- exchange activity

The apical Cl^--HCO_3^- exchange activity present in OA-treated Calu-3 cells was not blocked by pretreatment with the PKA-inhibitor H-89 (50 μM) under 'basal' conditions (Figure 7A & B). However, following forskolin stimulation, both the mean change in pH_i produced by apical Cl^- removal (Figure 7A) and the rate of acidification upon the subsequent re-addition of apical Cl^- (Figure 7B), were significantly reduced by H-89, consistent with forskolin enhancing apical AE activity through a PKA-dependent mechanism (compared with Figure 5B & C).

CFTR-dependence of the OA 'switch' in Calu-3 Cl^--HCO_3^- exchange activity

The apical anion exchange activity observed in OA-treated cells (Figure 5) was found not to be affected by GlyH-101 (10 μM) suggesting that it was independent of CFTR (Figure 8). In contrast, the enhancement of this OA-activated anion exchanger by forskolin (depicted in Figure 5), was prevented by GlyH-101 (Figure 8A & B, and compare to Figure 5A & B), suggesting that CFTR was essential. To investigate this CFTR-dependence further, we also studied the effect of OA in CFTR KD Calu-3 cells (Figure 8C). Like normal (WT) Calu-3 cells, OA-treated CFTR KD cells also exhibited an apical Cl^--HCO_3^- exchange activity under basal conditions (mean alkalinization of 0.23 ± 0.04 pH_i units in response to apical $0Cl^-$; $n = 4$), which again could be further enhanced by forskolin (0.44 ± 0.03 pH_i units; $P < 0.05$ compared with response under basal conditions; $n = 4$). However, the rate of re-acidification after forskolin stimulation was significantly less than in the OA-treated WT Calu-3 cells ($P < 0.05$; $n = 4$; Figure 8D), supporting the hypothesis that this enhancement of apical Cl^--HCO_3^- exchange activity by forskolin is through a CFTR-dependent mechanism.

CFTR expression also appeared to impact on the regulation of the basolateral anion exchanger by OA, because

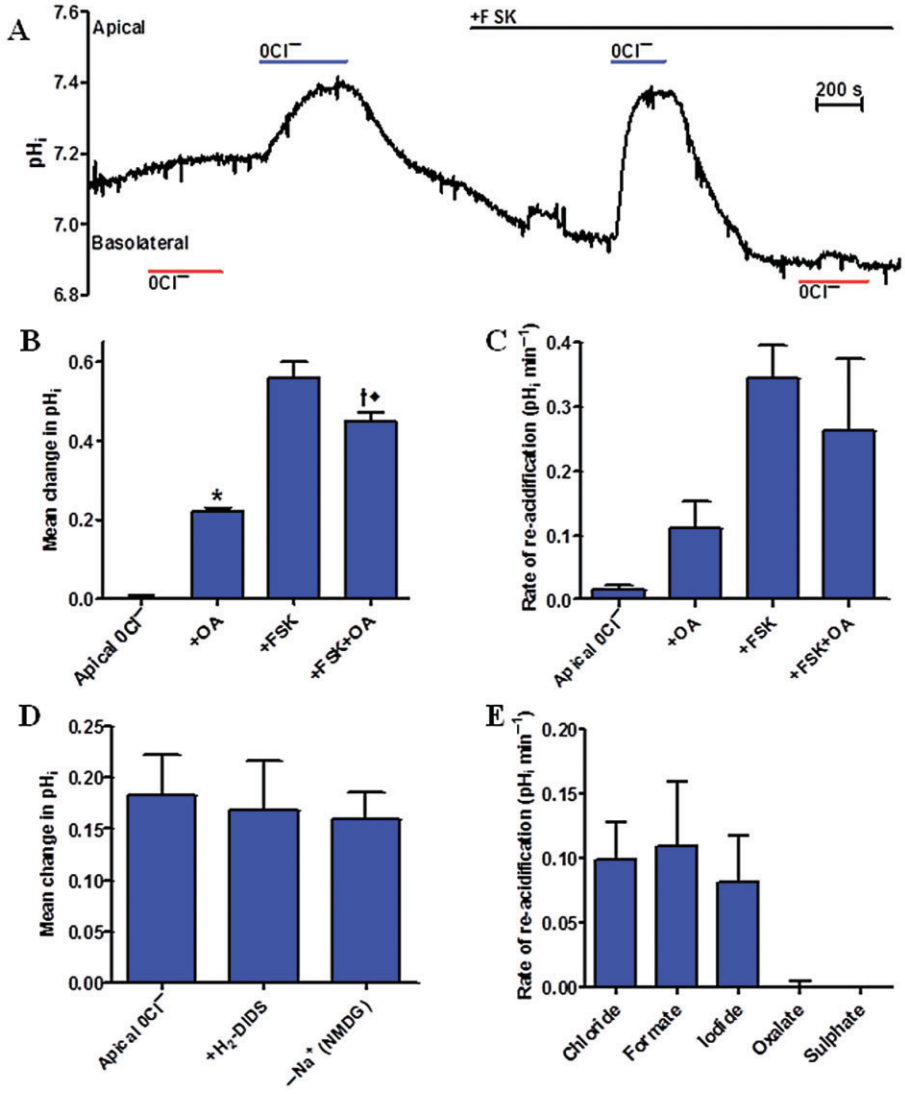


Figure 5

OA-dependent 'switch' in Calu-3 Cl^--HCO_3^- exchange activity. A: Representative pH_i trace illustrating the effect of pretreating Calu-3 cells with OA (100 nM) on the pH_i response to basolateral and apical Cl^- removal, in the absence and presence of forskolin (FSK). B: The effect of OA (100 nM) on the mean change in pH_i following the removal of apical Cl^- in unstimulated and forskolin-stimulated (5 μM) Calu-3 cells ($n = 5$; OA-treated and untreated Calu-3 cell experiments were run in parallel. *$P < 0.001$ compared with apical 0Cl^-. †$P < 0.05$ compared with +FSK. ♦$P < 0.001$ compared with +OA). C: The effect of OA on the mean rate of re-acidification in pH_i upon the re-addition of apical Cl^- in unstimulated and forskolin-stimulated WT Calu-3 cells ($n = 5$). D: The effects of apical H_2-DIDS (500 μM) and apical Na^+ removal (replaced with NMDG) on the mean alkalinization in pH_i following the removal of apical Cl^- in OA-treated (100 nM) Calu-3 cells ($n = 3$). E: Anion selectivity of the apical AE in OA-treated Calu-3 cells. The rate of recovery of pH_i by the introduction of monovalent (iodide, formate and chloride) and divalent (oxalate and sulphate) anions in zero Cl^- conditions, in okadaic acid (100 nM) treated Calu-3 cells ($n = 4$; paired observations).

OA-treated CFTR KD cells displayed some residual basolateral Cl^--HCO_3^- activity which was not observed in OA-treated WT cells (Figure 8C and compare to 5A). However, this residual basolateral Cl^--HCO_3^- exchange activity in OA-treated CFTR KD cells was still significantly less than in untreated CFTR KD cells but, interestingly, was not affected by forskolin (Figure 8E). Collectively, these results in CFTR KD cells further illustrate the importance of CFTR in regulating the 'switch' in AE activity and, in particular, its role in the inhibition of the basolateral exchanger.

Okadaic acid stimulates HCO_3^- but not fluid secretion

We have previously demonstrated that cAMP agonists increase the rate of transepithelial liquid secretion as well as the pH ($[HCO_3^-]$) of the secreted fluid from Calu-3 cells, through the coordinated activation of CFTR and pendrin at the apical membrane (Garnett *et al.*, 2011). Because OA treatment appeared to mimic the effect of cAMP agonists we speculated that this may also alter HCO_3^- secretion. Figure 9A

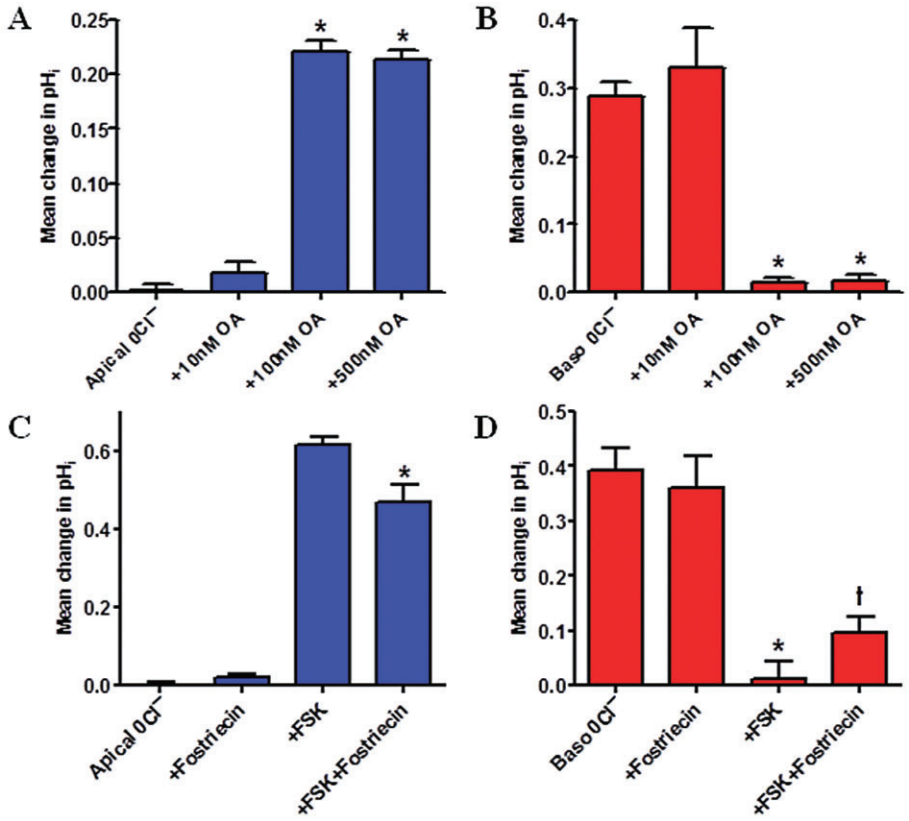


Figure 6

Okadaic acid dose–response and fostriecin experiments reflect PP1-dependence of OA-mediated 'switch' in Calu-3 AE activity. A: Dose–response effect of okadaic acid (0, 10, 100 and 500 nM) on the mean change in pH_i following the removal of apical Cl^- in unstimulated Calu-3 cells ($n = 4$; OA treatments were carried out in separate experiments that were run in parallel. *$P < 0.001$ compared with no OA and 10 nM OA). B: Dose–response effect of OA on mean change in pH_i following the removal of basolateral Cl^- in non-stimulated Calu-3 cells ($n = 3$; *$P < 0.01$ compared with no OA and 10 nM OA). C: The effect of fostriecin (100 nM) on the mean change in pH_i following the removal of apical Cl^- in unstimulated and forskolin (FSK)-stimulated Calu-3 cells ($n = 4$; fostriecin treated and untreated Calu-3 cell experiments were run in parallel. *$P < 0.01$ compared with apical $0Cl^-$ + FSK). D: The effect of fostriecin (100 nM) on the mean change in pH_i following the removal of basolateral Cl^- in non-stimulated and forskolin-stimulated Calu-3 cells ($n = 4$; *$P < 0.001$ compared with Baso $0Cl^-$ and +Fostriecin. †$P < 0.01$ compared with Baso $0Cl^-$ and +Fostriecin).

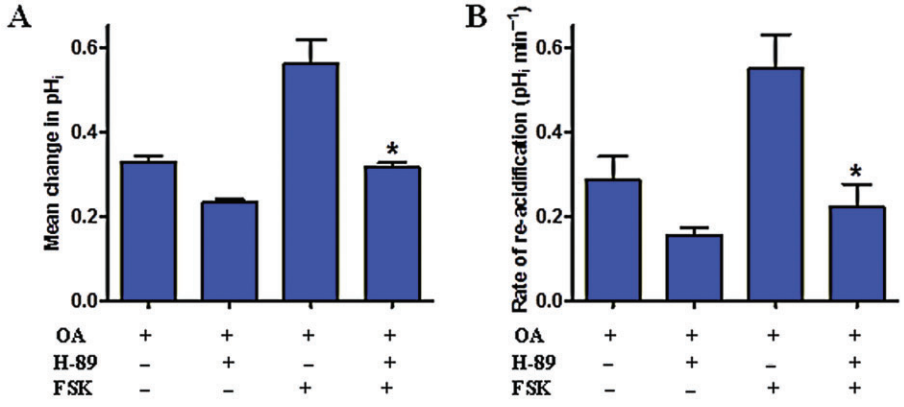


Figure 7

PKA-dependence of OA-activated apical Cl^--HCO_3^- exchange in Calu-3 cells. A: The effect of the PKA-inhibitor H-89 on the mean alkalinization in pH_i following the removal of apical Cl^- in unstimulated and forskolin-stimulated (5 μM) cells treated with okadaic acid (100 nM) ($n = 4$; *$P < 0.001$ compared with +OA + forskolin (FSK); paired observations). B: The effect of the PKA-inhibitor H-89 on the mean rate of re-acidification following the re-addition of apical Cl^-, in unstimulated and forskolin-stimulated cells treated with okadaic acid ($n = 4$; *$P < 0.01$ compared with +OA + FSK).

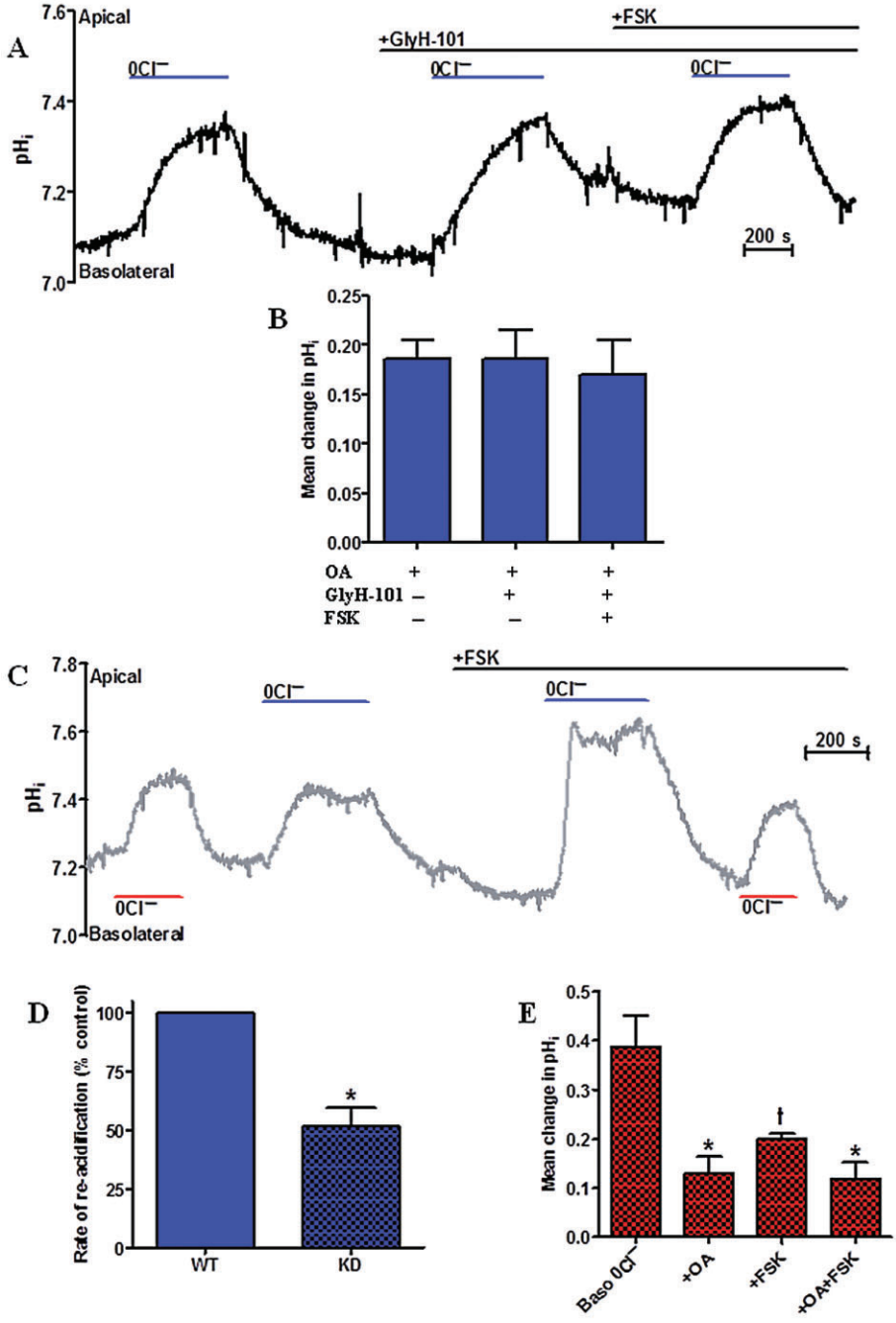


Figure 8

CFTR-dependence of OA-mediated 'switch' in normal and CFTR KD Calu-3 AE activity. A: Representative trace illustrating the effect of the CFTR inhibitor GlyH-101 (10 μM) present in the apical perfusate, on the change in pH_i following the removal of apical Cl^- in unstimulated and forskolin-stimulated (5 μM) WT Calu-3 cells treated with OA (100 nM). B: The effect of apical GlyH-101 on the mean alkalinization in pH_i following the removal of apical Cl^-, in unstimulated and forskolin-stimulated WT Calu-3 cells treated with OA ($n = 4$; paired observations). C: Representative trace illustrating changes in pH_i following the removal of apical Cl^- in unstimulated and forskolin-stimulated (5 μM) CFTR KD Calu-3 cells treated with OA (100 nM). D: The mean percentage rate of re-acidification following the re-addition of apical Cl^- in OA-treated, forskolin-stimulated CFTR KD cells, compared with the same response in WT Calu-3 cells ($n = 4$; OA-treated and untreated CFTR KD Calu-3 cell experiments were run in parallel. *$P < 0.01$ compared with WT). E: The effect of OA on the mean change in pH_i following the removal of basolateral Cl^- in non-stimulated and forskolin-stimulated CFTR KD Calu-3 cells ($n = 4$; *$P < 0.01$ compared with Baso $0Cl^-$. †$P < 0.05$ compared with Baso $0Cl^-$).

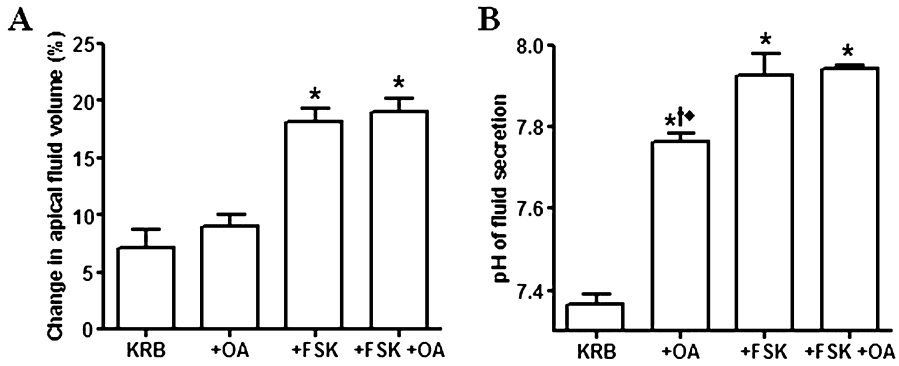


Figure 9

OA increases the pH but not the volume of fluid secreted by Calu-3 cells. Apical fluid volume (% change in volume; (A) and pH (B) in untreated and OA-treated Calu-3 cells under basal and forskolin (FSK)-stimulated conditions measured after 24 h ($n = 4$; fluid volume and pH measurements represent paired observations from the same transwells. (*$P < 0.0001$ compared with KRB. †$P < 0.05$ compared with +FSK. ♦$P < 0.01$ compared with OA + FSK).

shows that OA treatment had no effect on the volume of secreted fluid ($P > 0.05$; $n = 4$), but it did significantly enhance the pH of the apical fluid from 7.37 ± 0.02 to 7.77 ± 0.02 (Figure 9B), consistent with the effects of OA on apical and basolateral AEs. Furthermore, forskolin stimulation further increased both the volume and pH of apical fluid from OA-treated Calu-3 cells to levels similar to untreated cells ($P > 0.05$; $n = 4$; Figure 9A & B).

Discussion

In this study we have investigated the properties and regulation of Cl^--HCO_3^- anion exchangers on the basolateral and apical membranes of polarized cultures of Calu-3 cells. On the basolateral membrane our results showing that Cl^--dependent changes in pH_i were inhibited by the AE inhibitor H_2-DIDS, as well as by acetazolamide, supports the presence of the DIDS-sensitive anion exchanger, SLC4A2 (Loffing *et al.*, 2000; Huang *et al.*, 2012). Previous RT-PCR analysis of cells from various segments of the tracheobronchial tree detected the expression of SLC4A2 and brain-A3 isoforms, but not A1 or cardiac-A3 isoforms (Al-Bazzaz *et al.*, 2001). The levels of SLC4A2 mRNA expression were shown to be similar from the trachea all the way down to the small bronchi, with immunohistochemical analysis indicating that SLC4A2 is specific to airway epithelial cells.

Regulation of the basolateral Cl^--HCO_3^- exchanger in Calu-3 cells

SLC4 Cl^--HCO_3^- exchangers have previously been found to be active under cAMP-stimulated conditions in several tissues, such as SLC4A2 in the murine proximal colon (Gawenis *et al.*, 2010). However, the H_2-DIDS sensitive basolateral Cl^--HCO_3^- exchanger in guinea pig pancreatic duct cells was inhibited by an elevation of intracellular cAMP (Ishiguro *et al.*, 2002), similar to the basolateral AE in Calu-3 cells. Somewhat surprisingly, the inhibition by cAMP in Calu-3 cells appeared not to involve PKA, nor other potential downstream targets such as Epac and PI3K. Furthermore, it could not be explained by a change in pH_i, which has previously been shown to regulate SLC4A2 (Zhang *et al.*, 1996; Stewart *et al.*, 2001; 2007). Nonetheless, because pretreating cells with okadaic acid abolished SLC4A2 activity, the data strongly suggest that phosphorylation of SLC4A2 (or a regulatory protein) by an unknown kinase, is necessary for its inhibition. Based on the differential effects of okadaic acid and fostriecin, this regulation involves PP1, but not PP2A. As PPs can be activated by increases in cAMP, through a PKA-independent pathway (Feschenko *et al.*, 2002), they represent a potential mechanism for regulating HCO_3^- transport in Calu-3 cells.

PP1 inhibition reveals a CFTR-independent 'switch' in Calu-3 AE activity

In addition to regulating the basolateral AE, PP1 was also found to play a critical role in controlling the apical Cl^--HCO_3^- exchanger, pendrin. In this case, inhibiting PP1 led to pendrin activation in the absence of cAMP stimulation. Previous studies in Calu-3 cells have shown that the basolateral Na^+-K^+-$2Cl^-$ cotransporter (NKCC1) and apical CFTR are regulated by PP1/2A (Liedtke *et al.*, 2005; Thelin *et al.*, 2005), and that PP2A regulatory subunits physically associate with the carboxyl terminus of CFTR (Thelin *et al.*, 2005). PP1 therefore appears to be a key molecule in regulating HCO_3^- transport at both the apical and basolateral membranes.

The PP1-activated apical AE had a similar profile to that seen under forskolin-stimulated conditions, consistent with pendrin-mediated Cl^--HCO_3^- exchange (Garnett *et al.*, 2011). Importantly, in OA-treated Calu-3 cells, the CFTR pore blocker GlyH-101 could not inhibit this apical AE under non-stimulated conditions. Apical AE activity was also present in non-stimulated OA-treated CFTR KD Calu-3 cells, providing further support that the observed changes in pH; were not due to CFTR. Because we have previously found that pendrin was constitutively active when expressed in polarized cultures of FRT cells (Garnett *et al.*, 2011), these OA experiments suggest that pendrin is normally kept inactive under non-stimulated conditions through dephosphorylation by PP1. Importantly, cAMP stimulation still enhanced

apical AE activity in OA-treated cells, but this was, in contrast, through a CFTR-dependent mechanism, although the level of AE activity was still lower than in untreated Calu-3 cells. This enhanced AE activity following cAMP stimulation could be due to phosphorylation of pendrin by PKA, resulting in increased pendrin insertion into the apical membrane, as seen in thyrocytes following forskolin addition (phosphorylation site on pendrin STAS domain, RKDT 714–717; Bizhanova *et al.*, 2011). This hypothesis is supported by the fact that H-89 inhibited the forskolin-stimulated increase in apical AE activity in OA-treated Calu-3 cells.

Collectively these results indicate that in the absence of cAMP stimulation, inhibition of PP1 (by OA) produces a switch in Calu-3 anion exchange activity, fully inhibiting basolateral SLC4A2 and partially stimulating apical pendrin, through a process that is independent of CFTR. The similarity of the switch in AE activity in response to OA and cAMP agonists further suggests that PP1 is a downstream target of cAMP. The potential pathways involved in regulating the basolateral and apical AEs in Calu-3 cells are summarized in Figure 10. Consistent with a switch in Cl^--HCO_3^- exchange activity, treating Calu-3 cells with OA lead to an increase in fluid pH, signifying enhanced HCO_3^- secretion. However, because there was no change in the fluid volume secreted by OA-treated cells, compared with untreated Calu-3 cells, this result suggests that HCO_3^- and fluid secretion are not obligatory coupled.

PP1 is known to play a key role in the regulation of pancreatic ductal HCO_3^- secretion (Yang *et al.*, 2011) through its interaction with IRBIT (IP_3 receptor binding protein released with IP_3). Due to the similarities between pancreatic and airway SMG HCO_3^- secretion (HCO_3^- enters across the basolateral membrane through the NBCe1B and exits across the luminal membrane via the coordinated action of SLC26 Cl^--HCO_3^- exchange and CFTR), PP1 may regulate Calu-3 transepithelial HCO_3^- transport through a comparable mechanism. However, in the pancreatic studies, inhibiting PP1 pharmacologically reduced fluid/HCO_3^- secretion (Yang *et al.*, 2011), whereas it stimulated HCO_3^- secretion in Calu-3 cells. Recent studies by Lee and colleagues in *Xenopus oocytes* suggest that PP1 may dephosphorylate IRBIT itself, thereby restricting its ability to bind to and regulate NBC (Lee *et al.*, 2012). In this model, inhibition of PP1 could lead to stimulation of HCO_3^- secretion by IRBIT. However, because IRBIT does not appear to directly regulate ductal SLC26 anion exchange, but does so through its effects on CFTR activity (Yang *et al.*, 2011), it seems unlikely that the CFTR-independent effects we observed with okadaic acid in Calu-3 cells are due to changes in PP1 and IRBIT interaction. Further investigation will be required to elucidate how PP1 regulates Calu-3 HCO_3^- secretion.

Implications for CF

It is now well established that in CF tissues the lack of functional CFTR impairs airway HCO_3^- and fluid secretion producing an acidic ASL and dehydrated mucus unable to maintain airway sterility (Smith and Welsh, 1992; Choi *et al.*, 2001; Coakley *et al.*, 2003; Song *et al.*, 2006; Quinton, 2008; Muchekehu and Quinton, 2010; Cho *et al.*, 2011a; Gustafsson *et al.*, 2012; Pezzulo *et al.*, 2012), which suggests that failure in ASL pH homeostasis may underlie airway pathology

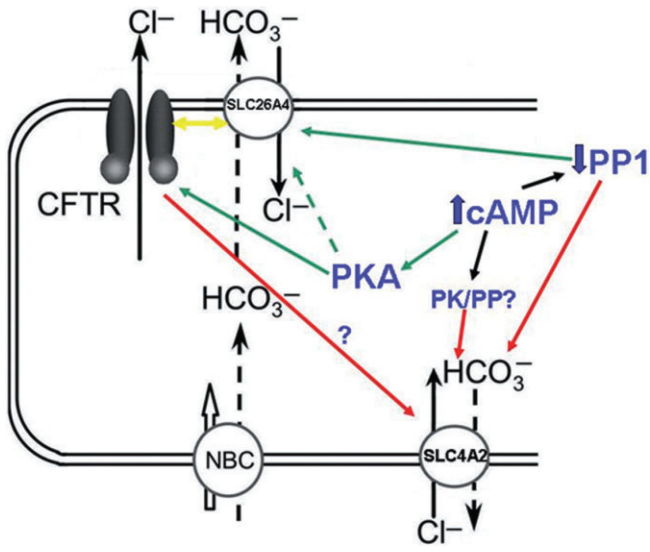


Figure 10

Model to explain the coordinated regulation of apical and basolateral Cl^--HCO_3^- exchangers in Calu-3 cells. Summary of the potential mechanisms involved in the reciprocal regulation ('switch') of the apical Cl^--HCO_3^- exchangers SLC26A4 (pendrin) and SLC4A2 (AE2) as determined by PK and PP inhibitor studies. Note this model assumes that the location of the two types of exchangers as well as the ability to secrete Cl^- and HCO_3^- originate from the same cell type, even though it is well documented that Calu-3 cells are not homogenous and contain both CFTR expressing (secretory) and goblet-like mucin granule containing cells in ~60–40% ratio (Kreda *et al.*, 2007). Green arrows indicate stimulatory regulation – cAMP elevation activates PKA, which in turn increases CFTR activity as well as pendrin, through an unknown mechanism, but which could involve interaction between the R domain of CFTR and the STAS domain of pendrin (Ko *et al.*, 2004). As such it is not clear whether PKA directly activates pendrin or indirectly activates it via CFTR (dashed green arrow). PP1 inhibition stimulates apical Cl^--HCO_3^- exchange independently of CFTR. Yellow arrow indicates the potential inhibitory effect of CFTR on pendrin in the absence of cAMP stimulation. Red arrows indicate inhibitory regulation – cAMP elevation inhibits the basolateral Cl^--HCO_3^- exchanger, SLC4A2, indirectly through stimulation of CFTR, or directly by an unknown PKA-independent mechanism (black arrows), such as inhibition of PP1. Inhibiting PP1 by OA mimics the effect of cAMP elevation, but whether this is through the same mechanism is not known.

in CF, and possibly other diseases such as asthma and chronic obstructive pulmonary disease (Cho *et al.*, 2011a). Therefore, restoring ASL pH through a CFTR-independent mechanism could represent a potential therapeutic target in the treatment of CF. The enhanced HCO_3^- secretion we have observed after inhibiting PP1 may point to such a mechanism, although fluid secretion was not increased at the same time. However, if activation of the apical AE was also linked to opening of calcium-activated Cl^- channels (CaCC), which are known to be functional in CF airway epithelial cells (Tarran *et al.*, 2002), this may provide a route to restore both HCO_3^- and fluid secretion. Furthermore, opening of CaCCs would also help support apical Cl^--HCO_3^- exchange, by acting as a shunt pathway for Cl^- ions that would prevent accumulation of intracellular Cl^- (due to cycling of the exchanger), in a manner analogous to CFTR.

Our study also highlights a potential dysregulation of basolateral Cl^--HCO_3^- exchange in CF cells, which could contribute to impaired transepithelial HCO_3^- secretion and is therefore an area that requires consideration in treatments that intend to improve base secretion in CF cells. In summary, our studies point to the potential for restoring ASL pH via targeting phosphorylation-dependent regulation of apical and basolateral anion exchangers of airway epithelial cells.

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Conflicts of interest

The authors have no conflicts of interest to report.

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