NOVEL ROLE FOR PENDRIN IN ORCHESTRATING BICARBONATE SECRETION IN CFTR-EXPRESSING AIRWAY SEROUS CELLS

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Running title: Role of pendrin in HCO\textsubscript{3} secretion in the airways

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In most HCO\textsubscript{3} secreting epithelial tissues, SLC26 CI/HCO\textsubscript{3} transporters work in concert with the cystic fibrosis transmembrane conductance regulator (CFTR) to regulate the magnitude and composition of the secreted fluid, a process that is vital for normal tissue function. By contrast, CFTR is regarded as the only exit pathway for HCO\textsubscript{3} in the airways. Here we show that CI/HCO\textsubscript{3} anion exchange makes a major contribution to transcellular HCO\textsubscript{3} transport in airway serous cells. Real time measurement of intracellular pH from polarised cultures of human Calu-3 cells demonstrated cAMP/PKA-activated CI-dependent HCO\textsubscript{3} transport across the luminal membrane via CFTR-dependent coupled CI/HCO\textsubscript{3} anion exchange. The pharmacological and functional profile of the luminal anion exchanger was consistent with SLC26A4 (pendrin), which was shown to be expressed by quantitative RT-PCR, Western blot and immunofluorescence. Pendrin-mediated anion exchange activity was confirmed by shRNA pendrin knockdown (KD) which markedly reduced cAMP-activated CI/HCO\textsubscript{3} exchange. To establish the relative roles of CFTR and pendrin in net HCO\textsubscript{3} secretion, transepithelial liquid secretion rate and liquid pH were measured in wild type, pendrin KD and CFTR KD cells. cAMP/PKA increased the rate and pH of the secreted fluid. Inhibiting CFTR reduced the rate of liquid secretion but not the pH, whereas decreasing pendrin activity lowered pH with little effect on volume. These results establish that CFTR predominately controls the rate of liquid secretion while pendrin regulates the composition of the secreted fluid and identifies a critical role for this anion exchanger in transcellular HCO\textsubscript{3} secretion in airway serous cells.

HCO\textsubscript{3} is a vital component of epithelial secretions. Despite the growing awareness of its importance in epithelial function, the molecular mechanism of HCO\textsubscript{3} secretion remains incompletely understood. Via its buffering role, HCO\textsubscript{3} controls the pH of the luminal microenvironment, a function particularly important to the physiology of many epithelial tissues, including the airways. Consistent with a role for HCO\textsubscript{3} secretion in airway function, a previous study found the airway surface liquid (ASL) to be acidic in cystic fibrosis (CF) compared to normal cell cultures (1) and a similar finding was made in secretions from nasal submucosal glands (SMGs) from CF patients (2). Aberrant pH/HCO\textsubscript{3} secretion likely contributes to CF lung pathogenesis in a number of fundamental ways. HCO\textsubscript{3} is a chaotropic anion that facilitates efficient solubilisation and transport of macromolecules such as mucus (3). Also the recent finding that HCO\textsubscript{3} secretion is required for mucus secretion (4-6) and that mucin expansion and viscosity are regulated by HCO\textsubscript{3} (7,8), strongly suggests that adequate HCO\textsubscript{3} is required for proper mucus homeostasis. In addition, acidic pH has been shown to reduce ciliary beat frequency (9), and impede bacterial killing by phagocytic cells (10,11). Collectively, these consequences of inadequate HCO\textsubscript{3} transport predispose the lungs to mucus blockage, bacterial infection and disease, all hallmarks of the CF lung.
Current studies suggest that HCO$_3^-$ exit (secretion) across the luminal plasma membrane of airway epithelial cells is mediated solely by the cystic fibrosis transmembrane conductance regulator (CFTR), the ion channel which is mutated in CF. Exactly how CFTR dysfunction leads to aberrant HCO$_3^-$ secretion is unclear. In primary cultures of surface bronchial and tracheal epithelial cells from CF human and pig, a lack of CFTR is associated with reduced electrogenic HCO$_3^-$ secretion (12,13). Likewise, in ex vivo studies of liquid/mucus secretion from intact submucosal glands (SMGs) from a range of species, cAMP-stimulated fluid secretion depends on both Cl$^-$ and HCO$_3^-$ and was absent in CF glands (14-16). Furthermore, detailed studies of anion and fluid secretion from polarised cultures of human airway Calu-3 cells, a model of human tracheobronchial SMG serous cells, also concluded that CFTR was the sole mediator of apical Cl$^-$ and HCO$_3^-$ secretion (17-19). However, in many HCO$_3^-$ secreting epithelia, including the pancreas (20), salivary glands (21), gastrointestinal (22) and reproductive tracts (23), HCO$_3^-$ secretion is mediated by CFTR and one or more Cl$^-$/HCO$_3^-$ exchangers belonging to the SLC26 gene family. This family comprises ten members, and their functional characterisation indicates distinct patterns of anion specificity and transport modes. Furthermore, structural analyses indicate that CFTR and the SLC26 transporters can physically interact through their regulatory (R) and STAS domains, respectively (24), a process that is enhanced by PKA phosphorylation of the R domain (24). In most cases, these molecular interactions synergise the transport activity of CFTR and the SLC26 exchanger, resulting in enhanced HCO$_3^-$ and fluid secretion from epithelial tissues.

Therefore, SLC26 anion exchangers have a well-documented role in HCO$_3^-$ secretion in non-airway tissues. Human tracheal airway epithelial cells express abundant SLC26A3, while delF508 cells do not (25). Furthermore RNA analysis has shown that human lungs also express SLC26A9 (26) which appears to act as a constitutively active CFTR-regulated Cl$^-$ channel in cultured human bronchial epithelial cells (27). However, to date it is unknown whether SLC26 proteins are involved in HCO$_3^-$ secretion in the airways. Hence, the purpose of this study was to investigate the potential role of SLC26 Cl$^-$/HCO$_3^-$ exchangers in transcellular HCO$_3^-$ secretion in airway epithelial cells. Our results show for the first time that human airway serous cells possess a luminal cAMP/PKA-activated Cl$^-$/HCO$_3^-$ exchanger that exhibits functional properties consistent with those of SLC26A4 (pendrin). Short hairpin RNA (shRNA)-mediated knock-down of pendrin expression significantly reduced Cl$^-$/HCO$_3^-$ exchange activity and markedly lowered the HCO$_3^-$ content of the secreted fluid. These studies therefore identify for the first time a critical role for pendrin in transepithelial HCO$_3^-$ secretion by airway serous cells.

**EXPERIMENTAL PROCEDURES**

*Calu-3 cell culture.* The human adenocarcinoma-derived cell line, Calu-3 (passage 20-50; (28)), was grown in Eagle’s minimal essential medium (EMEM) plus 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% non-essential amino acids (Sigma), and incubated in humidified air containing 5% CO$_2$ at 37 °C. CFTR KD Calu-3 cells (29), SLC26A4 and cyclophilin B KD Calu-3 cells were cultured in the same media supplemented with geneticin (CFTR KD, 400 µg/ml; G418; Sigma) or puromycin (10 µg/ml; Sigma), respectively. For experiments using polarised cells, Calu-3 cells were seeded onto clear Costar Transwell® inserts (0.45 µm pore size, 1.12 cm$^2$ surface area), at 250,000 cells cm$^{-2}$, respectively. Calu-3 cells generally formed a confluent monolayer with a stable transepithelial resistance ($V_o$) of 700-900 Ω.cm$^2$ after 5 days growth on Transwell inserts. Experiments were carried out 7-14 days post-seeding.

*FRT cell culture.* Non-transfected Fischer rat thyroid (FRT) cells and FRT cells stably transfected with Pendrin or CFTR were kindly provided by Drs. L. Galietta and O. Zegarra-Moran (University of Genoa, Italy) and generated as previously described (30). The cells were cultured in Coon’s modified Ham’s F-12 media supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% non-essential amino acids. For CFTR-transfected FRT cells, media was supplemented with 0.75 mg/ml geneticin (G418; Sigma) and 0.6 mg/ml zeocin (Sigma). Pendrin-transfected FRT cells were grown in media supplemented with 1 mg/ml geneticin and 0.5 mg/ml hygromycin (Sigma).

*shRNA knockdown of SLC26A4 in Calu-3 cells.* Individual SLC26A4 and cyclophilin B (control)-deficient Calu-3 cell lines were
produced using lentiviral-mediated delivery of shRNA (Sigma MISSION) knockdown with a set of 4 different shRNA sequences (supplemental Table S1). Lentiviral transduction particles were applied at a multiplicity of infection (MOI) ratio of 1. Transduced cells were selected 48 hours post-transduction using 10 μg/ml puromycin. Knockdown (KD) cell lines used for this study had V_0 and growth patterns similar to that of WT Calu-3 cells.

Measurement of intracellular pH. Cells were loaded with the pH-sensitive fluorescent dye, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM; 10 μM) for 45-60 mins at 37 ºC, in a 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES)-buffered salt solution which consisted of (in mM): 130 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 Na-HEPES and 10 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 HCO₃⁻-buffered Krebs solution (KRB) which consisted of (in mM): 115 NaCl, 5 KCl, 25 NaHCO₃, 1 MgCl₂, 1 CaCl₂ and 10 D-glucose, and adjusted to pH 7.4 by bubbling with a 95% O₂, 5% CO₂ mixture at 37 ºC. 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. Intracellular pH (pHᵢ) was measured from 15-20 cells as previously described (31), using a Life Sciences Microfluorimeter System (Life Sciences Resources, UK). Ratio values were calibrated to pHᵢ using the high K⁺-nigericin method (10 μM), using K⁺ solutions of various pH values from 5.6-8.6 (31). Mean changes in pHᵢ were estimated by calculating the average pH over 60 s (120 data points). The initial rate of pH_change (ΔpH/Δt) was calculated by linear regression fitted to a minimum of 40 data points. Total buffering capacity was estimated by the ammonium pulse technique, using the Henderson-Hasselbalch equation as previously described (31). The ΔpH/Δt values were converted to transmembrane efflux of HCO₃⁻ (-J(B)) using the equation: -J(B)= rate of pHᵢ change x total buffering capacity. For high K⁺ KRB, KCl was increased to 115 mM and NaCl reduced to 5 mM. For Cl⁻-free KRB, NaCl was substituted for Na-gluconate, with 6 mM Ca-gluconate replacing 1 mM CaCl₂ to compensate for the Ca²⁺ buffering capacity of gluconate and 5 mM KCl replaced with 2.5 mM K₂SO₄. For Na⁺-free KRB, 115 mM NMDG-Cl, replaced NaCl and 25 choline-HCO₃ replaced NaHCO₃. Atropine (10 μM) was included to block muscarinic receptors. Cl⁻-free HEPES-buffered solution consisted of (in mM): 130 Na-glucnaconate, 2.5 K₂SO₄, 1 Mg-gluconate, 6 Ca-gluconate, 10 HEPES (free acid) and 10 D-glucose. All general chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd., UK), except forskolin (Tocris), GlyH-101 (Calbiochem) and BAPTA-AM (Fluka).

Transepithelial liquid secretion rates and pH measurements. After washing confluent monolayers with PBS, to remove mucus, the rate of liquid secretion (Jᵥ) was determined by applying 0.2 ml and 1 ml of Krebs solution to the apical and basolateral surfaces of the cells, respectively, with the desired agonist or inhibitors. Cells were placed in a humidified CO₂ incubator at 37 ºC and the volume of the apical fluid measured using a calibrated micropipette, after 24 hours. For HCO₃⁻-free experiments, Transwells were bathed in HEPES-buffered solution and maintained in a humidified incubator at 37 ºC without CO₂ gassing. Correction for evaporative apical volume loss (0.08 ± 0.02 μl/cm²/h; n=12) was determined empirically by measuring the reduction in apical volume from Transwells coated with silicone gel to stop fluid leakage across the membrane. The pH of 5% CO₂-saturated apical fluid was measured using a micro-pH electrode (Hamilton, Switzerland) within 60 s of removing individual Transwells from the incubator. Control experiments showed that pH drifted by 0.04 pH units min⁻¹ using this approach.

Quantitative RT-PCR analysis of SLC26 gene expression. Total RNA was isolated using the Qiagen RNeasy mini-prep kit and quantified using the Bioanalyser 2100 and RNA total nano chips and reagents (Agilent Technologies). cDNA synthesis was performed with 20 ng/ml total RNA/sample using the GeneAmp RT-PCR kit (Applied Biosystems). A mixed tissue standard was generated from total RNA (liver, spleen, kidney, lung, testis, pancreas, brain, and foetal brain – purchased individually from Clontech). Quantitative RT-PCR (qRT-PCR) (Taqman) analysis was used to measure SLC26 gene expression, using previously optimized primers and probes (Applied Biosystems, Assays-on-Demand; supplemental Table S2). Amplicons were designed to span intron-exon boundaries to preclude possible amplification of genomic DNA sequences. Reactions were performed in 96-well optical plates and analysed
on the ABI PRISM 7700 (Applied Biosystems) using the following programme: 50 °C for 2 min and 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C 1 min. Data were quantified relative to the standard curve for each gene. Expression was normalised to GAPDH and expressed as percentage relative to the standard curve.

Immunocytochemistry. Calu-3 cells grown on Transwells were fixed and permeabilised with cold 100% methanol (-20 °C) for 15 mins on ice. Samples were blocked with 3 % horse serum for 1 h at room temperature, then incubated with a mouse polyclonal anti-Pendrin (Abnova) at 1:200 overnight at 4 °C. After blocking with 3 % goat serum for 1 h at room temperature, samples were incubated with a goat Alexa Fluor 488-coupled anti-mouse secondary antibody (Invitrogen) at 1:1000 in 3 % goat serum. The samples were also stained for zona occludens 1 (ZO-1), using a rabbit anti-ZO-1 antibody (1:500: Invitrogen), and a goat anti-rabbit secondary antibody (1:1000: Red Alexa Fluor 568, Invitrogen). Negative controls omitted primary antibody. Images were collected by confocal laser scanning microscopy, using a Leica TCS-NT system (Leica UK Ltd.) equipped with a x100 oil immersion lens, using appropriate excitation and emission filter sets for dual fluorophore detection. Leica software was used to capture images, under identical conditions of imaging, illumination intensity and photo-multiplier settings.

Statistical analysis. Results are presented as mean ± SEM, where n indicates the number of experiments. Statistical analysis was performed with GraphPad Prism software (GraphPad Software Inc, USA.), 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

Effects of asymmetrical Cl- removal in unstimulated cells. Under control conditions, removal of Cl from the apical perfusate did not change intracellular pH (pHi) indicating little resting apical anion exchange (AE) activity (Fig. 1A), which would be expected to cause an alkalinisation. In marked contrast, basolateral Cl- removal produced a large, mono-phasic alkalinisation of 0.43 ± 0.01 pH units (P<0.001, n=35). Cl- re-addition reversed this response at an initial rate of 0.52 ± 0.03 pH units min⁻¹ (J(B) = 49.1 ± 7.0 mM B min⁻¹). These results indicate functional AE activity in the basolateral, but not apical, membrane of Calu-3 cells under resting conditions, probably mediated by anion exchanger 2 (AE2)(32,33).

Effects of Cl- removal subsequent to cAMP elevation. Exposure of cells to forskolin (fsk; 5 µM) under symmetrical high Cl- conditions significantly acidified pHi by 0.13 ± 0.01 units (Fig. 1A), at a rate of 0.08 ± 0.01 pH units min⁻¹. Subsequent apical Cl- removal markedly alkalinised fsk-treated cells by 0.64 ± 0.03 pH units (P<0.001, n=35, Fig. 1B). The pH response was typically biphasic, consisting of a fast initial alkalinisation followed by a slower secondary rise in pHi that reached a steady state after 3-4 mins (Fig. 1A). On Cl- re-addition, pHi recovered to baseline values at an initial rate of 0.77 ± 0.08 pH units min⁻¹ (J(B)= 67.3 ± 13.3 mM B min⁻¹; Fig. 1B). Interestingly, under fsk-stimulation, removal of basolateral Cl- produced no change in pHi (Fig. 1A), which indicates that fsk treatment both inhibits basolateral AE and stimulates apical AE activity. Dideoxyforskolin, an inactive analogue of fsk, did not mimic these effects, thus confirming that a change in intracellular cAMP provided the fsk-induced ‘switch’ in the Cl- dependent apical and basolateral pHi responses (P>0.05 compared to non-stimulated apical and basolateral Cl- dependent changes in pHi; n=3; data not shown). Furthermore, both vasoactive intestinal peptide and adenosine, physiological, cAMP-mediated agonists in Calu-3 cells (34,35), changed pHi in response to apical Cl- removal, similar to the fsk-stimulated response (P>0.05; n=4; Fig. 1C). Consistent with a cAMP/PKA-dependent activation of an apical AE, the Cl- dependent pHi responses were reduced by the PKA inhibitor H-89, as well as by the general PK inhibitor staurosporine (Fig. 1D; Calu-3 cells were somewhat insensitive to H-89 and required pre-incubation at a relatively high concentration (50 µM), which may have PKA-independent effects). The changes in pHi caused by apical Cl- removal in fsk-treated cells depended entirely on HCO₃⁻; replacement of NaHCO₃ with Na-HEPES markedly reduced the Cl- dependent alkalinisation and pHi recovery (Fig. 1E). Finally, and in contrast to the results with cAMP agonists, raising cytosolic Ca²⁺ had no effect on apical AE activity (supplemental Fig. S1). Taken together, these results suggest that the observed changes in pHi are due to cAMP/PKA activation of an apical Cl- dependent HCO₃⁻ transporter which strongly selects for HCO₃⁻ over OH⁻.
Properties of cAMP-stimulated responses to apical Cl⁻ removal. The ability of different anions to support re-acidification following Cl⁻ withdrawal was next measured. Recovery of pHᵢ was supported by a range of monovalent but not divalent anions. A clear selectivity exists amongst the monovalent anions, with I⁻ and Br⁻ exhibiting the highest rate of re-acidification compared to the other anions (P<0.01; n=5). The anion/Cl⁻ selectivity ratio sequence was HCO₃⁻/Cl⁻ 0.6: NO₃⁻ 0.8: SCN⁻ 0.8: Cl⁻ 1: Br⁻ 1: I⁻ 1.3 (Fig. 2A). Removal of Na⁺ had no significant effect on the rate of re-acidification (P>0.05; n=4; Fig. 2B). Similarly hyperpolarisation of the resting membrane potential (Vₘ) using the K⁺ channel opener 1-ethyl-2-benzimidazolinone (EBO, 1 mM) likewise was without effect (Fig. 2C). However, clamping Vₘ to 0 mV through a combination of high extracellular K⁺ plus EBO did reduce the rate of re-acidification (ΔpHᵢ) by 23.7 ± 2.3% (P<0.05; n=4; Fig. 2C), indicating some dependence on Vₘ. The ΔpHᵢ was also insensitive to the general anion transporter inhibitor 4,4'-dismethoxybenzene-2,2'-disulfonate (H₂-DIDS) (P>0.05; n=4; Fig. 2D). The observed changes in pHᵢ depicted in Figs. 1-2 are therefore due to the activity of a Na⁺-independent, DIDS-insensitive, electrogenic, monovalent anion transporter, consistent with the known properties of CFTR.

Contribution of CFTR and basolateral Cl⁻/HCO₃⁻ exchange to pHᵢ responses. To determine whether CFTR contributes to the alkalinisation caused by apical Cl⁻ removal, the effect of CFTR inhibition was tested. GlyH-101 (10 μM) abolished the pHᵢ response to apical Cl⁻ removal in fsk-stimulated Calu-3 monolayers (lack of alkalinisation in the presence of GlyH-101, shown by black trace). CFTRₘₐₐₜ172 produced similar results (10 μM; data not shown). Note that the initial exposure to GlyH-101 caused an alkalinisation that is likely due to inhibition of HCO₃⁻ efflux from the cells.

In the absence of a CFTR blocker, the activity of a basolateral H₂-DIDS-sensitive ‘base’ transporter strongly influenced both the magnitude of the pHᵢ response to apical Cl⁻ removal and the ΔpHᵢ (supplemental Fig. S2). To ascertain whether a basolateral H₂-DIDS-sensitive transporter also affects the Cl⁻-dependent pHᵢ response in the presence of GlyH-101, Calu-3 monolayers were first exposed to basolateral H₂-DIDS before GlyH-101. This manoeuver restored apical Cl⁻-induced pHᵢ changes to GlyH-101 treated cells (Fig. 3A; blue trace) and indicate the presence of an apical Cl⁻/HCO₃⁻ exchanger. In contrast to the rates of alkalinisation with Cl⁻ removal, the ΔpHᵢ upon apical Cl⁻ re-addition was significantly reduced under these conditions (P<0.001; n=4; Fig. 3C). These data suggest that apical Cl⁻/HCO₃⁻ exchange is functionally coupled to Cl⁻ transport by CFTR, or that CFTR itself directly contributes to pH changes, in addition to the exchanger.

Knockdown of CFTR provides additional evidence for a role of both CFTR and a coupled anion exchanger. The role of CFTR in regulating apical Cl⁻/HCO₃⁻ exchange was next investigated using CFTR knockdown (KD) Calu-3 cells (29). CFTR content of KD cells was determined to be ~ 28 ± 5 % that of wild-type (WT) cells (supplemental Fig. S3A & B). Compared to WT cells, cAMP-stimulated secretory capacity is these CFTR KD cells was also reduced by ~ 25 % (29). Mean pHᵢ values did not differ significantly between WT (7.43 ± 0.03; n=50) and CFTR KD (7.39 ± 0.05; n=32; P>0.05) Calu-3 cells. The switch from basolateral to apical AE activity in response to cAMP is preserved in CFTR KD cells (Fig. 4A). However, in CFTR KD cells the basolateral anion exchanger was not fully inhibited under fsk-stimulated conditions (compare Figs. 1A and 4A). The reduced expression of CFTR in the CFTR KD Calu-3 cells was reflected in a reduced ΔpHᵢ (~48 %), which decreased from 0.75 ± 0.09 pH units min⁻¹ (J(B)= 97.6 ± 13.3 mM B min⁻¹) in WT cells to 0.39 ± 0.02 pH units min⁻¹ (J(B)= 65.9± 9.8 mM B min⁻¹) in KD cells (P<0.01; n=4, Fig 4B). These values are similar to those obtained in GlyH-101/H₂-DIDS–treated WT Calu-3 cells (Fig. 3B). Although ΔpHᵢ was slower, the profile of the apical Cl⁻-dependent change in pHᵢ in CFTR KD Calu-3 monolayers broadly resembled that of WT cells, being H₂-DIDS-insensitive, abolished by GlyH-101 addition in the absence of basolateral H₂-DIDS (data not shown) and supported only by monovalent anions. However, the I⁻/Cl⁻ selectivity ratio significantly increased from 1.3 in WT cells to 2.1 in CFTR KD cells (P<0.001; n=4; Fig. 4C; see Discussion). Taken together, these results suggest that both CFTR and an apical Cl⁻/HCO₃⁻ exchanger contribute to Cl⁻-induced pHᵢ changes in Calu-3 cells.
Pendrin is expressed in Calu-3 and human airway cells. Apical SLC26A family Cl/HCO₃⁻ exchangers contribute to transepithelial HCO₃⁻ secretion in other HCO₃⁻-secreting epithelia. To identify the apical AE, qRT-PCR was performed for all 10 members of the SLC26A family (36) using RNA extracted from polarised Calu-3 cells. Figure 5A shows that a number of SLC26A transporters are expressed in Calu-3 cells, several of which are known to function as Cl/HCO₃⁻ exchangers (SLC26A4, A6, A7 and A9). Of these four candidates, only pendrin (SLC26A4) has properties which are consistent with our results; a monovalent anion transporter with high affinity for iodide and insensitivity to H₂-DIDS (37-40). A mouse polyclonal anti-SLC26A4 antibody detected a band migrating at ~100 kDa in immunoblot analysis of Calu-3 whole cell lysates (supplemental Fig. S4). The size of the detected species is consistent with that of the band detected using heterologously expressed pendrin fusion proteins, as well as previous reports (41). Confocal immunofluorescence images revealed a punctate localisation of pendrin near the apical membrane of Calu-3 monolayers (Fig. 5C; top; green), when compared to ZO-1 expression (Fig. 5C; bottom; red). Immunohistochemical studies on native human tissue showed that pendrin is highly expressed in surface ciliated cells, is not present in mucous-secreting cells of submucosal glands, and is present, albeit at a lower level than ciliated cells, in serous-like cells from SMGs (supplemental Fig. S5). This work provides additional support for pendrin expression in human airway serous cells and also confirms that pendrin is expressed in surface bronchial epithelial cells (42).

Pendrin knockdown provides insights into its function in Calu-3 cells. To establish if pendrin functions in HCO₃⁻ transport in Calu-3 cells, pendrin (SLC26A4) and cyclophilin B (control) KD Calu-3 cells were produced by shRNA inhibition. Knockdown was verified using both qRT-PCR and immunodetection methods. Pendrin mRNA expression in these KD cells was reduced to 8.5 ± 0.7% compared to cyclophilin B (control) KD Calu-3 cells (P<0.05; n=3; Fig. 5B). Confocal immunofluorescence imaging verified pendrin expression in both control KD Calu-3 monolayers and CFTR KD Calu-3 cells, whereas no signal was detected in the pendrin KD cells (Fig. 5C).

Although pendrin KD cells showed a fsk-stimulated apical Cl⁻-dependent change in pHᵢ, the ΔpHᵢ was reduced by 47.6 ± 2.4%, compared to control KD cells (P<0.001; n=5; Fig. 5D). Like CFTR KD cells, the profile of the apical Cl⁻-dependent change in pHᵢ in pendrin KD cells was reminiscent of WT Calu-3 cells. It was only supported by monovalent anions (Fig 5E), was H₂-DIDS-insensitive (Fig. 5F) and abolished by GlyH-101 addition (data not shown). Interestingly, unlike WT as well as CFTR-KD cells, the pendrin KD cells showed no significant difference in the ΔpHᵢ when iodide replaced chloride (Fig. 5E, P>0.05; n=4). This observation is consistent with the KD of an AE having a high affinity for iodide. Neither pendrin nor control KD Calu-3 cells showed significant differences in CFTR protein expression (P>0.05; n=3; supplemental Fig. S3 A & B). Thus, these differences in apical Cl⁻-induced changes in pHᵢ in pendrin KD cells do not reflect a change in CFTR expression. In addition, short-circuit current (Iₛᶜ) measurements demonstrated a similar GlyH-101-sensitive fraction of fsk-stimulated Iₛᶜ (indicative of CFTR-dependent anion transport) in pendrin and control KD Calu-3 monolayers (P>0.05; n=9; supplemental Fig. S3C). The H₂-DIDS insensitivity of the apical Cl⁻-dependent changes in pHᵢ in WT and pendrin KD Calu-3 cells suggest that other SLC26 members highly expressed in these cells, such as SLC26A2 or SLC26A6 (both are sensitive to DIDS;43,44), are unlikely to be involved in these responses.

pHᵢ response to Cl⁻ removal by Fisher Rat Thyroid cells expressing CFTR or pendrin. The significant changes in I/CFᵢ ratio for the ΔpHᵢ across the different cell types (Figs. 2A, 4C & 5E), together with expression data and pharmacological responses to H₂-DIDS and GlyH-101, indicate that both CFTR and pendrin contribute to the Cl⁻-induced pHᵢ responses. As a further test, the pHᵢ responses to Cl⁻ removal and re-addition were evaluated in polarised FRT monolayers stably expressing either CFTR or pendrin alone (30). Fig. 6A shows that FRT cells expressing CFTR alkaliniised in response to apical Cl⁻ removal in the presence of fsk, supporting the notion that CFTR can conduct HCO₃⁻ into the cell under an imposed outwardly-directed Cl⁻ gradient. The pHᵢ response to apical Cl⁻ was significantly augmented after fsk treatment. This increase could be completely inhibited by GlyH-101; thus, CFTR mediated the changes in pHᵢ (Fig. 6A & B). Anion substitution studies revealed that only
monovalent anions supported re-acidification and I' and Cl'-selectivity did not differ (Fig. 6C). Pendrin-expressing FRT cells responded to apical Cl' removal very differently. Significant Cl'-induced pH responses were observed in the absence of cAMP stimulation (Fig. 6D). This Cl'/HCO₃⁻ AE activity was not enhanced by forskolin or inhibited by GlyH-101 (Fig. 6D & E). Importantly, the ΔpHₕ was markedly higher for I' compared to Cl' (Fig. 6F; I'/Cl' = 3.2), with no ability to transport divalent anions, results that are entirely consistent for pendrin (39,40).

Anion transport is critical for fluid secretion. We next measured and compared both the rate of transepithelial liquid secretion (Jᵥ) and the pH of secreted fluid samples from WT, CFTR KD and pendrin KD monolayers. WT Calu-3 monolayers, submerged in HCO₃⁻-KRB, increased the apical fluid volume from 200 µl to 210 ± 1 µl over 24 h (P<0.05; n=6; Fig. 7A), corresponding to a Jᵥ of 0.42 ± 0.02 µl/cm²/h. Forskolin increased Jᵥ, approximately five-fold to 2.23 ± 0.11 µl/cm²/h (P<0.001; n=6). Bilateral Cl' removal abolished basal liquid secretion and reduced forskolin-stimulated secretion by 88 ± 22% (P<0.001; n=6). No significant fluid secretion was observed from Calu-3 monolayers bathed in HEPES-buffered KRB under either non-stimulated or forskolin-stimulated conditions (P>0.05; n=6). These results show that both basal and forskolin-stimulated Calu-3 liquid secretion is entirely Cl'- and HCO₃⁻-dependent.

To determine the role played by CFTR in transepithelial HCO₃⁻ and liquid secretion, experiments were performed with the CFTR blocker, GlyH-101. In the presence of apical GlyH-101, non-stimulated WT Calu-3 cells absorbed rather than secreted fluid (-0.18 ± 0.01 µl/cm²/h; P<0.001; n=3; Fig. 7B), suggesting that all basal liquid secretion involves CFTR. Forskolin-stimulated fluid secretion was substantially inhibited by GlyH-101 (60 ± 7% inhibition; P<0.001; n=3), but nonetheless achieved levels significantly greater than control (P<0.001; n=3). Assuming complete block of CFTR in these experiments, these results suggest that although the majority of the cAMP-stimulated liquid secretion depends on CFTR, a significant CFTR-independent, cAMP-stimulated, component of liquid secretion does exist. The pH of the apical fluid from non-stimulated Calu-3 monolayers did not differ significantly from that of the bathing Krebs solution (compared to pH 7.4; P>0.05; n=3; Fig. 7C). However, apical fluid pH significantly increased to 7.8 ± 0.1 (~ 60 mM HCO₃⁻) following forskolin stimulation (P<0.001; n=3). Surprisingly, despite reducing the rate of liquid secretion, GlyH-101 addition had no effect on the pH of the secreted fluid under either non-stimulated or forskolin-stimulated conditions (P>0.05; n=3).

The CFTR-dependence of Calu-3 liquid secretion was further explored using CFTR KD Calu-3 cells. Under non-stimulated conditions, CFTR KD Calu-3 cells failed to increase the volume of apical fluid over 24 hours (P>0.05; n=4; Fig. 7D), and secreted significantly less liquid compared to WT cells under forskolin-stimulated conditions (34 ± 3% compared to WT; P<0.001; n=4). Like GlyH-101 addition in WT Calu-3 cells, CFTR KD had no significant effect on the pH of secreted apical fluid under either non-stimulated or forskolin-stimulated conditions (P>0.05; n=3; Fig. 7E). These results establish a critical role for CFTR in regulating the rate of transepithelial liquid secretion, but not in controlling the pH ([HCO₃⁻]) of the secreted fluid.

In contrast, pendrin knockdown did not significantly affect either the rate of transepithelial liquid secretion or the pH of secreted fluid under basal conditions, compared to control KD Calu-3 cells (P>0.05; n=3; Fig. 7D & E). However, although pendrin KD Calu-3 cells produced an increase in fluid volume (P<0.05; n=3) and pH (P<0.01; n=3) in response to forskolin, comparatively less fluid was secreted (P<0.05; n=3; Fig. 7D). Importantly, the secreted fluid was markedly less alkaline (pH 7.54 ± 0.01; P<0.001; n=3; Fig. 7E). Taken together, these results are consistent with pendrin mediating the majority of HCO₃⁻ secretion (exit) across the luminal membrane of Calu-3 monolayers via coupled Cl'/HCO₃⁻ exchange. Furthermore, because the osmolarity of the secreted fluid from all treatment groups did not significantly differ from the Krebs buffer (data not shown), any increase in [HCO₃⁻] indicates that the [Cl'] of the secreted fluid must decrease proportionally to maintain constant osmolarity. From the [Cl'] and volume of fluid secreted it is possible to estimate total Cl' secretion capacity for the different cell types (supplemental Table S3). The results show that CFTR KD substantially reduced, while pendrin KD increased total Cl' secretion, as would be predicted from their proposed roles in transepithelial salt and fluid secretion in Calu-3 cells (see Fig. 8).
DISCUSSION

Our findings demonstrate for the first time the presence of an apical Cl/HCO₃⁻ exchange activity in cAMP-stimulated Calu-3 cells. This cAMP-activated apical AE activity is Na⁺-independent, H₂-DIDS-insensitive and capable of transporting a broad range of monovalent, but not divalent, anions in exchange for HCO₃⁻. The selectivity profile is I⁻>Br⁻>Cl⁻=HCOO⁻, consistent with the pH-regulatory role of pendrin for HCO₃⁻ transport capability from pH₃, regulatory (via basolateral AE) to HCO₃⁻ secretory modes (via activation of an apical AE) in response to cAMP elevation and activation of PKA (see Fig. 8 for discussion). This dual role of CFTR is particularly evident in the CFTR KD Calu-3 cells, where the reduced CFTR expression in these cells not only lowered the rate of cAMP-stimulated apical AE activity, but also led to partial relief of basolateral AE inhibition normally seen in stimulated cells. Taken together, these observations suggest that CFTR participates in both processes. Furthermore, FRT cells expressing pendrin alone (Fig. 6) show a basal level of AE activity which could not be further enhanced by cAMP elevation (Fig. 6 D&E). These results may indicate that in Calu-3 cells, CFTR tonically inhibits pendrin activity until a cAMP stimulus is received. Exactly how this occurs requires further clarification. Previous studies on CFTR and SLC26A3 and A6 transporters postulate physical interaction between the phosphorylated R-domain of CFTR with the STAS domain of the SLC26 transporters which promote enhanced channel and AE activity (24). Co-expression studies in HEK cells showed that CFTR expression led to a marked increase in Cl⁻/OH⁻ exchange mediated by SLC26A3, A4 and A6 (48). It is also possible that, under resting conditions, association between CFTR and pendrin blocks AE activity, a phenomenon recently described for Slc26a9 and the R-domain of CFTR in Xenopus oocytes co-expression studies (49).

The inhibition of the basolateral Cl⁻/HCO₃⁻ exchanger under cAMP-elevated conditions fits with a primary role of this AE in maintaining pH₃. It is likely that the basolateral AE is also important for Cl⁻ accumulation under resting conditions, as has already been suggested for Calu-3 cells (33) as well as for HCO₃⁻-secreting pancreatic duct cells (50). Our results also suggest that GlyH-101 inhibition of CFTR in stimulated cells, leads to the re-activation of the basolateral AE, possibly via the resulting alkalisation. This may help prevent an intracellular alkali load by shunting HCO₃⁻ accumulation by the basolateral Na⁺-HCO₃⁻ cotransporter (NBC; Fig. 8). The exact mechanisms involved in the cAMP/CFTRD-independent inhibition of the basolateral AE presently are not clear. AE2 is known to be very
pH-sensitive (51). Thus, intracellular acidification produced by the efflux of $\text{HCO}_3^-$ across the apical membrane upon fsk stimulation could provide the basis of one potential mechanism.

We obtained an insight into the role of the apical Cl/$\text{HCO}_3^-$ exchanger in Calu-3 cells by examining the composition of the secreted fluid (Fig. 7). Our pH measurements predict fsk-stimulated apical $\text{Cl}^-$ secretion facilitated by pendrin-mediated Cl/$\text{HCO}_3^-$ exchange. Forskolin-stimulated pendrin-KD Calu-3 cells showed reduced apical AE activity and produced a fluid of lower pH, consistent with these predictions. Although CFTR knockdown or inhibition by GlyH-101 had no effect on the pH of fsk-stimulated secreted fluid, both manoeuvres reduced the volume of secreted fluid. These findings are consistent with electrogenic Cl efflux via CFTR-driven transcellular fluid secretion, and oppose the inhibition by glyH-101 of the pH-sensitive (51). Thus, intracellular acidification produced by the efflux of $\text{HCO}_3^-$ across the apical membrane upon fsk stimulation could provide the basis of one potential mechanism.

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mucus release, suggesting that HCO₃⁻ is required for proper mucus production (4-6). In addition, recent work indicates that HCO₃⁻ ions play an essential role in determining the final rheological properties of mucus via competing with fixed anions in mucins for Ca²⁺. This ultimately regulates mucin swelling, cross-linking and gel formation. Lack of HCO₃⁻ leads to the formation of thick, sticky, mucus which strongly adheres to the lining epithelium (7,8). In the context of the SMG and CF, lack of HCO₃⁻ would therefore predispose the glands to mucus blockage and damage, a characteristic sign of CF airway disease, and an important mediator of lung pathophysiology. The recent finding that pendrin expression is coupled to mucin gene expression in response to inflammatory cytokines is important in that it suggests that mucus production and mucus expansion may be tightly co-regulated (42).

In conclusion, our results are consistent with pendrin mediating the majority of HCO₃⁻ secretion across the apical membrane of Calu-3 monolayers via coupled exchange of Cl⁻ for HCO₃⁻. This work establishes a critical, and novel, role for this anion exchanger in transporting HCO₃⁻ across human airway serous epithelial cells.

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The abbreviations used are: AE, anion exchange; BCECF-AM, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; CFTR, cystic fibrosis transmembrane conductance regulator; cystic fibrosis (CF); EBIO, 1-ethyl-2-benzimidazolinone; Fsk, forskolin; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; H2DIDS, 4,4'-diisothiocyanato-1,2-diphenylethane-2,2'-disulfonate; Jv, rate of liquid secretion; KD, knock down; KRB, HCO3--buffered Krebs solution; NKCC, Na+-K+-2Cl- cotransporter; NBC, Na+-HCO3- cotransporter; pH, intracellular pH; ΔpHRA; rate of re-acidification; shRNA, short hairpin; (Vm, membrane potential; Vte, transepithelial resistance.

FIGURE LEGENDS

Fig. 1. cAMP activates apical Cl--dependent HCO3- transport in polarised cultures of Calu-3 cells. A: Representative experimental trace showing the effect of Cl- removal (0Cl-) from the HCO3- -buffered bathing solution on intracellular pH (pHi), in non-stimulated and forskolin-stimulated (FSK, 5 µM) conditions. B: Mean ΔpHRA following apical Cl- re-addition under non-stimulated and fsk-stimulated (FSK) conditions. (n=35; paired observations.*P<0.001 compared to apical 0Cl-. †P<0.001 compared to basolateral 0Cl-). C: The effect of basolateral vasoactive intestinal peptide (VIP; 150 nM) and bilateral adenosine (ADO; 10 µM) on the ΔpHRA following apical Cl- re-addition. Data are expressed as percentage (%) of the rate obtained with fsk-treated monolayers (n=4). All agonists performed in parallel using separate monolayers. D: Effect of H-89 (50 µM) and staurosporine (1 µM) on ΔpHRA following apical Cl- re-addition in fsk-treated Calu-3 cells. (n=8). Cell cultures were pre-treated with either inhibitor for 60 mins. Inhibitors were present in all solutions throughout the experiment. Inhibitor-treated and untreated Calu-3 cells ran in parallel. (*P<0.001 compared to apical 0Cl+FSK). E: Effect of external HCO3- on mean ΔpHRA following apical Cl- re-addition in fsk-stimulated Calu-3 cells. HCO3- -free solutions were buffered to pH 7.4 with NaHEPES. (n=35; paired observations. *P<0.001 compared to apical 0Cl. †P<0.001 compared to apical 0Cl+FSK).

Fig. 2. Ionic dependence of apical Cl--dependent HCO3- transport in forskolin-stimulated Calu-3 cell monolayers. A: Anion selectivity of the apical Cl--dependent pH changes measured by the ability of the replacement anion (for Cl-) to support re-acidification under fsk-stimulated conditions. Anions were added iso-osmotically to replace Cl-. (n=5; Paired observations; *P<0.01 compared to chloride. †P<0.001 compared to formate, nitrate and thiocyanate. B: The effect of bilateral Na+ removal on ΔpHRA upon apical Cl- re-addition in fsk-treated cells. Data expressed as a % of the rate obtained with fsk-treated monolayers in the presence of bilateral Na+. (n=4; Na+-containing and Na+-free Krebs solutions applied in separate experiments, performed in parallel). C: The effect of bilateral high K+ (115 mM) and K+ channel opener EBIO (1 mM) on the %ΔpHRA upon apical Cl- re-addition in fsk-stimulated cells. pHi responses in high K+ and 1-EBIO compared to control apical 0Cl+FSK responses. Each condition measured in separate experiments performed in parallel. (n=4; *P<0.05 compared to apical 0Cl+FSK). D: The effect of apical H2-DIDS (500 µM) on theΔpHRA upon apical Cl- re-addition in fsk-stimulated cells. (n=4; paired observations).
Fig. 3. Demonstration of apical Cl/HCO₃⁻ exchange activity in the presence of apical GlyH-101, solely or in combination with basolateral H₂-DIDS. A: Experimental traces of the effect of apical GlyH-101 (10 µM) on apical Cl⁻-induced changes in pHᵢ, in fsk-stimulated (5 µM) Calu-3 cells, in the presence (blue trace) and absence (black trace) of basolateral H₂-DIDS (500 µM). B: The mean change in pHᵢ produced by apical Cl⁻ removal under each condition (n=4; *P<0.001 compared to apical 0Cl⁻ +FSK. †P<0.01 compared to +GlyH-101). C: The ΔpHᵢ upon apical Cl⁻ re-addition under each condition. (n=4; paired observations; *P<0.001 compared to apical 0Cl⁻ +FSK. †P<0.01 compared to +GlyH-101).

Fig. 4. Apical Cl⁻-dependent HCO₃⁻ transport in Calu-3 cells is partially CFTR-dependent. A: Trace showing the effect of Cl⁻ removal (0Cl⁻) on pHᵢ, under non-stimulated and fsk-stimulated (FSK, 5 µM) conditions in CFTR KD Calu-3 cells. B: Comparison of the mean ΔpHᵢ upon apical Cl⁻ re-addition in wild type (WT) and CFTR knockout (KD) Calu-3 cells. WT and CFTR KD experiments carried out in parallel (n=4; *P<0.01 compared to WT). C: The mean rate of pHᵢ recovery in CFTR KD Calu-3 cells upon introduction of monovalent (chloride, formate, and iodide) and divalent (oxalate and sulphate) anions following apical Cl⁻ removal under fsk-stimulated conditions. Anions were added iso-osmotically to replace Cl⁻. (n=4; paired observations; *P<0.001 compared to chloride and formate).

Fig. 5. Pendrin knockdown Calu-3 cells show reduced apical Cl⁻-dependent HCO₃⁻ transport. A: Quantitative RT-PCR (Taqman) analysis of SLC26 mRNA expression in WT Calu-3 cells grown on Transwell supports, relative to standard curve and normalised to GAPDH (%)(n=3 separate cell cultures). B: qRT-PCR analysis of the % pendrin expression in pendrin KDCalu-3 cells normalised to GAPDH, compared to that of cyclophilin B KD (control KD) Calu-3 cells(n=3). C: Confocal micrographs showing pendrin (green; upper panels) and ZO-1 (red; lower panels) staining in wild type (WT), CFTR knockdown (CFTR KD), pendrin KD (A4 KD) and cyclophilin B KD (control KD) Calu-3 cell monolayers. WT no 1°, represents immunofluorescence from WT Calu-3 cells with only the secondary antibodies applied. Note that cells treated with either antibody are separate cultures). D: Comparison of the mean ΔpHᵢ upon re-addition of Cl⁻ in cyclophilin B (control KD) and pendrin KD Calu-3 cells. Control KD and pendrin KD cell experiments carried out in parallel. (n=5; *P<0.001 compared to Control KD). E: The mean ΔpHᵢ in pendrin KD Calu-3 cells upon introduction of monovalent (chloride, formate, and iodide) and divalent (oxalate and sulphate) anions following apical Cl⁻ removal, under fsk-stimulated conditions (n=4; paired observations). F: The effect of apical H₂-DIDS (500 µM) on theΔpHᵢ upon apical Cl⁻ re-addition in fsk-stimulated pendrin KD cells. Data expressed as a % of the rate obtained with control fsk-treated monolayers. (n=4; paired observations).

Fig. 6. Properties of Cl⁻-dependent changes in pH in CFTR and Pendrin-transfected FRT cells. A & D: Experimental traces showing the effect of forskolin (5 µM) on changes in pHᵢ following the removal of apical Cl⁻ in (A) CFTR-transfected and (D) pendrin-transfected FRT cell monolayers. Note that untransfected FRT cells produced no response to this manoeuvre (data not shown). B and E: The effect of fsk and fsk plus GlyH-101 (10 µM) on ΔpHᵢ following apical Cl⁻ re-addition in CFTR-transfected (B) and pendrin-transfected (E) FRT cells (n=4; Paired observations;*P<0.05 compared to apical 0Cl⁻. †P<0.01 compared to +FSK+GlyH-101). C & F: The mean ΔpHᵢ produced by the introduction of monovalent (iodide, formate and chloride) and divalent (oxalate and sulphate) anions following the removal of apical Cl⁻ in (C) CFTR-transfected and (F) Pendrin-transfected FRT cells (n=4; Paired observations;*P<0.05 compared to Cl⁻ and formate).

Fig. 7. Assessment of transepithelial fluid secretion rates and fluid pH. A: Changes in the rate of transepithelial fluid secretion by wild-type Calu-3 cell monolayers in response to bilateral Cl⁻ and HCO₃⁻ removal, using Cl⁻-free HCO₃⁻-buffered (0Cl⁻) and HEPES-buffered Krebs (HEPES) solutions under non-stimulated and forskolin-stimulated (FSK) conditions, compared to liquid secretion from monolayers bathed in the standard HCO₃⁻-buffered Krebs solution (KRB). Forskolin (5 µM) was
Figure 8. Proposed model for HCO$_3^-$ secretion in human airway serous cells. A & B: Diagram illustrating the movement of Cl$^-$ and HCO$_3^-$ ions across the basolateral and luminal membrane of non-stimulated (A) and cAMP-stimulated (B) Calu-3 cells, based on pH$_i$ and transepithelial fluid secretion studies. Under non-stimulated conditions Cl$^-$ is accumulated across the basolateral membrane by the combined action of the basolateral Na$^+$-K$^+$-2Cl$^-$ cotransporter, NKCC1, together with parallel activity of the Na$^+$-HCO$_3^-$ cotransporter (NBC) and anion exchanger 2 (AE2) (32,33). Under these conditions a basal level of CFTR activity drives a small amount of a Cl$^-$-rich secretion with a pH of ~ 7.4 (25 mM HCO$_3^-$) via electrogenic Cl$^-$ efflux through CFTR. Elevation of cAMP inhibits basolateral AE2, and stimulates NBC (21). At the apical membrane cAMP/PKA further increases CFTR activity, as well as activates pendrin (SLC26A4). Stimulation of CFTR activity leads to a marked rise in net transepithelial fluid secretion driven by electrogenic Cl$^-$ efflux through CFTR, while the co-activation of pendrin increases the HCO$_3^-$ content of this secreted fluid to ~ 75 mM (pH 7.9), through coupled Cl$^-$/HCO$_3^-$ exchange, with Cl$^-$ cycling across the apical membrane via CFTR and SLC26A4.
Figure 1

A

Apical 0 Cl

+ Forskolin

Basolateral 0 Cl

B

Rate of re-acidification (pH i min⁻¹)

Apical 0 Cl

Basolateral 0 Cl

Apical 0 Cl + Forskolin

C

% rate of re-acidification (pH i min⁻¹)

+ Vip

+ ADO

D

% rate of re-acidification (pH i min⁻¹)

+ H-89

+ Stauroporine

E

Rate of re-acidification (pH i min⁻¹)

Apical 0 Cl

+ Forskolin

+ Forskolin (HERES)
Figure 2

A

Rate of re-acidification (pH \text{min}^{-1})

Bromide | Chloride | Formate | Iodide | Nitrate | Thiocyanate | Oxalate | Sulphate

B

% rate of re-acidification (pH \text{min}^{-1})

Apical IGF-IR

C

% rate of re-acidification (pH \text{min}^{-1})

Apical IGF-IR + FSK

EBIO

D

Rate of re-acidification (pH \text{min}^{-1})

Apical IGF-IR + FSK

EBIO
Figure 3

A

B

C

Figure 4

A

B

C
Figure 5

A

Expression Relative to GAPDH

B

Pendrin expression (% of WT normalised to GAPDH)

C

WT

WT no 1°

CFTR KD

A4 KD

control KD

D

Rate of re-acidification (pH min⁻¹)

E

Rate of re-acidification (pH min⁻¹)

F

% rate of re-acidification (pH min⁻¹)

-0.05 -0.00 0.05 0.10 0.15 0.20 0.25 0.30 0.35 0.40 0.45 0.50 0.55 0.60

Chloride Formate Iodide Oxalate Sulphate

Apico IQF F9K H₂O₉₃
Figure 6

A

**Apical**

- **Basolateral**

- + Forskolin

B

**Rate of re-acidification (pH min⁻¹)**

- **Cl⁻**
- **Forskolin**
- **Cl⁻**

C

**Rate of re-acidification (pH min⁻¹)**

- **Cl⁻**
- **Forskolin**
- **Iodide**
- **Oxalate**
- **Sulphate**

D

**Apical**

- **Basolateral**

- + Forskolin

E

**Rate of re-acidification (pH min⁻¹)**

- **Cl⁻**
- **Forskolin**
- **Cl⁻**

F

**Rate of re-acidification (pH min⁻¹)**

- **Cl⁻**
- **Forskolin**
- **Iodide**
- **Oxalate**
- **Sulphate**
Figure 7

A

B

C

D

E

Figure 8

A

B
Novel role for pendrin in orchestrating bicarbonate secretion in CFTR-expressing airway serous cells
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