Paracellular bicarbonate flux across human cystic fibrosis airway epithelia tempers changes in airway surface liquid pH

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Key points
- Cl− and HCO3− had similar paracellular permeabilities in human airway epithelia.
- PCl/PNa of airway epithelia was unaltered by pH 7.4 vs. pH 6.0 solutions.
- Under basal conditions, calculated paracellular HCO3− flux was secretory.
- Cytokines that increased airway surface liquid pH decreased or reversed paracellular HCO3− flux.
- HCO3− flux through the paracellular pathway may counterbalance effects of cellular H+ and HCO3− secretion.

Abstract Airway epithelia control the pH of airway surface liquid (ASL), thereby optimizing respiratory defences. Active H+ and HCO3− secretion by airway epithelial cells produce an ASL that is acidic compared with the interstitial space. The paracellular pathway could provide a route for passive HCO3− flux that also modifies ASL pH. However, there is limited information about paracellular HCO3− flux, and it remains uncertain whether an acidic pH produced by loss of cystic fibrosis transmembrane conductance regulator anion channels or proinflammatory cytokines might alter the paracellular pathway function. To investigate paracellular HCO3− transport, we studied differentiated primary cultures of human cystic fibrosis (CF) and non-CF airway epithelia. The paracellular pathway was pH-insensitive at pH 6.0 vs. pH 7.4 and was equally permeable to Cl− and HCO3−. Under basal conditions at pH ~6.6, calculated paracellular HCO3− flux was weakly secretory. Treating epithelia with IL-17 plus TNFα alkalinized ASL pH to ~7.0, increased paracellular HCO3− permeability, and paracellular HCO3− flux was negligible. Applying IL-13...
Introduction

The acid-base status of airway surface liquid (ASL) is tightly regulated (Fischer & Widdicombe, 2006). Active proton secretion produces an ASL pH that is acidic compared with the interstitial space (Jayaraman et al. 2001a; Coakley et al. 2003; McShane et al. 2003; Pezzulo et al. 2012; Garland et al. 2013; Abou Alaiwa et al. 2014a; Schultz et al. 2017; Abou Alaiwa et al. 2018). H⁺ secretion is neutralized, in part, by HCO₃⁻ secretion by the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel, Ca²⁺-activated Cl⁻ channels, and pendrin-mediated Cl⁻/HCO₃⁻ exchange (Coakley et al. 2003; Fischer & Widdicombe, 2006; Shah et al. 2016; Lennox et al. 2018; Simonin et al. 2019). In cystic fibrosis (CF), loss of CFTR-mediated HCO₃⁻ secretion decreases the ASL pH (Pezzulo et al. 2012; Garland et al. 2013; Abou Alaiwa et al. 2014a; Garnett et al. 2016; Haggie et al. 2016; Shah et al. 2016; Abou Alaiwa et al. 2018; Simonin et al. 2019). The acidic ASL pH impairs at least two host-defence mechanisms: antimicrobial activity (Pezzulo et al. 2012; Abou Alaiwa et al. 2014b; Shah et al. 2016; Simonin et al. 2019) and mucociliary transport (Clary-Meinesz et al. 1998; Hoegger et al. 2014; Tang et al. 2016; Ostedgaard et al. 2017).

In parallel with the cellular pathway, with its channels, transporters and pumps, lies the paracellular pathway, which allows passive ion flux (Diamond, 1978; Anderson & Van Itallie, 2009). The paracellular pathway could provide a route for HCO₃⁻ secretion or absorption, and thus it could modify ASL pH. However, HCO₃⁻ flux through the paracellular pathway is rarely considered, perhaps because the use of the short-circuit technique negates the influence of the paracellular pathway.

HCO₃⁻ flux through the paracellular pathway might perturb ASL pH in disease. As an example, the ASL of newborn humans with CF, newborn CF pigs, and differentiated cultures of human and pig CF airway epithelia is more acidic than non-CF ASL (Coakley et al. 2003; Pezzulo et al. 2012; Garland et al. 2013; Abou Alaiwa et al. 2014a; Garnett et al. 2016; Haggie et al. 2016; Shah et al. 2016; Abou Alaiwa et al. 2018; Simonin et al. 2019). However, over the course of months and years, the in vivo pH of CF ASL alkalinizes (Abou Alaiwa et al. 2014a; Schultz et al. 2017; Abou Alaiwa et al. 2018). These findings suggest that an in vivo factor might contribute to age-dependent alkalinization. Airway inflammation develops over a similar time course (Khan et al. 1995; Muhlebach et al. 1999; Dakin et al. 2002; Sly et al. 2009). Consistent with the hypothesis that inflammation may increase ASL pH, previous reports suggest that proinflammatory cytokines alter ASL pH in primary cultures of CF epithelia (Kreindler et al. 2009; Gorrieri et al. 2016; Haggie et al. 2016; Lennox et al. 2018; Scudieri et al. 2018; Kim et al. 2019; Rehman et al. 2020).

Thus, further knowledge of the HCO₃⁻ permeability and flux via the paracellular pathway could aid understanding of how ASL pH is controlled. To assess paracellular HCO₃⁻ permeability, we first tested whether pH alters the paracellular permeability of airway epithelia. We then evaluated the paracellular HCO₃⁻ permeability and calculated the paracellular HCO₃⁻ flux in the absence and presence of cytokines that are associated with airway inflammation.

Methods

Airway epithelial cells were obtained from CF and non-CF tissue obtained from the Iowa Donor Network with CF donor information supplied in Table 1 and studies were approved by the University of Iowa Institutional Review Board and conform to the principles and regulations of The Journal of Physiology (Grundy, 2015). Epithelial cells were cultured according to a previous protocol (Karp et al. 2002). Briefly, donor tissue was digested with pronase then seeded upon collagen-coated semi-permeable membranes (0.33 cm² polycarbonate filters, Costar #3413) and grown at an air–liquid interface. Cultures were used after complete cellular differentiation (>21 days) and resistances >166 Ω.cm².

Solutions

All chemicals were from Sigma-Aldrich unless otherwise stated. All dilution potential solutions consisted of the same minor salts and glucose in mM; 5 glucose, 1.2 calcium gluconate, 1.2 magnesium gluconate, and were buffered with 5 Hepes (pH 7.4 solutions) or 5 MES (pH 6.0

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solutions) and titrated at 37°C to their respective pH value with N-methyl-D-glucamine (NMDG), a cell- and junctional-impermeant sugar, that acts as a strong base (i.e. >99% dissociation) at pH 7.4 and 6.0 (pKa 9.6; 22°C). The [NMDG+] added was < 1 mM and was therefore excluded from ionic strength calculations. All major salts (e.g. NaCl) were made at the following concentrations in mM: 150, 112.5, 75, 37.5 or 18.75 and were gassed with compressed air. All solutions were made to 310 ± 5 mOsm by mannitol addition and verified by a vapour pressure osmometer (Wescor Inc.) each time a solution was made. Solutions were made on the day of each experiment.

For solutions containing HCO3−, the [HCO3−] was computed using the Henderson–Hasselbalch equation. 5% CO2 partial pressure was calculated using the average atmospheric pressure of Iowa City (765.5 mmHg; Iowa City Municipal Airport) and correcting for the vapour pressure of water (47.10 mmHg). For dilution potential experiments, NaHCO3 and respective NaCl control solutions were (in mM) 22 or 11. The basolateral solution was 22 mM NaHCO3 or 22 mM NaCl for their respective experiments. The NaCl control solution for NaHCO3 experiments was titrated to the same pH value as its corresponding NaHCO3 solution to control for possible pH-dependent effects. Calculated pH values for these experiments were pH 7.4 and pH 7.1. However, empirical pH values for these NaHCO3 solutions equilibrated with 5% CO2 were 7.58 (22 mM NaHCO3) and 7.21 (11 mM HCO3), which may be attributed to the increased pKa of HCO3− (Hastings & Sendroy, 1925) and decreased CO2 solubility (Van Slyke et al. 1928) for low ionic strength solutions. Therefore, control NaCl solutions were titrated to these empirical pH values with NMDG. The paracellular NaCl permeability for pH 7.58 or pH 7.21 low ionic strength experiments did not differ from values obtained from pH 7.4 or pH 6.0 experiments performed at higher ionic strengths. All NaHCO3 solutions were gassed with 5% CO2/21%O2 balanced with nitrogen and all NaCl solutions were gassed with air.

For open-circuit experiments performed to estimate paracellular HCO3− current, three different solutions were used. The basolateral solution contained in mM: 5 glucose, 104.8 sodium chloride, 22 sodium bicarbonate, 5.2 potassium chloride, 18.2 sodium gluconate, 1.2 calcium gluconate, 1.2 magnesium gluconate, 2.2 NMDG and 2.2 gluconic acid. Physiological ASL solutions contained 5 glucose, 70 sodium chloride, 20 potassium chloride, 1.2 calcium gluconate, 1.2 magnesium gluconate, 26.3 NMDG, 26.3 gluconic acid and either 22 sodium bicarbonate/13 sodium gluconate (pH 7.4) or 4 sodium bicarbonate/31 sodium gluconate (pH 6.6). These solutions approximate the ionic activity of native human ASL cultured at the air–liquid interface (Knowles et al. 1997; Jayaraman et al. 2001b; Namkung et al. 2009).

Pharmacological reagents

The following drugs and final concentrations were used in this study: 100 μM amiloride (Sigma-Aldrich), 100 μM DIDS (Sigma-Aldrich), 100 μM GlyH-101 (Cystic Fibrosis Foundation Therapeutics and Robert Bridges), and 1 mM acetazolamide (Sigma-Aldrich). All drugs were dissolved in DMSO (Thermo Fisher Scientific).

Cytokine treatment

For the cytokine studies, the media was changed every 2 days. For the IL-13 experiments, 20 ng/ml IL-13 (R&D Systems) or DMSO vehicle was added to the basolateral compartment of differentiated epithelia, then 20 μl of the basolateral solution was added to the apical surface and experiments were performed 21 days after initial treatment. 20 ng/ml IL-13 is sufficient to increase goblet cell abundance in many laboratories (Laoukili et al. 2001; Atherton et al. 2003; Zhen et al. 2007; Kanoh et al. 2011; Thavagnanam et al. 2011; Dickinson et al. 2016; Pezzulo et al. 2019). For IL-17/TNFα experiments, 20 ng/ml IL-17 (R&D Systems) and 10 ng/ml TNFα (R&D Systems) or DMSO was added to the basolateral media and experiments were performed 2 days later based on preliminary dose–response studies and previous reports.

| Donor | Age | Sex | Cystic fibrosis transmembrane conductance regulator mutations |
|-------|-----|-----|------------------------------------------------------------|
| 1     | 27  | Female | ΔF508/1717-1G→A                                                |
| 2     | 36  | Female | ΔF508/R347P                                                   |
| 3     | 29  | Female | ΔF508/3876delA                                                 |
| 4     | 36  | Female | ΔF508/ΔF508                                                   |
| 5     | 22  | Male   | ΔF508/1717-1G→A                                                |
| 6     | 21  | Female | ΔF508/2622 + 1G→A                                              |
| 7     | 36  | Male   | ΔF508/R553X                                                   |
| 8     | 64  | Male   | ΔF508/L1254X                                                  |
| 9     | 37  | Male   | ΔF508/deletions of exons 2–3                                   |
| 10    | 38  | Male   | ΔF508/ΔF508                                                   |
| 11    | 19  | Female | ΔF508/G85E                                                   |
| 12    | 31  | Female | ΔF508/ΔF508                                                   |
| 13    | 24  | Female | ΔF508/unknown                                                 |
| 14    | 31  | Female | ΔF508/G551D                                                   |
| 15    | 42  | Female | ΔF508/ΔF508                                                   |
| 16    | 34  | Female | ΔF508/3659delC                                                 |
| 17    | 54  | Female | ΔF508/I336K                                                   |
| 18    | 35  | Female | ΔF508/2184insA                                                |
| 19    | 24  | Female | ΔF508/G551D                                                   |
| 20    | 29  | Female | ΔF508/3849 + 10kbC→T                                           |

Table 1. Cystic fibrosis donors used in this study
Electrophysiology

Epithelia were assayed in Ussing chambers (Physiologic Instruments) with 3 M KCl agar bridges connected to amplifiers (VCC-MC8, Physiologic Instruments) recording open-circuit transepithelial voltage ($V_t$). A 5 µA bipolar current pulse was applied across the epithelium periodically. The current-induced change in $V_t$ was used to calculate the transepithelial conductance ($G_t$). Data were acquired with Acquire & Analyse software (version 2.3.8, Physiologic Instruments).

Importantly, junction potentials induced in the bilateral solution. Junction potentials induced in the chamber. After the experiment, cells were lysed by distilled water and electrode drift was assessed in the original bilateral solution. Junction potentials induced in the voltage probes by ionic dilutions were then assessed without an epithelium and subtracted from the obtained dilution potentials.

Calculations

Activity coefficients were calculated using the ionic strength of each solution and the extended Debye–Hückel equation (Robinson & Stokes, 1959), which is applicable for physiological concentrations:

$$\log \gamma = \frac{-0.509z^2 \sqrt{\mu}}{1 + 3.29 \xi \gamma}$$  \hspace{1cm} (1)

Where $\gamma$ is the activity coefficient of the ion, $z$ is the ionic charge, $\mu$ is the ionic strength of the solution, and $\xi$ is the effective diameter of the hydrated ion taken from Keilland (1937). The constants $-0.509$ and $3.29$ were used because experiments were performed at 37°C (Manov et al. 1943).

Ion activity was calculated by the equation:

$$a_{ion} = \gamma \times [c]$$  \hspace{1cm} (2)

Where $a$ is the ion activity, $\gamma$ is the activity coefficient, and $[c]$ is the ion concentration.

Relative permeability ($P_{A\text{ion}}/P_{Na}$) was calculated using the Goldman–Hodgkin–Katz equation (Goldman, 1943; Hodgkin & Katz, 1949):

$$\Delta V_t = \frac{RT}{F} \ln \left( \frac{\alpha_{Na_{basolateral}} + P_{A^-}/Na^+ \times \alpha_{A_{apical}}}{\alpha_{Na_{apical}} + P_{A^-}/Na^+ \times \alpha_{A_{basolateral}}} \right)$$  \hspace{1cm} (3)

Approximating the constants at 37°C simplifies to:

$$\Delta V_t = 61.5 \log \left( \frac{\alpha_{Na_{basolateral}} + P_{A^-}/Na^+ \times \alpha_{A^-}}{\alpha_{Na_{apical}} + P_{A^-}/Na^+ \times \alpha_{A_{basolateral}}} \right)$$  \hspace{1cm} (4)

Where $F$ is Faraday’s constant, $R$ is the gas constant, $T$ is temperature, $\Delta V_t$ is the dilution potential, $a$ is the ionic activity, and $P_{A^-}/Na^+$ is the relative permeability of anion $A^-$ to Na$^+$. When bi-ionic potentials were measured (potential generated by replacing apical NaCl with an equimolar monovalent chloride salt), we used Eqn 4 and substituted $\alpha_{Na_{apical}}$ with $P_{Na^+}/Na^+$ × the substituted cation’s activity.

Partial conductance ($G_{ion}$) was computed using the following formula (Schultz, 1980; Sten-Knudsen, 2002):

$$\Delta V_t = \left( \frac{G_{Na}}{G_p} \times E_{Na} \right) + \left( \frac{G_p - G_{Na}}{G_p} \times E_{anion} \right)$$  \hspace{1cm} (5)

Where $\Delta V_t$ is the dilution potential, $G_p$ is the paracellular conductance in the corresponding symmetrical salt solution, and $E_{Na}$ and $E_{anion}$ are the computed Nernst potential for the ion during dilution. The value $G_p - G_{Na}$ is the partial conductance of the anion ($G_{anion}$). The quantities $G_{Na}$ and $G_p - G_{Na}$ represent the transference numbers for Na$^+$ and its corresponding anion.

Absolute paracellular permeabilities were calculated using the following equation (Schultz, 1980; Sten-Knudsen, 2002):

$$P_{ion} = \frac{G_{ion} \times RT}{(zF)^2 V_t \left[ a_{basolateral} - a_{apical} \right]}$$  \hspace{1cm} (6)

Where $P_{ion}$ is the ion’s absolute permeability, $G_{ion}$ is the ion’s partial conductance, $z$ is the ion’s charge, $a$ is the ion’s activity, $F$ is Faraday’s constant, $R$ is the gas constant, $T$ is temperature, $V_t$ is the transepithelial voltage and $E_{ion}$ is the ion’s Nernst potential. We obtained similar values for $P_{ion}$ using the approach taken by Kimizuka and Koketsu (Kimizuka & Koketsu, 1964), using total $G$ rather than $G_{ion}$ (not shown).

Ionic mobilities ($\mu$) at 37°C were derived from reported limiting equivalent conductivities ($\lambda$) at 35°C (Robinson & Stokes, 1959) by the relationship:

$$\lambda^{\circ}_{35^\circ} = |z| \times \mu \times F$$  \hspace{1cm} (7)

Where $z$ is the ion’s charge and $F$ is Faraday’s constant. A $\mu_\text{HCO}_3^-$ of $4.11 \times 10^{-4}$ cm$^2$ sec$^{-1}$ V$^{-1}$ was derived by the Nernst–Einstein relationship:

$$\lambda^{\circ}_{\text{HCO}_3^-} = D_\text{HCO}_3, 35^\circ \times \frac{z^2 R T}{\lambda^{\circ}_{35^\circ}}$$  \hspace{1cm} (8)

Where $z$ is the ion’s charge, $F$ is Faraday’s constant, and $D$ is the HCO$_3^-$ diffusion coefficient at 35°C, $1.10 \times 10^{-5}$ cm$^2$ s$^{-1}$ (Voipio, 1998). We then substitute $\lambda^{\circ}_{\text{HCO}_3^-}$ into Eqn 7 to obtain $\mu_\text{HCO}_3^-$. Ionic conductance $G_{\text{HCO}_3^-}$ for non-symmetrical solutions were obtained by using estimated $P_{ion}$ values, known ionic concentrations, calculated Nernst
potentials, and empirical transepithelial voltages substituted into Eqn 6. To estimate $P_{ion}$ for a given paracellular conductance, we used the relationship between donor-matched $P_{HCO3}$ estimated from 22 mM NaHCO3 vs. conductance in 150 mM NaCl solution.

With the solved $G_{ion}$, the ion-specific current ($I_{ion}$) was calculated using the driving-force derivation of Ohm’s law:

$$I_{ion} = G_{ion} (V_t - E_{ion})$$ (9)

Ion-specific current was transformed to ion-specific fluxes using Faraday’s constant and were scaled to hours. Paracellular flux is directly related to paracellular current by $I_{Paracellular} = zFJ_{Paracellular}$. Therefore, we use the term paracellular flux and paracellular current interchangeably. For the discussion, we refer to the ion concentration, $[HCO_3^-]$, and ion activity, $aHCO_3^-$, interchangeably. Ion activities were used for all calculations.

**Data analysis**

Electrophysiological data were analysed using a custom graphical user interface coded in MATLAB version R2018b (Mathworks). Equations were solved in MATLAB version R2018b (Mathworks) using custom code. All codes were written by Ian M. Thornell and are freely available upon request and the data that support the findings of this study are available from the corresponding authors upon reasonable request.

**Statistics**

An initial $P_{Cl/Na}$ experiment ($n = 8$ donors) was performed and data were used to perform an a priori power analysis for the remainder of the study using GraphPad Prism 7.0d (GraphPad) and G*Power 3.1 software (Faul et al. 2007). For this initial experiment, relative permeabilities were normally distributed (the Shapiro–Wilk test) and had equal variance (F test). For the obtained effect size ($d = 1.64$), six donors were used in subsequent experiments to detect a 0.1 change in relative permeability ($\alpha = 0.05$, $\beta > 0.80$). Data were compared using either a paired Student’s t test, a one-way ANOVA with Bonferroni correction, or a Wilcoxon matched-pairs signed rank test. Linear fits were obtained using the least-squares method and compared using an extra sum-of-squares F test. For all tests, statistical significance was defined as $P \leq 0.05$. Any outliers were identified by a Grubb’s test, $\alpha \leq 0.01$.

**Results**

**Paracellular ion permeability is pH-insensitive**

It is unknown whether physiological changes in apical pH alter the paracellular permeability of airway epithelia. To evaluate paracellular permeability, we first eliminated electrogenic transcellular Na+ transport by adding 100 µM amiloride to the apical solution (Fig. 1A). To eliminate electrogenic transcellular Cl− transport, we used CF epithelia to eliminate CFTR anion channels and added 100 µM DIDS to the apical solution to inhibit Ca2+-activated Cl− channels (Ousingsawat et al. 2009). This combination resulted in a small apical-positive transepithelial voltage ($V_t$), consistent with a previous study (Coakley et al. 2003). We then determined the relative paracellular Cl− to Na+ permeability ($P_{Cl/Na}$) by measuring changes in $V_t$ in response to a series of apical NaCl dilutions. We set pH to 7.4 or 6.0 bilaterally; the pH buffer was Hepes or MES. At both pH 7.4 and 6.0, apical NaCl dilutions depolarized $V_t$, indicating that Na+ was more permeable than Cl− through the paracellular pathway (Fig 1A and B). $P_{Cl/Na}$ was not altered at pH 6.0 (Fig. 1C) suggesting that physiological pH does not alter the paracellular ion permeability of airway epithelia. We also estimated the paracellular electrical conductance ($G_p$) after adding amiloride and DIDS to CF epithelia and before the measurement of dilution potentials. $G_p$ was not affected by the changes in pH (Fig. 1D). By comparing epithelia from different donors, we found that $P_{Cl/Na}$ was independent of $G_p$ (Fig. 1E), and $P_{Na}$ and $P_{Cl}$ increased in parallel with $G_p$ (Fig. 1F). These data suggest that epithelia with low and high $G_p$ contain similar paracellular permeation pathways.

**Paracellular HCO3− permeability is similar to paracellular Cl− permeability**

Because $P_{Cl/Na}$ was unaffected by changing from pH 7.4 to pH 6.0 solutions, we were able to assess paracellular HCO3− permeability with dilution potentials, which at a constant CO2 concentration will impose a concomitant pH change. Decreasing NaHCO3 from 22 mM to 11 mM revealed relative HCO3− permeabilities, $P_{HCO3/Na}$, that were similar to pH-matched reductions in NaCl from 22 mM to 11 mM, $P_{Cl/Na}$ (Fig. 2A). Paracellular HCO3− permeability was unaffected by inhibiting carbonic anhydrase with 1 mM acetazolamide, indicating that HCO3− permeated as an ion rather than carbonic anhydrase-mediated CO2 reconversion reactions (Fig. 2B).

To further assess paracellular ion permeability, we tested several monovalent ions of different size. Cations were more permeant than anions (Fig. 2C). However, ionic size had minimal effects on permeation. Thus, the paracellular pathway was more selective for charge vs. ion size.

Paracellular Cl− and HCO3− fluxes are determined by $P_{Cl}$ and $P_{HCO3}$, the [Cl−] and [HCO3−], and $V_t$. To obtain the data necessary for calculating paracellular HCO3− flux, open-circuit $V_t$ and $G_p$ were recorded from human CF and non-CF epithelia. Figure 3A shows the
sequence of additions and timing in a non-CF epithelium. The relationships between \( P_{\text{Cl}} \) and \( P_{\text{HCO}_3} \) and \( G_p \) are shown in Fig. 3B and C. Epithelia were bathed in solutions with an ASL-like composition (Knowles et al. 1997; Jayaraman et al. 2001b; Namkung et al. 2009) titrated to pH 6.6 and also to 7.4. For epithelia from each donor, we determined \( P_{\text{Cl}} \) and \( P_{\text{HCO}_3} \) (Fig. 3D and G). The paracellular permeabilities of these two anions were similar. We then used Eqn 6 to calculate the paracellular \( \text{Cl}^- \) and \( \text{HCO}_3^- \) conductances for CF and non-CF epithelia (Fig. 3E and H). Because the [\( \text{HCO}_3^- \)] was less than the [\( \text{Cl}^- \)], the paracellular conductance for \( \text{HCO}_3^- \) was less than for \( \text{Cl}^- \). Raising the apical [\( \text{HCO}_3^- \)] increased the paracellular \( \text{HCO}_3^- \) conductance.

We also calculated the paracellular \( \text{Cl}^- \) and \( \text{HCO}_3^- \) fluxes from the driving-force derivation of Ohm’s law (Eqn 9) and Faraday’s constant (Fig. 3F and I). These calculations yielded two main observations. First, the

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**Figure 1.** Human airway epithelia cultured at the air–liquid interface have cation-selective tight junctions that are not affected by low physiological pH

Red is pH 6.0, black is pH 7.4, squares are Na\(^+\) data and triangles are Cl\(^-\) and all error bars represent standard deviations of the mean. One donor was omitted because a 11.43 mS cm\(^{-2}\) baseline conductance was identified as an outlier; Grubb’s test \( \alpha \leq 0.01 \). A, representative dilution potential experiment not corrected for junction potentials. B, junction potential-corrected dilution potential summary data for experiments containing identical solutions titrated to pH 7.4 or 6.0; \( n = 7 \) donors. The dashed lines represent theoretical dilution potentials calculated by substituting average relative permeability data from panel C into the Goldman–Hodgkin–Katz equation. C, relative paracellular permeability, each circle represents a single human donor. \( P = 0.54 \); two-tailed paired Student’s \( t \) test; \( n = 7 \) donors. D, bilateral 150 mM NaCl paracellular conductance. Each circle represents a single human donor. \( P = 0.63 \); paired Student’s \( t \) test; \( n = 7 \) donors. E, relative paracellular permeability vs. paracellular conductance, each circle represents a single human donor. We cannot reject the null hypothesis that the slopes were zero; pH 7.4 \( P = 0.15 \), pH 6.0 \( P = 0.25 \); \( F \) test; \( n = 7 \) donors each. F, paracellular ion permeability vs. paracellular conductance. Data were fit with linear regressions, \( r^2 > 0.98 \). We cannot reject the null hypothesis that the slopes were equal; \( P = 0.12 \); \( F \) test; \( n = 7 \) donors each ion and pH value.
paracellular HCO$_3^-$ flux was smaller in magnitude than the paracellular Cl$^-$ flux. Second, for an apical pH of 6.6, the calculated paracellular fluxes were absorptive for Cl$^-$ and slightly secretory for HCO$_3^-$.

When we raised the apical pH to 7.4, the calculated HCO$_3^-$ flux became absorptive. Thus, at an apical pH of 7.4, the calculated paracellular fluxes were absorptive for direction of active transcellular Cl$^-$ and HCO$_3^-$ secretion observed for airway epithelia.

Discussion

Our results indicate that human airway epithelia have a paracellular pathway that is as permeable to HCO$_3^-$ as to Cl$^-$. The HCO$_3^-$ permeability indicates that the

Proinflammatory cytokines increased ASL pH and eliminated or reversed paracellular HCO$_3^-$ secretion

Inflammation involves airway epithelia in many lung diseases. Given the importance of ASL pH to respiratory defences, we asked whether cytokines might change the paracellular HCO$_3^-$ permeability of airway epithelia and thereby alter ASL pH. IL-13 is a cytokine that is an important mediator of allergic diseases and drives the TH2-high asthma phenotype (Wills-Karp et al. 1998; Wesołowska-Andersen & Seibold, 2015; Svenningsen & Nair, 2017). IL-13 may also play an important role in CF (Hauber et al. 2003). In a previous study, we applied IL-13 for 21 days to primary cultures of human airway epithelia and induced goblet cell metaplasia (Pezzulo et al. 2019). Here we asked whether IL-13 changes paracellular anion permeability and calculated transepithelial HCO$_3^-$ fluxes. Compared with the vehicle control, IL-13 increased ASL pH in CF airway epithelia from 6.6 to 7.4 (Table 2). However, IL-13 did not significantly change Gp, or the permeability of HCO$_3^-$, Cl$^-$, Na$^+$ or K$^+$ (Table 2, Fig. 4A). The calculated paracellular HCO$_3^-$ conductance increased with the increase in apical [HCO$_3^-$] (Fig. 4B).

In vehicle-treated epithelia, we calculated a small secretory HCO$_3^-$ flux (Fig. 4C). IL-13 reversed that to a small absorptive HCO$_3^-$ flux. These results indicate that the IL-13-induced ASL alkalinization was not likely due to HCO$_3^-$ secretion through the paracellular pathway.

We also tested IL-17 and TNFα because they are commonly elevated in CF airways. They are involved in neutrophil recruitment (Smart & Casale, 1994; Lukacs et al. 1995; Laan et al. 1999; Ferretti et al. 2003; Stopplelenburg et al. 2013; Michel et al. 2014), a prominent feature of CF airway disease (Conese et al. 2003; Cantin et al. 2015; Russell et al. 2016; Liu et al. 2017). We asked whether these cytokines alter paracellular HCO$_3^-$ permeability and HCO$_3^-$ secretion and thereby contribute, at least in part, to the increased ASL pH. Compared with the vehicle control, IL-17/TNFα increased ASL pH (Table 3). These cytokines increased: P$_{Na}$, P$_{Cl}$ and P$_{HCO3}$ (Table 3, Fig. 4A). However, the increase was not the result of a non-specific leak because they decreased P$_{Cl/Na}$ and P$_{K/Na}$, whereas a non-specific leak would be predicted to increase P$_{Cl/Na}$ and P$_{K/Na}$. Paracellular HCO$_3^-$ conductance increased due to the increased P$_{HCO3}$ and the increased apical [HCO$_3^-$] (Fig. 4B). With vehicle-treated control epithelia, the calculated paracellular HCO$_3^-$ flux was small and secretory (Fig. 4C). After IL-17 and TNFα, the paracellular HCO$_3^-$ flux was not different from zero.

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paracellular pathway could influence ASL pH. Indeed, under basal conditions, we calculated a small secretory paracellular HCO$_3^-$ flux. This flux would tend to counterbalance, in part, the acidic ASL pH produced by H$^+$ secretion (Coakley et al. 2003; Fischer & Widdicombe, 2006; Shah et al. 2016; Lennox et al. 2018; Simonin et al. 2019). After treating epithelia with the proinflammatory cytokines, IL-13 and IL-17/TNF$\alpha$, ASL pH increased. However, at a pH of 7.0, the calculated paracellular HCO$_3^-$ fluxes were negligible, and at a pH of 7.4, paracellular HCO$_3^-$ fluxes were absorptive. Thus, as the pH of ASL increases towards that of basolateral liquid, paracellular HCO$_3^-$ flux becomes absorptive, tempering the alkaline pH generated by transcellular HCO$_3^-$ secretion.

Figure 3. Human airways have small paracellular HCO$_3^-$ fluxes
Panels D, E and F; n = 5 cystic fibrosis (CF) donors. 1 of 6 CF donors was excluded from these studies because cultures were unresponsive to amiloride. Black circles represent Cl$^-$ data and red circles represent HCO$_3^-$ data, each for a single donor. Conditions listed at the top of panel D also apply to aligned data points in panels E and F, and conditions listed at the top of panel G also apply to aligned data points in panels H and I. A, representative transepithelial voltage recording for non-CF epithelia bathed in symmetrical solutions. Non-CF summary data are in panels G–I. The same protocol was performed on CF epithelia to generate subsequent panels. B, paracellular P$_{Cl}$ vs. donor-matched 150 mM G$_p$ from Fig. 1 and 2 data, circles represent 11 donors; $r^2 = 0.57$. C, paracellular P$_{HCO3}$ from Fig. 2 data vs. donor-matched 150 mM NaCl G$_p$ from Fig. 1 and 2 data, circles represent 11 donors; $r^2 = 0.84$. D, paracellular P$_{anion}$ calculated from G$_p$ and either Panel B or Panel C; one-way ANOVA. Conditions for groups in panels D–F are shown at top. E, G$_{anion}$ calculated using Eqn 6. Paracellular G$_{HCO3}$ increased when apical [HCO$_3^-$] increased; Bonferroni-corrected P values shown, one-way ANOVA. F, paracellular anion flux calculated using Eqn 9 and Faraday’s constant. Minimal paracellular HCO$_3^-$ secretion became HCO$_3^-$ absorption with increased apical [HCO$_3^-$]; Bonferroni-corrected P values shown, one-way ANOVA. G, paracellular P$_{anion}$ calculated from G$_p$ and either Fig. 3B or 3C; one-way ANOVA. H, G$_{anion}$ calculated using Eqn 6. Paracellular G$_{HCO3}$ increased when apical [HCO$_3^-$] increased; Bonferroni-corrected P values shown, one-way ANOVA. I, paracellular anion flux calculated using Eqn 9 and Faraday’s constant. Minimal paracellular HCO$_3^-$ secretion became HCO$_3^-$ absorption with increased apical [HCO$_3^-$]; Bonferroni-corrected P values shown, one-way ANOVA.
Table 2. IL-13 increases airway surface liquid pH of human cystic fibrosis airway epithelia, but does not alter paracellular ion permeabilities

|                               | 21 days vehicle (PBS) | 21 days IL-13 | P value |
|-------------------------------|-----------------------|---------------|---------|
| Native airway surface liquid (ASL) experiments; n = 6 |                        |               |         |
| ASL pH                        | 6.58; [6.51, 6.63]     | 7.40; [7.20, 7.76] | <0.0001 |
| 22 mM NaCl dilution potential experiments; n = 6 |                        |               |         |
| Gp (mS cm\(^{-2}\))           | 0.802 ± 0.355          | 0.755 ± 0.259  | 0.75    |
| \(P_{\text{Cl}/Na}\) \((x10^{-6} \text{ cm s}^{-1})\) | 0.73 ± 0.06            | 0.61 ± 0.16    | 0.06    |
| \(P_{\text{Na}}\) \((x10^{-6} \text{ cm s}^{-1})\) | 6.87 ± 2.92            | 7.08 ± 1.70    | 0.49    |
| \(P_{\text{Cl}}\) \((x10^{-6} \text{ cm s}^{-1})\) | 5.11 ± 2.39            | 4.48 ± 2.19    | 0.15    |
| 22 mM NaHCO\(_3\) dilution potential experiments; n = 5\(^{[2]}\) |                        |               |         |
| Gp (mS cm\(^{-2}\))           | 0.664 ± 0.292          | 0.749 ± 0.158  | 0.51    |
| \(P_{\text{HCO}_3/Na}\)       | 0.74 ± 0.07            | 0.61 ± 0.18    | 0.12    |
| \(P_{\text{Na}}\) \((x10^{-6} \text{ cm s}^{-1})\) | 5.44 ± 2.21            | 6.52 ± 1.57    | 0.41    |
| \(P_{\text{HCO}_3}\) \((x10^{-6} \text{ cm s}^{-1})\) | 4.43 ± 2.14            | 4.61 ± 0.85    | 0.67    |
| 150 mM NaCl/KCl bi-ionic potential experiments; n = 5\(^{[3]}\) |                        |               |         |
| \(P_{\text{K}/Na}\)           | 1.09 ± 0.08            | 1.11 ± 0.12    | 0.67    |

Data reported as means ± standard deviation

\(^{[1]}\)Statistical analyses, including mean and standard deviation, were performed using [H\(^+\)]. For presentation, the [H\(^+\)] were converted to pH, and hence the standard deviations are shown as intervals.

\(^{[2]}\)Donor excluded from analysis; Grubb’s test α/2264

Table 3. IL-17/TNF\(_\alpha\) increases airway surface liquid pH of human cystic fibrosis airway epithelia and alters paracellular ion permeabilities

|                               | 48 h vehicle (PBS) | 48 h IL-17/TNF\(_\alpha\) | P value |
|-------------------------------|--------------------|---------------------------|---------|
| Native airway surface liquid (ASL) experiments; n = 6 |                        |                           |         |
| ASL pH                        | 6.57; [6.54, 6.59]  | 7.04; [6.93, 7.19]        | <0.0001 |
| 22 mM NaCl dilution potential experiments; n = 6 |                        |                           |         |
| Gp (mS cm\(^{-2}\))           | 0.585 ± 0.158       | 1.026 ± 0.472             | 0.03    |
| \(P_{\text{Cl}/Na}\)       | 0.79 ± 0.04         | 0.62 ± 0.09               | 0.01    |
| \(P_{\text{Na}}\) \((x10^{-6} \text{ cm s}^{-1})\) | 4.85 ± 1.17         | 8.90 ± 3.75              | 0.03    |
| \(P_{\text{Cl}}\) \((x10^{-6} \text{ cm s}^{-1})\) | 3.83 ± 0.96         | 5.61 ± 2.92              | 0.03    |
| 22 mM NaHCO\(_3\) dilution potential experiments; n = 6 |                        |                           |         |
| Gp (mS cm\(^{-2}\))           | 0.597 ± 0.193       | 1.344 ± 0.390             | 0.002   |
| \(P_{\text{HCO}_3/Na}\)       | 0.74 ± 0.08         | 0.69 ± 0.05               | 0.31    |
| \(P_{\text{Na}}\) \((x10^{-6} \text{ cm s}^{-1})\) | 5.06 ± 1.52         | 11.72 ± 3.07             | 0.0009  |
| \(P_{\text{HCO}_3}\) \((x10^{-6} \text{ cm s}^{-1})\) | 3.82 ± 1.36         | 8.23 ± 2.75              | 0.007   |
| 150 mM NaCl/KCl bi-ionic potential experiments; n = 6 |                        |                           |         |
| \(P_{\text{K}/Na}\)           | 1.02 ± 0.04         | 0.87 ± 0.05               | 0.005   |

Data reported as means ± standard deviation

\(^{[1]}\)Statistical analyses, including mean and standard deviation, were performed using [H\(^+\)]. For presentation, the [H\(^+\)] were converted to pH, and hence the standard deviations are shown as intervals.

\(^{[2]}\)Values obtained using two-tailed paired Student’s t test or

\(^{[3]}\)Two-tailed Wilcoxon matched-pairs ranked sign

We considered the possibility that inflammation could disrupt the barrier function of airway epithelia. Because ASL has a pH and \([\text{HCO}_3^-]\) that is lower than that of basolateral liquid, barrier disruption might allow basolateral \(\text{HCO}_3^-\) to flow into and alkalinize ASL. Both IL-13 and IL-17/TNF\(_\alpha\) increased ASL pH, whereas IL-13 did not change Gp or \(P_{\text{HCO}_3}\), and IL-17/TNF\(_\alpha\) increased both. However, both sets of cytokines decreased \(P_{\text{Cl}/Na}\); the opposite of what would be expected for disruption of the epithelial barrier because the mobility of \(\text{Cl}^-\) in water is approximately 1.5 times that of \(\text{Na}^+\). Moreover, pH changes in the physiological range did not alter paracellular conductance or \(P_{\text{Cl}/Na}\), suggesting that effects of cytokines were not secondary to altered pH.
HCO₃⁻ permeated the paracellular pathway as an anion rather than as a CO₂ reconversion reaction as indicated by its carbonic anhydrase insensitivity. Moreover, HCO₃⁻ and other tested monovalent anions had similar permeabilities, and all were approximately 3/4 as permeable as Na⁺, K⁺, and the other tested monovalent cations. It is possible that the size and charge selectivity arose from the summation of many intercellular spaces with distinct size and charge selectivity. However, these data suggest that the net paracellular transport of airway epithelia can be generalized as weakly cation-selective with minimal size selectivity.

Our dilution potential experiments revealed that paracellular PCl and P_HCO₃ were equal. A previous report suggested that P_Cl was greater than P_HCO₃ (Coakley et al. 2003). A potential explanation for this difference is that the previous study used bi-ionic experiments, and the HCO₃⁻ concentration (125 mM HCO₃⁻, pH 8.15) might result in CO₃²⁻ formation and precipitation of extracellular Ca²⁺, which is required to maintain tight junction integrity (Cereijido et al. 1998; Wang et al. 2000). Disrupted barrier integrity would lead to an inflated P_Cl because Cl⁻ has a higher ionic mobility in free solution than HCO₃⁻. However, other culture or technical differences might be responsible.

The proximal tubule, another epithelium with luminal acidification, has a P_HCO₃ that is less than P_Cl (Cogan & Alpern, 1984). That arrangement may achieve maximal transepithelial HCO₃⁻ absorption as a result of transcellular HCO₃⁻ absorption with minimal HCO₃⁻ reflux through the paracellular pathway, while allowing paracellular Cl⁻ absorption. The factors that determine the ratio of P_HCO₃ to P_Cl in the proximal tubule or in airway epithelia are unknown. However, it may be relevant that absolute paracellular permeabilities in the proximal tubule are much greater than in airway epithelia.

![Figure 4. Proinflammatory cytokines increased airway surface liquid pH and induce HCO₃⁻ absorption or minimal HCO₃⁻ flux](image)

1 of 6 cystic fibrosis (CF) donors was excluded from the IL-13 analysis because of a high IL-13-induced paracellular HCO₃⁻ conductance of 0.868 mS cm⁻²; Grubb's test α ≤ 0.01. Red circles represent HCO₃⁻ data and black circles represent Cl⁻ data, each for a single donor. A, paracellular P_HCO₃ reported in Table 2 and Table 3; two-tailed paired Student's t test, P values shown. B, G_HCO₃ calculated using Eqn 6, paracellular G_HCO₃ increased due to increased apical [HCO₃⁻]; two-tailed paired Student's t test, P values shown. C, paracellular HCO₃⁻ flux calculated using Eqn 9 and Faraday's constant. Minimal HCO₃⁻ secretion was reduced or absorptive with cytokine treatments; two-tailed paired Student's t test, P values shown.

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Previous studies have reported that IL-13 increased (Lennox et al. 2018) and decreased (Haggie et al. 2016) ASL pH. Applying IL-17 to airway epithelia for two days was also reported to increase ASL pH (Kreindler et al. 2009). We found that treating epithelia for 21 days with IL-13 or two days with IL-17/TNFα increased ASL pH. Interestingly, IL-13 did not alter Gp, and in a separate study, one day of IL-17/TNFα treatment increased ASL pH but did not alter Gp (Rehman et al. 2020). These differences highlight the likely contribution of time of treatment and identity of cytokine for responses, as has been previously noted (Coyne et al. 2002). In addition to cytokines, second messengers might alter paracellular HCO$_3^−$ flux. Previous reports with differentiated airway cell lines suggested that cAMP alters the paracellular conductance (Nilsson et al. 2010; Weiser et al. 2011). Whether or not HCO$_3^−$ flux is appreciable under these conditions will depend on P$_b^{HCO_3}$, V$_t$, and the apical pH after cAMP stimulation.

Our study has limitations. We used the Goldman–Hodgkin–Katz equation, which assumes a constant electrical field, to obtain ion permeabilities. It is possible that along the lateral space the ion encounters more complex forces than a constant field. We did not consider the unstirred layer effect, which was likely nominal because the osmotic-induced water permeability of airway epithelia is independent of bath perfusion rates (Folkesson et al. 1996). We did not address paracellular proton permeability, which cannot be measured by electrophysiological methods due to its nanomolar concentration. However, the lumen-negative trans-epithelial voltages of airway epithelia predict paracellular proton secretion with inflammatory cytokines, which are not consistent with the alkalization observed. We performed dilution potential experiments with CF airway epithelia to reduce transcellular Cl$^-$ and HCO$_3^−$ secretion and extended the permeability values to non-CF airways. However, LeSimple et al. (LeSimple et al. 2010) found that CFTR expression in immortalized CF epithelial cells increased transepithelial resistance via tight junction assembly. Li et al. (2012) found that CFTR expression in MDCK cells decreased transepithelial resistance. Consistent with a role for CFTR in tight junction assembly, Ruan et al. (2014) found that CFTR co-localizes with ZO-1 in the trachea. However, our estimated Gp was similar for CF and non-CF epithelia.

These data suggest that the paracellular pathway acts as a HCO$_3^−$ shunt. Under physiological conditions, the reversal potential for HCO$_3^−$ is slightly hyperpolarized relative to V$_t$, and passive HCO$_3^−$ flux through the paracellular pathway opposes net transepithelial acidification. Under pathological conditions that increase the ASL pH, such as those modelled by cytokine treatment, the reversal potential for HCO$_3^−$ is depolarized relative to the V$_t$, and passive HCO$_3^−$ flux opposes net transepithelial alkalinization. This shunting mechanism may help to maintain an optimal ASL pH for antimicrobial activity and mucus rheology.

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Additional information

Author contributions

IMT, TR, AAP and MJW conceived and designed the studies. IMT, TR and AAP conducted the experiments and acquired the data. IMT, TR, AAP and MJW analysed the data. IMT and MJW wrote the manuscript. All authors revised the manuscript.

Competing interests

No conflicts of interest, financial or otherwise, are declared by the authors.

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Keywords

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Statistical Summary Document