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Bicarbonate transport of airway surface epithelia in luminally perfused mice bronchioles

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Abstract

HCO₃⁻ secretion in distal airways is critical for airway mucosal defense. HCO₃⁻/H⁺ transport across the apical membrane of airway surface epithelial cells was studied by measuring intracellular pH in luminally microperfused freshly dissected mice bronchioles. Functional studies demonstrated that CFTR, ENaC, Cl⁻-HCO₃⁻ exchange, Na⁺-H⁺ exchange, and Na⁺-HCO₃⁻ cotransport are involved in apical HCO₃⁻/H⁺ transport. RT-PCR of isolated bronchioles detected fragments from Cftr, α, β, γ subunits of ENaC, Ae2, Ae3, NBCe1, NBCe2, NBCn1, NDCBE, NBCn2, Nhe1, Nhe2, Nhe4, Nhe5, Slc26a4, Slc26a6, and Slc26a9. We assume that continuous decline of intracellular pH following alkaline load demonstrates time course of HCO₃⁻ secretion into the lumen which is perfused with a HCO₃⁻-free solution. Forskolin-stimulated HCO₃⁻ secretion was substantially inhibited by luminal application of CFTR_{inh}-172 (5 μM), H₂DIDS (200 μM), and amiloride (1 μM). In bronchioles from a cystic fibrosis mouse model, basal and acetylcholine-stimulated HCO₃⁻ secretion was substantially impaired, but forskolin transiently accelerated HCO₃⁻ secretion of which the magnitude was comparable to wild-type bronchioles. In conclusion, we have characterized apical HCO₃⁻/H⁺ transport in native bronchioles. We have demonstrated that cAMP-mediated and Ca²⁺-mediated pathways are involved in HCO₃⁻ secretion and that apical HCO₃⁻ secretion is largely mediated by CFTR and H₂DIDS-sensitive Cl⁻-HCO₃⁻ exchanger, most likely Slc26a9. The impairment of HCO₃⁻ secretion in bronchioles from a cystic fibrosis mouse model may be related to the pathogenesis of early lung disease in cystic fibrosis.

Keywords: Distal airway, HCO₃⁻ secretion, Bronchiole, Microperfusion, Surface epithelial cells, Intracellular pH

Introduction

The airway surface liquid (ASL) is a thin layer of fluid covering the luminal surface of airway epithelium. The ASL is composed of inner periciliary liquid layer (PCL) and outer single-layer thin mucus. Proper volume/depth, viscosity, and pH of ASL are required for efficient mucociliary clearance and antimicrobial activity [11, 38, 47].

It is widely accepted that the volume/depth of PCL is determined by Cl⁻ secretion via cystic fibrosis transmembrane conductance regulator (CFTR) and Ca²⁺-activated

Cl⁻ channel (CaCC) and Na⁺ absorption via epithelial Na⁺ channel (ENaC) [30, 33]. In proximal airways, Cl⁻ secretion is mostly derived from serous cells of submucosal glands [5, 8, 17]. In distal airways, submucosal glands are absent [10, 35] and concurrent Cl⁻ secretion and Na⁺ absorption was observed in surface epithelial cells [44]. Loss of CFTR function due to severe pathogenic variants in both alleles of the *CFTR* gene causes cystic fibrosis (CF). The initial event of CF lung disease is characterized by low PCL volume, which is thought to be achieved by defective CFTR-mediated Cl⁻ secretion and abnormally elevated Na⁺ absorption via ENaC [33].

Evidence has accumulated to indicate that HCO₃⁻ transport is important in airway mucosal defense. HCO₃⁻ concentration affects physical properties of mucus [4, 39]

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and mucociliary transport in ex vivo pig trachea under acetylcholine (ACh) stimulation was more dependent on HCO_3^- secretion than Cl^- secretion [13]. ASL pH in vivo newborn CF pigs was more acidic compared to wild-type and the impaired bacterial-killing activity of CF ASL was rescued by adding NaHCO_3 [38]. Cellular mechanisms for HCO_3^- transport in airways have been studied using cultured human nasal epithelial cells [36, 37] and Calu-3 cells, a model of serous cells of submucosal glands [20, 25, 28]. However, HCO_3^- transport in distal airways/bronchioles is not well understood. ASL pH was more alkaline in lower airways than in upper airways in human [34]. Thus, a balance of HCO_3^- and H^+ secretion may shift to HCO_3^- secretion in distal airways.

Distal airways contribute to 85–90% of the total epithelial surface area of conducting airways [10, 50]. Moreover, mucus plugging and obstruction of bronchioles are among the earliest events of CF lung disease, suggesting that regulation of epithelial ion transport in distal airways is critical for normal lung physiology [46]. However, the assessment of ion transport in distal airways/bronchioles has been limited because of the small size, complex anatomy and relative inaccessibility of structures [10]. Measurement of transmembrane potential in sheep, porcine, and human bronchioles identified Na^+ and Cl^- conductive pathways [2, 6, 9]. Aquaporin-mediated transepithelial water permeability was identified in guinea pig bronchioles [18]. Measurement of transepithelial potentials by a capillary-Ussing chamber revealed concurrent fluid secretion and absorption and HCO_3^- secretion in human bronchioles [45]. However, characteristics and cellular mechanisms of HCO_3^- transport in distal airways/bronchioles have not been fully investigated.

In the present study, HCO_3^- transport in surface epithelial cells of native bronchioles was studied by measuring intracellular pH (pH_i) in lumenally microperfused freshly dissected mice bronchioles. HCO_3^- transport in bronchioles from a CF mouse model was also studied.

Methods

Ethics approval

The study was approved by the Ethical Committee of Nagoya University on Animal Use for Experiment (approval No. M210457-003) and the Recombinant DNA Experiment Safety Committee of Nagoya University (approval No. 20-93).

Isolation of bronchioles from mice lung

A CF mouse model in which the F508del mutation was introduced in the mouse *Cftr* with the C57BL/6J genetic background (ΔF mouse) [53] was purchased from the Jackson Laboratory (Bar Harbor, ME). ΔF mice and their wild-type littermates were bled in Center for Research of

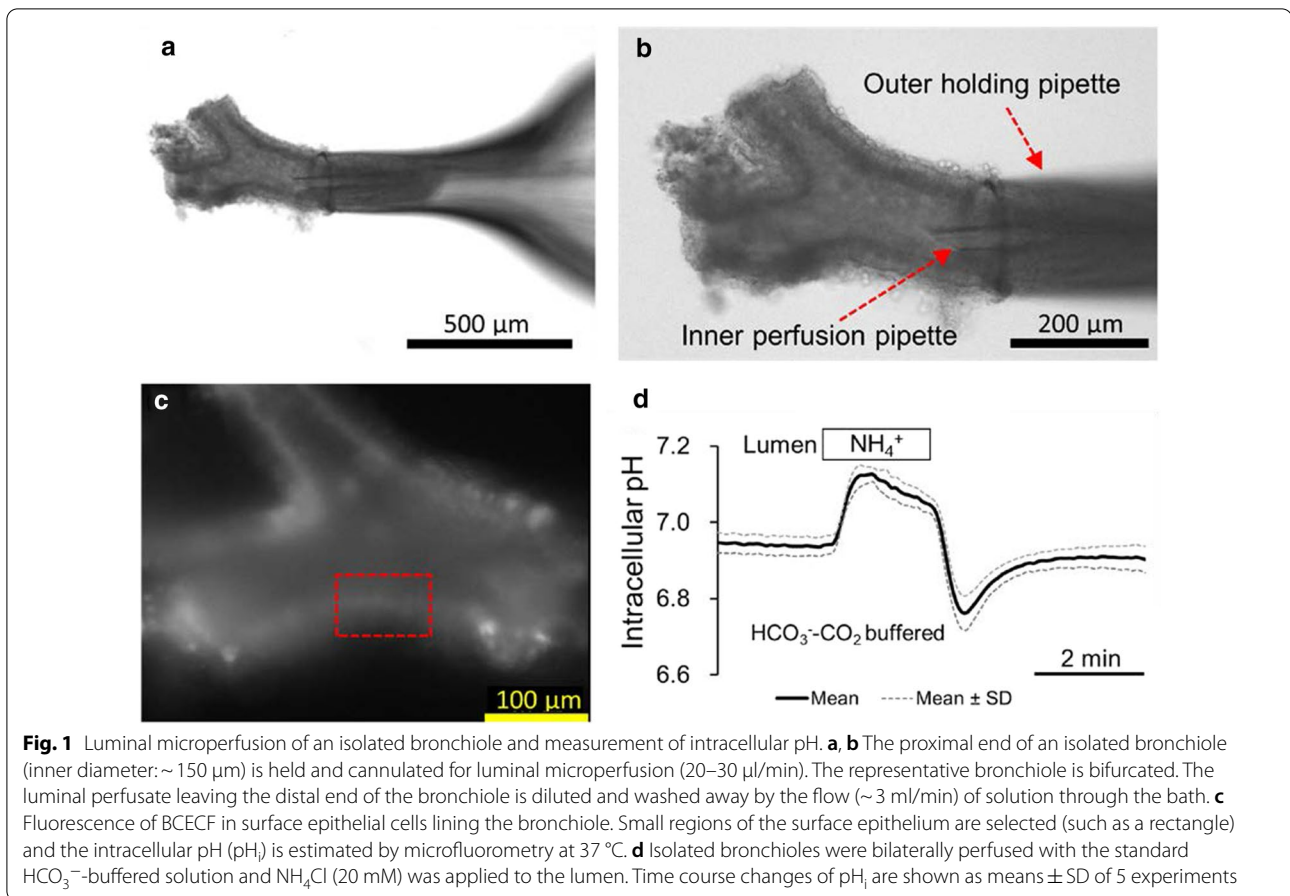
Laboratory Animals and Medical Research Engineering, Nagoya University. Mice (8–10 weeks of age) of either sex were suffocated with CO_2 . The thorax was opened and the ice-cold standard HCO_3^- -buffered solution was gently injected into the trachea to fill the lungs. The lungs were then removed and the segments of conducting bronchioles (the third or fourth branches, 150–180 μm in inner diameter) were micro-dissected using sharpened needles in the ice-cold standard HCO_3^- -buffered solution.

Solutions

The standard HCO_3^- -buffered solution contained (mM): 115 NaCl, 5 KCl, 1 CaCl_2 , 1 MgCl_2 , 10 D-glucose, and 25 mM NaHCO_3 , and was equilibrated with 95% O_2 –5% CO_2 . The 25 mM HCO_3^- –0% CO_2 solution was gassed with 100% O_2 (pH: ~7.8) and thus was nominally free of CO_2 . The standard Hepes-buffered solution contained (mM): 130 NaCl, 5 KCl, 1 CaCl_2 , 1 MgCl_2 , 10 D-glucose, and 10 Na-Hepes, and was equilibrated with 100% O_2 . The Cl^- -free HCO_3^- -buffered solution contained (mM): 115 Na-gluconate, 2.5 K_2HPO_4 , 1 CaSO_4 , 1 MgSO_4 , 10 D-glucose, and 25 mM NaHCO_3 , and was equilibrated with 95% O_2 –5% CO_2 . The Cl^- -free Hepes-buffered solution contained (mM): 130 Na-gluconate, 2.5 K_2HPO_4 , 1 CaSO_4 , 1 MgSO_4 , 10 D-glucose, and 10 Na-Hepes, and was equilibrated with 100% O_2 . The Na^+ -free HCO_3^- -buffered solution contained *N*-methyl-D-glucamine (NMDG) in place of NaCl, choline bicarbonate in place of NaHCO_3 , and 10 μM atropine to prevent the possible activation of muscarinic receptors by choline. The Na^+ -free HEPES-buffered solution contained NMDG-Cl in place of NaCl, and Hepes-acid in place of Na-Hepes. In the HCO_3^- -buffered solution containing 20 mM NH_4Cl , the concentration of NaCl was reduced to maintain osmolarity. All solutions, except for the 25 mM HCO_3^- –0% CO_2 solution, were adjusted to pH 7.4 at 37 °C.

Microperfusion of isolated bronchioles

The lumen of the isolated bronchiole segments was microperfused by applying a method to microperfuse isolated pancreatic ducts [22]. One end of bronchiole was cannulated for luminal microperfusion (Fig. 1a and b). The concentric pipette arrangement consisted of an outer holding pipette, an inner perfusion pipette, and a silica inner capillary for exchange of solutions. The combination of inner silica capillary and waste line enables rapid exchange of luminal solutions. The lumen was perfused at 20–30 $\mu\text{l}/\text{min}$ while the bath was perfused at ~3 ml/min and maintained at 37 °C. The luminal perfusate leaving the other end of the bronchiole was diluted and washed away by the much greater flow of solution



through the bath, which prevented the luminal perfusate from gaining access to the basal surface of the bronchiole.

Measurement of intracellular pH of bronchiole surface epithelium

The intracellular pH (pH_i) in the epithelial cells was estimated by microfluorometry using the pH-sensitive fluoroprobe 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF). After cannulating a bronchiole for luminal microperfusion, the epithelial cells were loaded with BCECF by perfusing the lumen with a solution containing the acetoxymethyl ester BCECF-AM (5 μM) for 10 min. Small regions of the bronchiole surface epithelium (Fig. 1c) were illuminated alternately at 430 and 480 nm and fluorescence was measured at 530 nm (F_{430} and F_{480}). Values of pH_i were calculated from the F_{480}/F_{430} ratio after correction for the endogenous tissue fluorescence measured prior to loading with BCECF. Calibration data were obtained by the high K^+ -nigericin technique [36, 48].

Reverse transcriptase-polymerase chain reaction

Messenger RNA expression of several ion transporters and channels was examined in isolated bronchioles and tracheal mucosa by polymerase chain reaction (PCR) (Table 1). Primers were derived from published sequences with GenBank accession numbers. The PCR protocol was: 96°C , 25 s; 60°C , 30 s; 72°C , 40 s; 35 cycles. Templates for positive controls were complementary DNAs (cDNAs) prepared from lung, kidney, heart, colonic mucosa, brain and stomach mucosa. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers (452 bp) were used for the positive controls.

Materials

BCECF-AM was obtained from Invitrogen (Carlsbad, USA); 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt hydrate (H_2DIDS) was from Molecular Probes (Eugene, USA); forskolin, amiloride, $\text{CFTR}_{\text{inh}}-172$ and other standard laboratory chemicals were from Sigma (St. Louis, USA).

Table 1 Primer pairs used to amplify ion transporters and channels

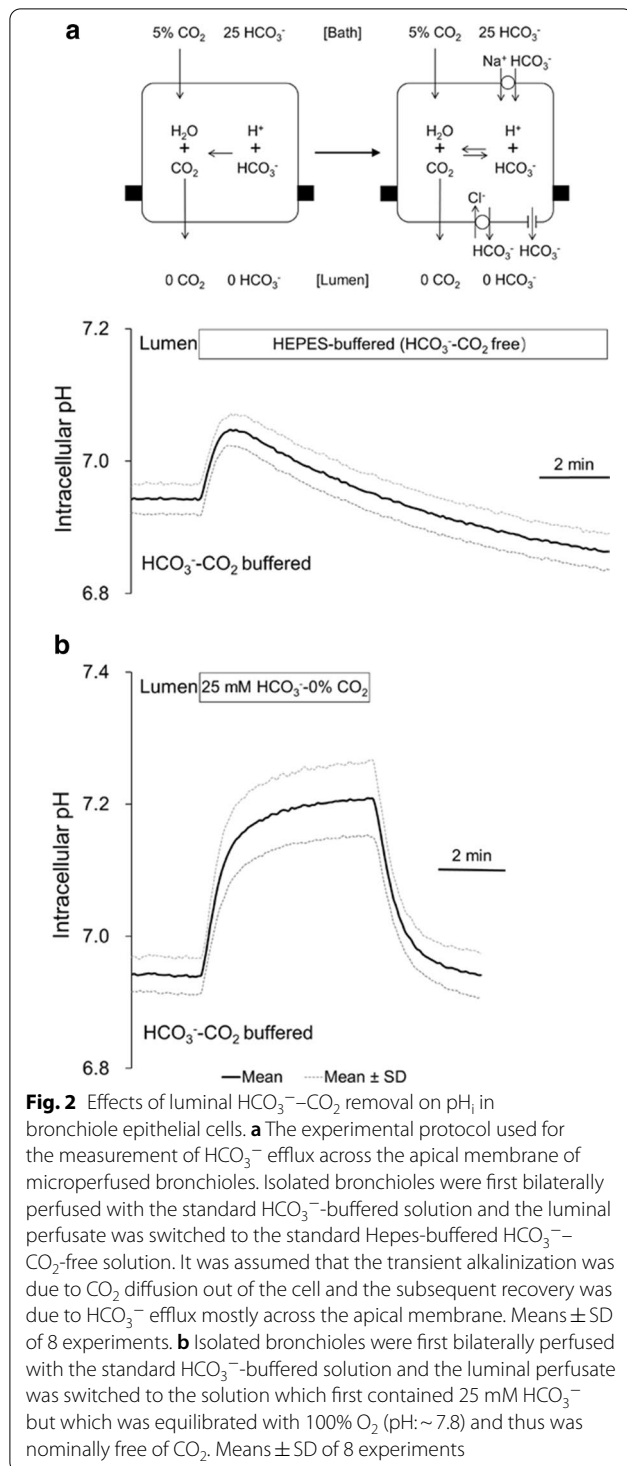
Name	Accession number	Primer	Sequence (5' → 3')	Size (bp)
Cftr	NM_021050.2	Forward	(4070) atggaaagttgcagatgaggtt	399
		Reverse	(4468) ctcatctttccgaggagctaa	
Slc26a3 (Dra)	NM_021353.3	Forward	(556) cctactttttctgggacatc	385
		Reverse	(940) accgactccaggactttgaata	
Slc26a4 (Pendrin)	NM_011867.4	Forward	(974) cggcatcctctccattatctac	465
		Reverse	(1438) gccacaaaacaggagaaaaatc	
Slc26a6 (Pat1)	NM_134420.4	Forward	(2455) tgaagagaagtgcggtgtaga	385
		Reverse	(2839) ttctcaggctcttaatgcaca	
Slc26a9	NM_177243.4	Forward	(1457) cactgaccctactactcttg	425
		Reverse	(1881) tggtttcatgaagagggactt	
α-ENaC	NM_011324.2	Forward	(1496) caggcgaattattctcagtcc	451
		Reverse	(1946) cctgggcttaggtagaagat	
β-ENaC	NM_001272023.1	Forward	(1004) acatcggtcaggagactatgt	282
		Reverse	(1285) ggtcttgaaacaggaatgaag	
γ-ENaC	NM_011326.3	Forward	(709) gaagaaactggtgggatttcag	367
		Reverse	(1075) gaaggggtgtactcatctcg	
Slc4a2 (Ae2)	NM_001253892.1	Forward	(3298) aaccaagattcaggaagcaaa	471
		Reverse	(3768) tctcgtgtactatccacacc	
Slc4a3 (Ae3)	NM_001357149.1	Forward	(1612) atgaccctgatgctaaggagaa	393
		Reverse	(2004) gaatcacaatgtaccatccaa	
Slc4a4 (NBCe1)	NM_001136260.1	Forward	(2206) aaaaccagtcgctattttccaa	412
		Reverse	(2617) gggcaatggagataacagtagc	
Slc4a5 (NBCe2)	NM_001166067.1	Forward	(1814) agcctcttatcatcctcagcag	315
		Reverse	(2128) tgtagtggtgatgaagtcagg	
Slc4a7 (NBCn1)	NM_001033270.2	Forward	(676) accctatgtggcaactctgtct	393
		Reverse	(1068) ttttctctgtcttccacttc	
Slc4a8 (NDCBE)	NM_001347102.1	Forward	(381) gtttgaagagatgtggaggac	434
		Reverse	(814) gatccacctgtcttagatccac	
Slc4a10 (NBCn2)	NM_001242378.1	Forward	(2967) gtgcttctctctcaaaggaat	445
		Reverse	(3412) cacatggcagctttgacattt	
Slc9a1 (Nhe1)	NM_134647.4	Forward	(951) gtgcctgatagcaggagagc	202
		Reverse	(1153) cctgtccttgagcagtgct	
Slc9a2 (Nhe2)	NM_001033289.2	Forward	(2088) gcacagttctcggaagtc	168
		Reverse	(2256) gtccgagtcgctgctatttc	
Slc9a3 (Nhe3)	NM_001081060.2	Forward	(2046) acagaagcggaggaatagca	199
		Reverse	(2245) tatcaattcctgccccagag	
Slc9a4 (Nhe4)	NM_177084.3	Forward	(2084) gaggaacctgcaaaaatcaa	162
		Reverse	(2246) ccacgtcttcaggagaaagc	
Slc9a5 (Nhe5)	NM_001323971.2	Forward	(970) ggacaggtgggaacagtttg	182
		Reverse	(1152) ggcatagagggcagagtgag	

Statistics

Data are presented as the means ± SD unless otherwise indicated. Tests for statistically significant differences were made with Student's *t*-test.

Results

Isolated bronchioles from CF mice ($\Delta F/\Delta F$ mice) were used in experiments shown in Fig. 8. Isolated bronchioles from wild-type mice were used in the other experiments (Figs. 1, 2, 3, 4, 5, 6, 7).



Basal pH_i in bronchiole epithelial cells and the response to luminal NH₄⁺

When isolated bronchioles were bilaterally (bath and lumen) perfused with the standard HCO₃⁻-buffered solution containing 25 mM HCO₃⁻ and 5% CO₂ (pH 7.4),

basal pH_i was 6.94 ± 0.03 (*n* = 64, mean ± SD). When isolated bronchioles were bilaterally perfused with the standard HEPES-buffered (HCO₃⁻-CO₂-free) solution (pH 7.4), basal pH_i was 6.77 ± 0.03 (*n* = 40). Basal pH_i in the presence of HCO₃⁻-CO₂ was significantly (*p* < 0.01) higher compared to that in the absence of HCO₃⁻-CO₂. When NH₄Cl (20 mM) was applied to the lumen in the presence of HCO₃⁻-CO₂, pH_i showed typical time-course changes by NH₄⁺ pulse [40]. Addition of NH₄Cl caused quick alkalization (NH₃ influx) followed by slower decline and removal of NH₄Cl caused quick acidification (NH₃ efflux) followed by slower recovery to the baseline (Fig. 1d). This suggests that H⁺/HCO₃⁻ transport is active in this preparation.

Effects of luminal HCO₃⁻-CO₂ removal on pH_i in microperfused bronchioles

When isolated bronchioles were first bilaterally perfused with the standard HCO₃⁻-buffered solution and the luminal perfusate was switched to the standard HEPES-buffered (HCO₃⁻-CO₂-free) solution (Fig. 2a), pH_i quickly increased from 6.94 ± 0.02 to 7.05 ± 0.02 (*n* = 8) and then gradually decreased towards a value (6.86 ± 0.03) lower than the baseline in 10 min. To distinguish between the separate effects of removal of CO₂ and HCO₃⁻ from the lumen, a solution was prepared which first contained 25 mM HCO₃⁻ but which was equilibrated with 100% O₂ (pH: ~7.8) and thus was nominally free of CO₂. When the luminal perfusate was switched to the 25 mM HCO₃⁻-0% CO₂ solution, pH_i quickly increased to 7.21 ± 0.06 (*n* = 8) and the alkalization was sustained (Fig. 2b). Thus, the transient alkalization and the subsequent recovery (acidification) by removal of luminal HCO₃⁻-CO₂ was most likely due to CO₂ diffusion out of the cell followed by HCO₃⁻ efflux (Fig. 2a). Most of the HCO₃⁻ efflux was probably via the apical membrane due to the steep HCO₃⁻ gradient between the cell and the lumen (HCO₃⁻ concentration was close to zero). H⁺ influx was not likely involved in the subsequent acidification because Na⁺-H⁺ exchanger would work for H⁺ extrusion in this condition.

Effects of luminal application of forskolin, CFTR_{inh}-172, H₂DIDS, and amiloride on apical HCO₃⁻ efflux in bronchiole epithelial cells

The mechanisms for HCO₃⁻ efflux across the apical membrane were examined using the protocol of Fig. 2a. After HCO₃⁻-CO₂ was removed from the luminal perfusate, forskolin (5 μM), the activator of adenylate cyclase, was applied to the lumen as indicated (Fig. 3a). Stimulation with forskolin transiently accelerated the pH_i decline by 97% (*p* < 0.01) (*n* = 8, Fig. 3a and f) of control (without forskolin stimulation: blue line in Fig. 3a). The late phase of pH_i decline (at midpoint pH_i of 6.95) was also accelerated by 47% (*p* < 0.05) (Fig. 3a and g) of control. The

data suggest that elevation of intracellular cAMP activates HCO_3^- secretion in a biphasic manner: initial large response followed by sustained activation, in mice bronchiole epithelial cells.

CFTR_{inh}-172 (5 μM) and H_2DIDS (200 μM) in the lumen inhibited the forskolin-stimulated transient pH_i decline by 69% ($n=8$, $p<0.01$) (Fig. 3b and f) and 65% ($n=8$, $p<0.01$) (Fig. 3c and f), respectively. Luminal CFTR_{inh}-172 and H_2DIDS also slowed down the late phase of pH_i decline (at midpoint pH_i of 6.95) by 54% ($p<0.01$) (Fig. 3b and g) and 33% ($p<0.05$) (Fig. 3c and g), respectively. The data suggest that both CFTR and H_2DIDS -sensitive HCO_3^- transporter and/or HCO_3^- -permeable anion channel partly mediate cAMP-stimulated HCO_3^- secretion. The forskolin-stimulated transient pH_i decline in the presence of both CFTR_{inh}-172 and H_2DIDS (Fig. 3d and f) was significantly ($p<0.05$) smaller compared to that in the presence of CFTR_{inh}-172 or H_2DIDS alone (Fig. 3b, c, and f). The late-phase of pH_i decline in the presence of both CFTR_{inh}-172 and H_2DIDS (Fig. 3d and g) was significantly ($p<0.05$) slower compared to that in the presence of H_2DIDS alone (Fig. 3c and g).

Luminal application of CFTR_{inh}-172 and H_2DIDS by themselves induced a transient small dip of pH_i (Fig. 3b and c). The transient pH_i dip largely disappeared when CFTR_{inh}-172 and H_2DIDS were simultaneously applied to the lumen (Fig. 3d). This suggests that CFTR and H_2DIDS -sensitive HCO_3^- transporter/channel compensate each other for apical HCO_3^- efflux. We speculate on the mechanisms as follows. CFTR inhibition would hyperpolarize the cell, which would induce transient HCO_3^- efflux via a HCO_3^- -permeable anion channel or an electrogenic HCO_3^- transporter (such as 1Cl^- - 2HCO_3^- exchanger). If H_2DIDS -sensitive HCO_3^- transport is electrogenic, luminal H_2DIDS would hyperpolarize the cell, which would induce transient HCO_3^- efflux via CFTR.

To examine the role of ENaC in HCO_3^- secretion, a relatively low concentration of amiloride (1 μM) [32] was applied to the lumen. Amiloride (1 μM) in the lumen inhibited the forskolin-stimulated transient pH_i decline by 63% ($n=8$, $p<0.01$) (Fig. 3e and f), but did not significantly affect the late phase of pH_i decline (Fig. 3e and g).

The data suggest that ENaC is involved in the regulation of HCO_3^- secretion. The transient dip of pH_i by luminal amiloride (Fig. 3e) likely indicates apical HCO_3^- efflux which is accelerated by membrane hyperpolarization.

When isolated bronchioles were bilaterally perfused with the standard HCO_3^- -buffered solution, application of CFTR_{inh}-172 (5 μM) to the lumen caused a transient increase of pH_i by 0.022 ± 0.003 unit ($n=8$, data not shown). The pH_i increase was not observed in the absence of HCO_3^- - CO_2 and enhanced by 77% ($p<0.05$) by stimulation with forskolin (5 μM) (data not shown). The data suggest that CFTR is involved in HCO_3^- secretion in a physiological condition.

Effects of luminal Cl^- removal on pH_i in bronchiole epithelial cells

To examine the activity of Cl^- - HCO_3^- exchange in the apical membrane, effects of luminal Cl^- removal on pH_i were examined. When isolated bronchioles were bilaterally perfused with the standard HEPES-buffered HCO_3^- - CO_2 -free solution, removal of luminal Cl^- (by replacement with gluconate) caused a slight decline of pH_i (Fig. 4a and e). In contrast, when isolated bronchioles were bilaterally perfused with the standard HCO_3^- -buffered solution, luminal Cl^- removal caused a reversible increase of pH_i by 0.14 ± 0.03 unit ($n=8$) over ~ 4 min period (Fig. 4b and e), most likely due to influx of luminal HCO_3^- in exchange for intracellular Cl^- . When the activity of apical Cl^- - HCO_3^- exchange is shown as the initial rate of pH_i increase upon luminal Cl^- removal, the activity is not affected by forskolin (5 μM) in the lumen (Fig. 4c and e) and largely ($p<0.01$) inhibited by H_2DIDS (200 μM) in the lumen (Fig. 4d and e). The data suggest that H_2DIDS -sensitive Cl^- - HCO_3^- exchanger is localized in the apical membrane.

Na^+ -dependent H^+ extrusion across the apical membrane of bronchiole epithelial cells

To examine whether Na^+ - H^+ exchanger (NHE) and Na^+ - HCO_3^- cotransporter (NBC) are localized in the apical membrane, luminal Na^+ -dependent H^+ extrusion was examined. In the absence of HCO_3^- - CO_2 , removal of luminal Na^+ (by replacement with NMDG) caused a continuous decline of pH_i and restoration of Na^+ to the

(See figure on next page.)

Fig. 3 Effects of luminal application of forskolin, CFTR_{inh}-172, H_2DIDS , and amiloride on apical HCO_3^- efflux. **a-e** Isolated bronchioles were first bilaterally perfused with the standard HCO_3^- -buffered solution and the luminal perfusate was switched to the standard HEPES-buffered HCO_3^- - CO_2 -free solution. After HCO_3^- - CO_2 was removed from the luminal perfusate, forskolin (5 μM) was applied to the lumen. Time course changes of pH_i in the absence (**a**) or presence of CFTR_{inh}-172 (5 μM) in the lumen (**b**), H_2DIDS (200 μM) in the lumen (**c**), both CFTR_{inh}-172 (5 μM) and H_2DIDS (200 μM) in the lumen (**d**), or amiloride (1 μM) in the lumen (**e**) are shown as means \pm SD of 8 experiments, respectively. The blue line in **a** indicates mean change of pH_i without forskolin stimulation as a reference. **f** Early-phase pH_i decline (ΔpH for 1 min) just after forskolin stimulation. $^{\#}p<0.01$ compared with control (without forskolin stimulation). $^*p<0.01$ compared with forskolin alone. $^{\$}p<0.05$. **g** Late-phase pH_i decline ($\Delta\text{pH}/\text{min}$ at midpoint pH_i of 6.95) under forskolin stimulation (red dashed lines in **a-e**). $^{\#}p<0.05$ compared with control (without forskolin stimulation). $^*p<0.01$, $^{**}p<0.05$ compared with forskolin alone. $^{\$}p<0.01$

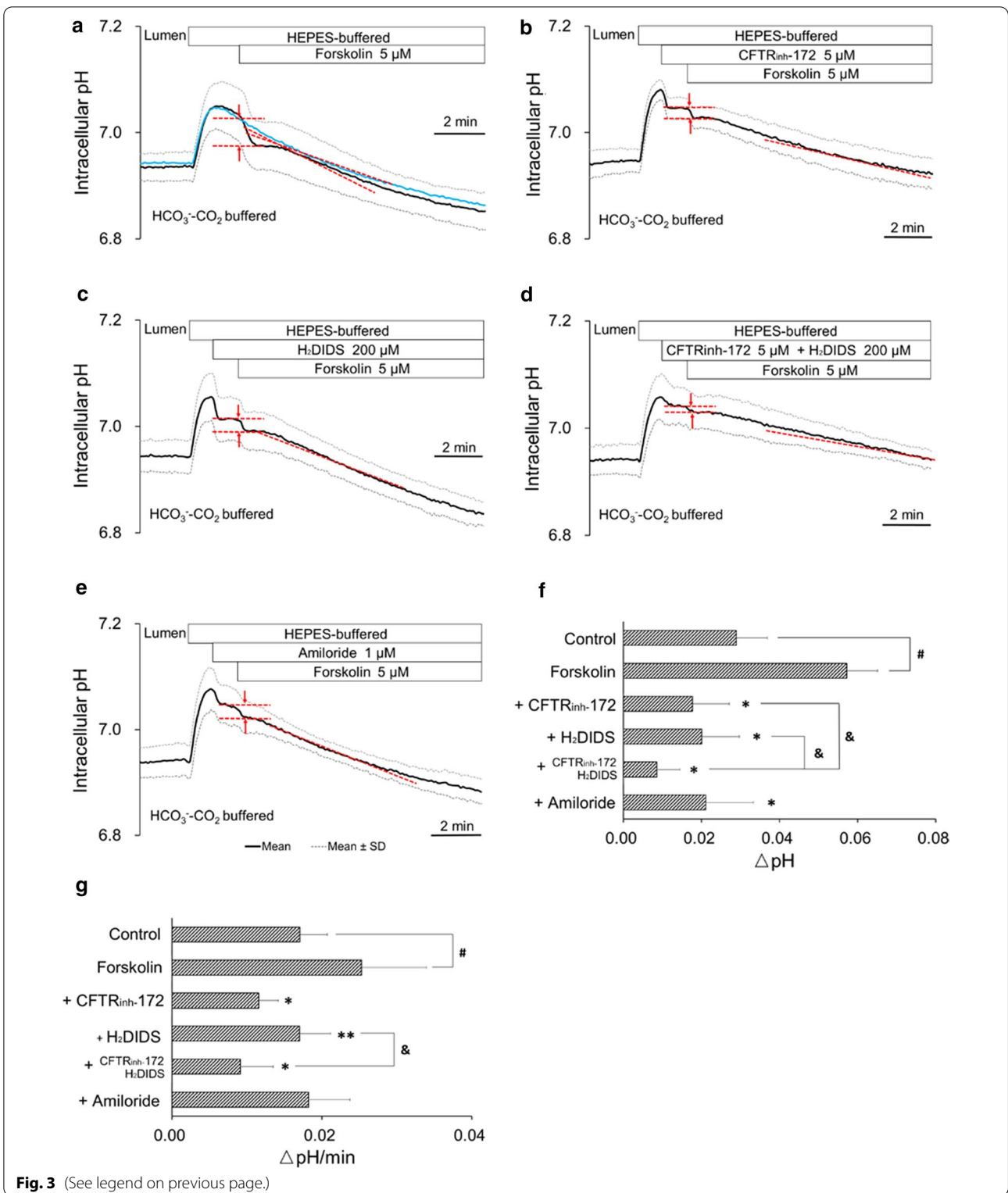
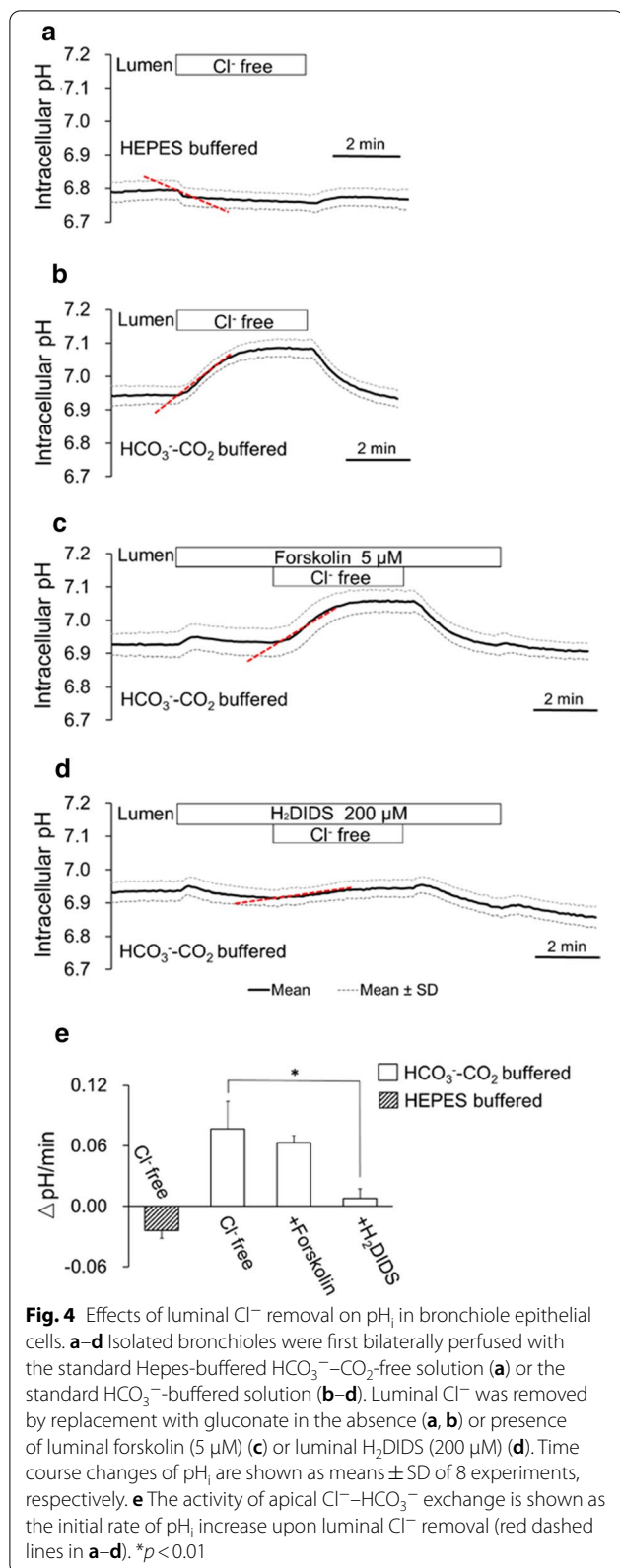


Fig. 3 (See legend on previous page.)

lumen caused a recovery (Fig. 5a). When the activity of luminal Na⁺-dependent H⁺ extrusion is shown as the initial pH_i increase (ΔpH for 1 min) upon restoration of

luminal Na⁺, the activity was completely ($p < 0.01$) inhibited by amiloride (100 μM) in the lumen (Fig. 5b and



f). The data suggest that NHE is localized in the apical membrane.

The activity of luminal Na^+ -dependent H^+ extrusion in the presence of HCO_3^- - CO_2 (Fig. 5c and f) was significantly ($p < 0.05$) greater compared to that in the absence of HCO_3^- - CO_2 (Fig. 5a and f), partially inhibited by amiloride ($100 \mu\text{M}$) in the lumen (Fig. 5d and f), and completely inhibited by a combination of amiloride ($100 \mu\text{M}$) and H_2DIDS ($200 \mu\text{M}$) in the lumen (Fig. 5e and f). The data suggest that NBC is localized in the apical membrane.

Effects of luminal amiloride on pH_i in bronchiole epithelial cells

While lower concentrations of amiloride inhibit ENaC with IC_{50} of $1 \mu\text{M}$ [32], higher concentrations of amiloride (0.5 – 1 mM) also inhibit apical NHE in human bronchial epithelium [49]. Figure 6 shows the effects of various concentrations of luminal amiloride (1 , 10 , and $100 \mu\text{M}$) on basal pH_i (Fig. 6). To examine the relative contribution of ENaC and apical NHE in H^+ / HCO_3^- transport in a physiological condition, we examined concentration-dependent effects of luminal amiloride rather than a more specific inhibitor of NHE such as ethylisopropyl amiloride (EIPA).

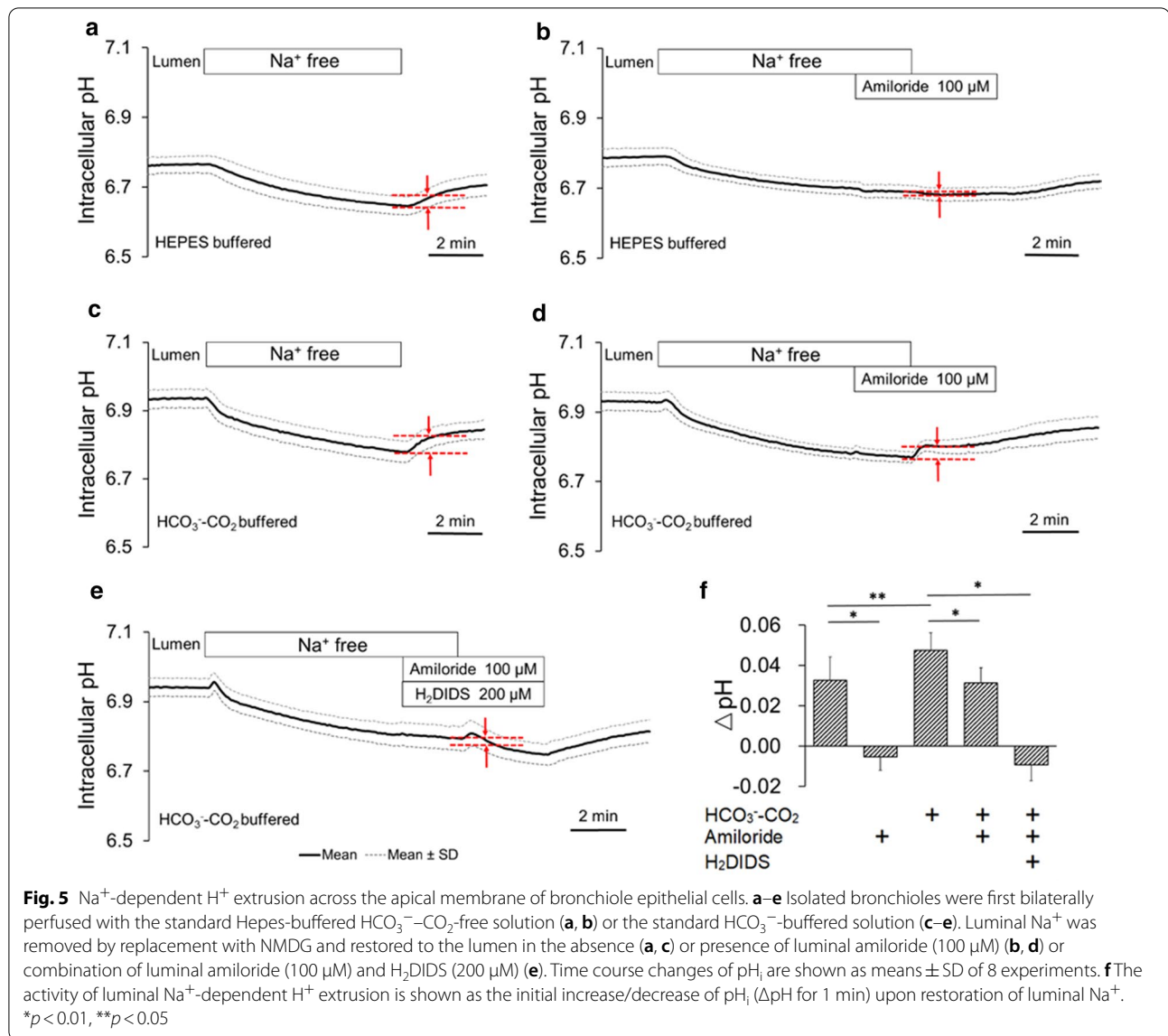
In the absence of HCO_3^- - CO_2 , luminal application of amiloride caused concentration-dependent decline of pH_i (Fig. 6a, c, e, g).

In the presence of HCO_3^- - CO_2 , luminal application of $1 \mu\text{M}$ amiloride caused an increase in pH_i by 0.03 ± 0.01 ($n = 8$, Fig. 6b and g). Luminal $100 \mu\text{M}$ amiloride caused a transient increase followed by a continuous decline in pH_i by 0.06 ± 0.01 ($n = 8$) in 5 min (Fig. 6f and g). Luminal $10 \mu\text{M}$ amiloride (Fig. 5d and g) caused an intermediate pattern of pH_i changes of those by $1 \mu\text{M}$ and $100 \mu\text{M}$ amiloride.

Thus, the effects of lower concentration of apical amiloride on basal pH_i were dependent on the presence of HCO_3^- - CO_2 , which suggests that ENaC is involved in the regulation of HCO_3^- transport. The data also indicate that apical NHE is involved in the regulation of basal pH_i .

Messenger RNA expression of ion transporters and channels in bronchiole epithelial cells

Expression of Cftr, ENaC subunits, and Slc4, Slc9, and Slc26 families of transporters in isolated bronchioles and tracheal mucosa was examined by RT-PCR (Fig. 7). Amplified fragments from Cftr, α , β , γ subunits of ENaC, Slc4a2 (Ae2), Slc4a3 (Ae3), Slc4a4 (NBCe1), Slc4a5



(NBCe2), Slc4a7 (NBCn1), Slc4a8 (NDCBE), Slc4a10 (NBCn2), Slc9a1 (Nhe1), Slc9a2 (Nhe2), Slc9a4 (Nhe4), Slc9a5 (Nhe5), Slc26a4 (Pendrin), Slc26a6 (Pat1), and Slc26a9 were detected in isolated bronchioles and tracheal mucosa. Fragments from Slc9a3 (Nhe3) and Slc26a3 (Dra) were not detected in isolated bronchioles and tracheal mucosa.

Basal pHi and apical HCO₃⁻ efflux in bronchiole epithelial cells from CF mice

Basal pHi in the presence of HCO₃⁻-CO₂ in isolated bronchioles from ΔF/ΔF mice (6.97 ± 0.02, *n* = 6) was slightly but significantly (*p* < 0.05) higher compared to bronchioles from wild-type mice (6.94 ± 0.02, *n* = 8, blue line) (Fig. 8a and d). Initial increase of pHi (ΔpHi)

by removal of luminal HCO₃⁻-CO₂ was also significantly (*p* < 0.01) greater in ΔF/ΔF bronchioles compared to wild-type bronchioles (Fig. 8a and e). The data suggest that basal HCO₃⁻ secretion is impaired in CF bronchioles. The rate of pHi decline at midpoint pHi of 6.95 was significantly (*p* < 0.05) slower in CF bronchioles compared to wild-type bronchioles (Fig. 8a and g).

Stimulation with luminal forskolin (5 μM) transiently accelerated pHi decline (apical HCO₃⁻ efflux) in ΔF/ΔF bronchioles (Fig. 8b and f) and the acceleration was comparable to wild-type bronchioles (blue line). Forskolin failed to accelerate the late phase of pHi decline (at midpoint pHi of 6.95) in ΔF/ΔF bronchioles (Fig. 8b and g). The data suggest that cAMP stimulation transiently activated HCO₃⁻ secretion in CF bronchioles probably

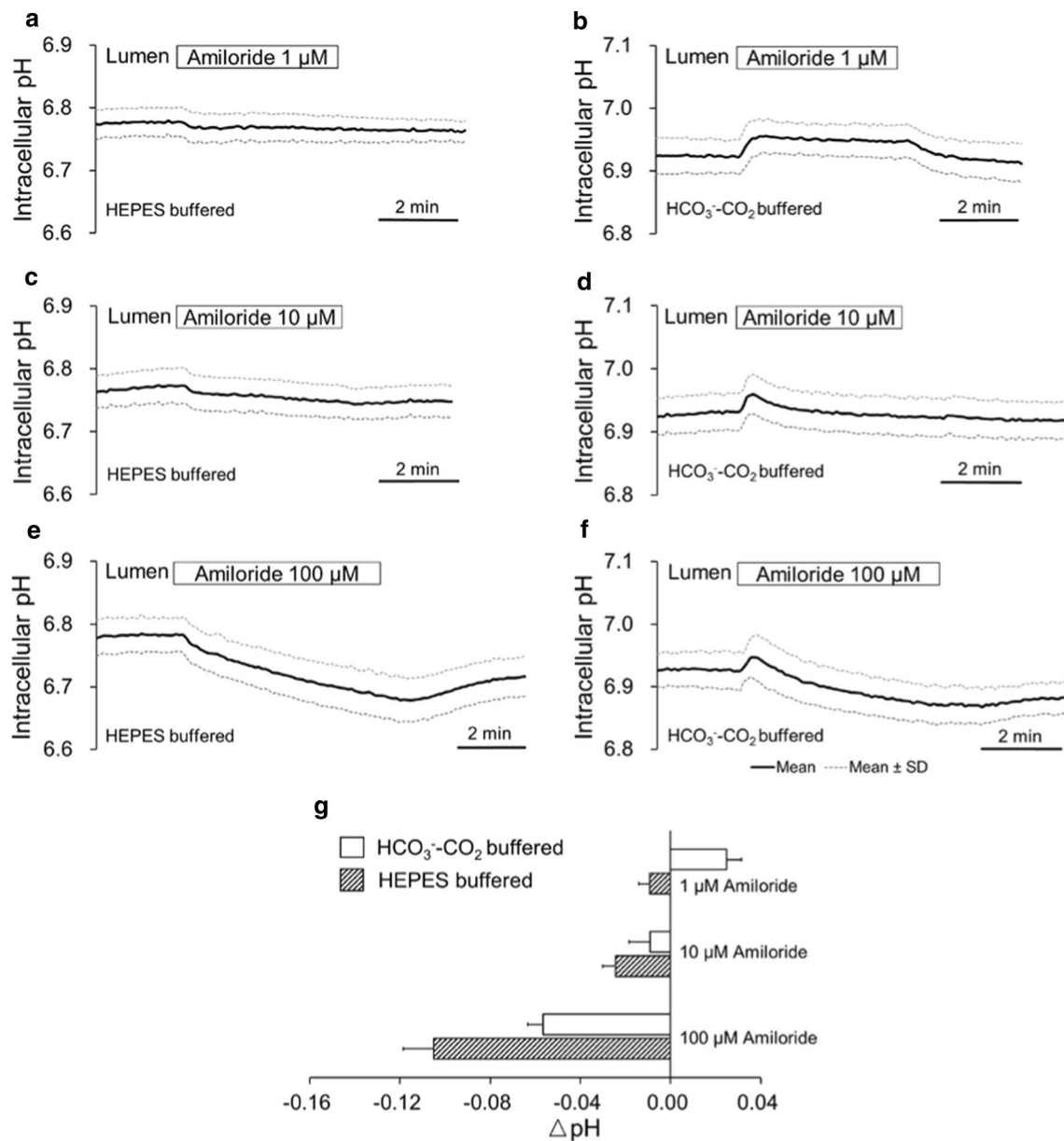


Fig. 6 Effects of luminal amiloride on pH_i in bronchiole epithelial cells. **a-f** Isolated bronchioles were first bilaterally perfused with the standard HEPES-buffered HCO_3^- - CO_2 -free solution (**a, c, e**) or the standard HCO_3^- -buffered solution (**b, d, f**). Amiloride was applied to the lumen as indicated at concentrations of 1 μM (**a, b**), 10 μM (**c, d**) or 100 μM (**e, f**). Time course changes of pH_i are shown as means \pm SD of 8 experiments. **g** Increase or decrease of pH_i (ΔpH for 5 min) by luminal application of amiloride at various concentrations

via activation of a HCO_3^- -permeable anion channel or a HCO_3^- transporter, but failed to induce sustained increase of HCO_3^- secretion.

Luminal application of ACh induced a transient increase of transepithelial ion current in mice and pig tracheal epithelium [16, 21]. Application of ACh (10 μM) to the lumen transiently accelerated pH_i decline (apical HCO_3^- efflux) in wild-type bronchioles (blue line in

Fig. 8c) and the acceleration was greater than forskolin (Fig. 8f, $p < 0.01$). The ACh-induced transient acceleration of pH_i decline was reduced by 45% ($p < 0.01$) in $\Delta F/\Delta F$ bronchioles (Fig. 8c and f). Luminal ACh did not affect the late phase of pH_i decline in both wild-type and $\Delta F/\Delta F$ bronchioles (Fig. 8c and g). The data indicate that ACh stimulation transiently activated HCO_3^- secretion in wild-type bronchioles and that the ACh-induced

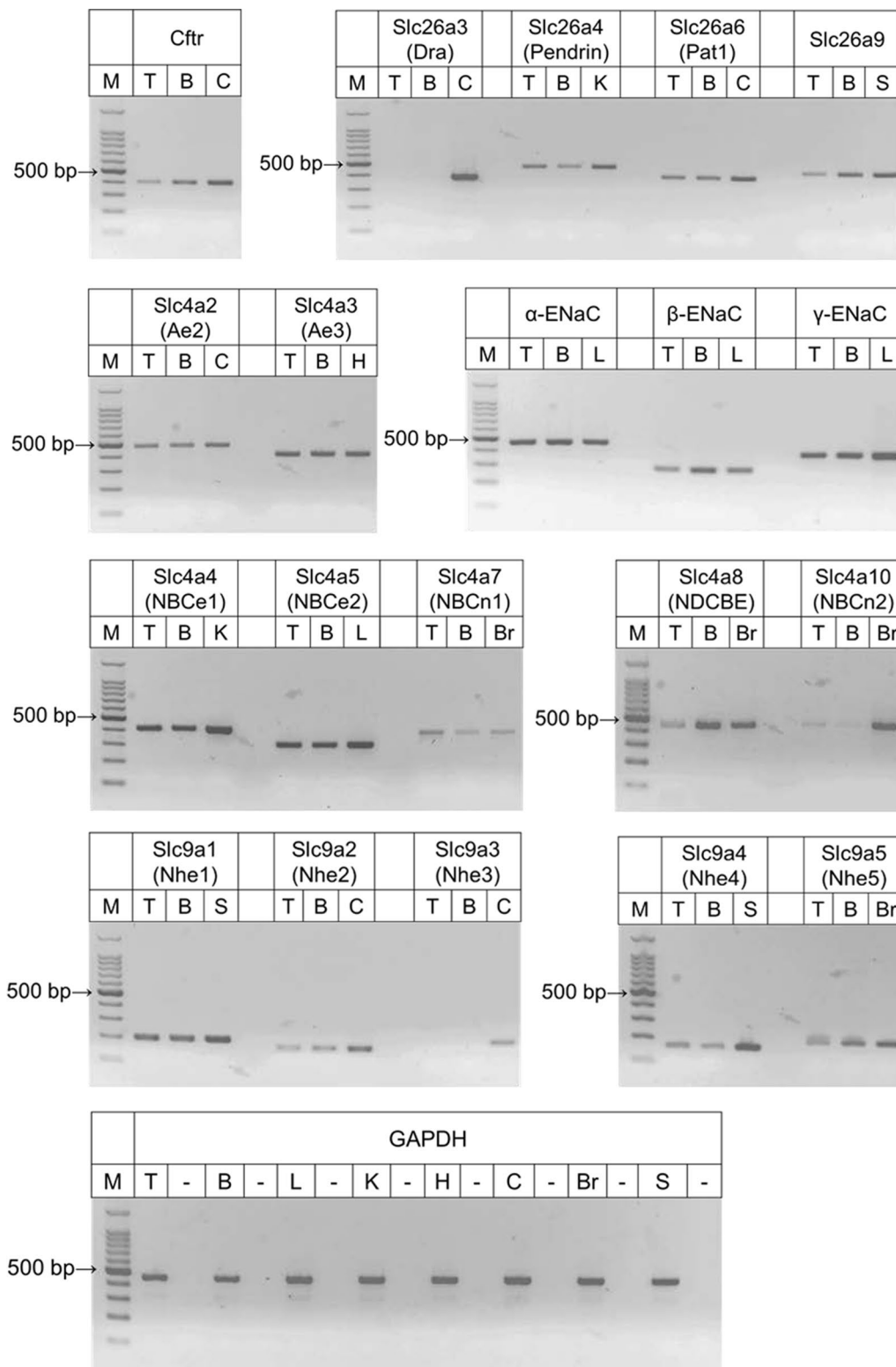
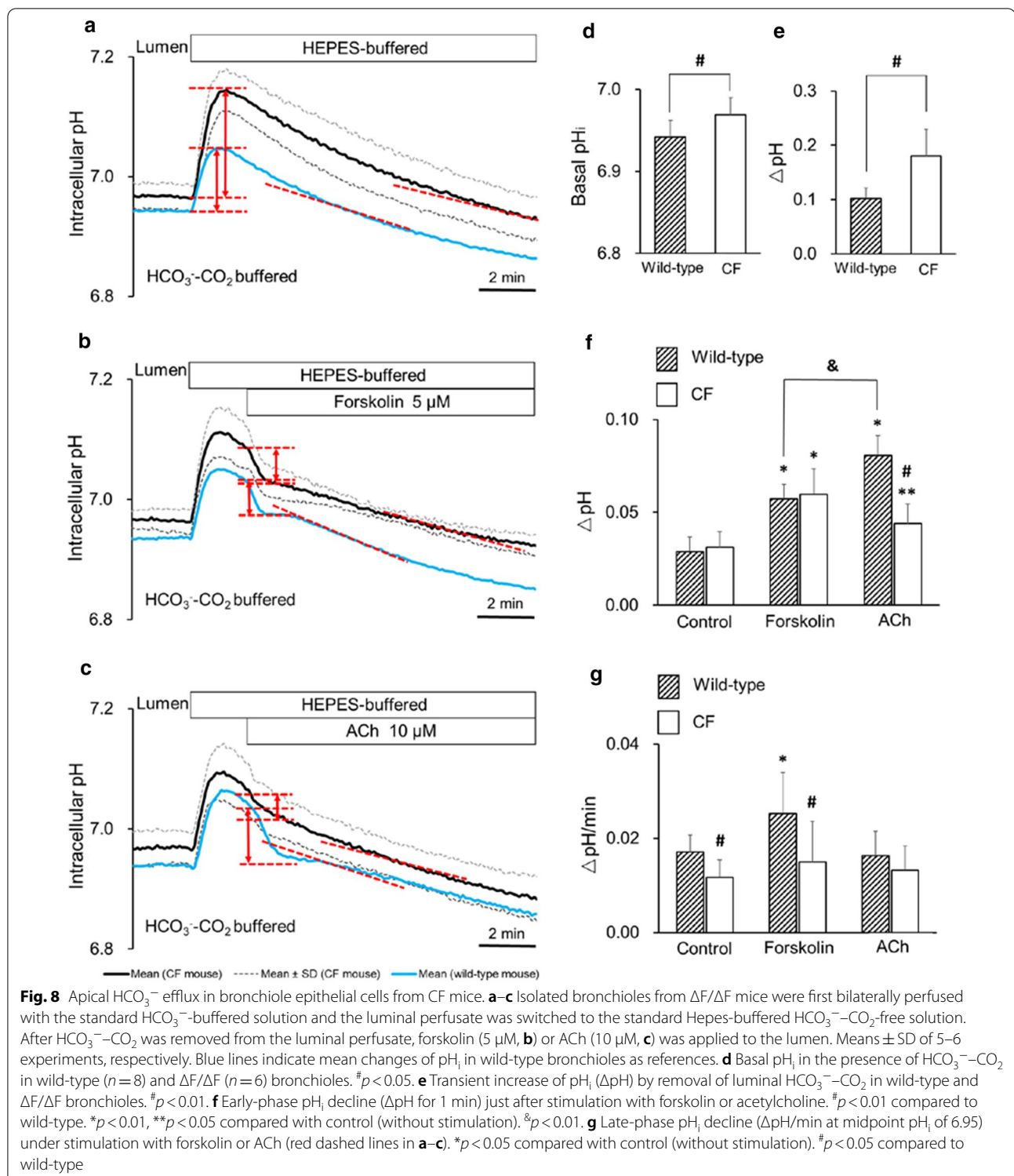


Fig. 7 Messenger RNA expression of ion transporters and channels in bronchiole epithelial cells. Messenger RNA was extracted from tracheal mucosa (T), isolated bronchioles (B), colon (C), kidney (K), stomach (S), heart (H), lung (L), and brain (Br) and reverse transcribed. PCR was performed using each cDNA as template and with gene-specific primers (Table 1). GAPDH was used as a reference. - : PCR was performed in the absence of RT enzyme. M: 100-bp DNA ladder



enhancement of HCO_3^- secretion was substantially reduced in CF bronchioles. This suggests that CFTR partly mediates ACh-induced HCO_3^- secretion in addition to CaCC in mice bronchiole epithelial cells.

Discussion

In the present study, HCO_3^- transport in surface epithelial cells of native bronchioles was studied by measuring pH_i in lumenally microperfused freshly dissected mice

bronchioles. HCO_3^- transport in bronchioles from CF mice was also studied. Although some connective tissue was attached to the outside of bronchioles (Fig. 1), surface epithelial cells were successfully loaded with BCECF from the lumen and pH_i was measured as long as 30 min. The present study focused on $\text{HCO}_3^-/\text{H}^+$ transport across the apical membrane, since rapid exchange of luminal solutions was achieved in our preparation [22].

Intracellular pH in surface epithelial cells of mice bronchioles

Human and rodent bronchioles are lined with columnar to cuboidal epithelium which is composed of ciliated and nonciliated (Clara) cells [35]. In the present study, basal pH_i of surface epithelial cells in isolated mice bronchioles was ~ 6.94 in bilateral (bath and lumen) presence of 25 mM HCO_3^- and 5% CO_2 . The value is similar to the basal pH_i of cultured human nasal epithelial cells (~ 6.94) in the same experimental condition [37]. The relatively low basal pH_i likely resulted from higher pCO_2 in the lumen compared to the physiological *in vivo* situation where the luminal side of the epithelial layer is exposed to air.

Ion transporters and channels localized in the apical membrane of bronchiole epithelial cells

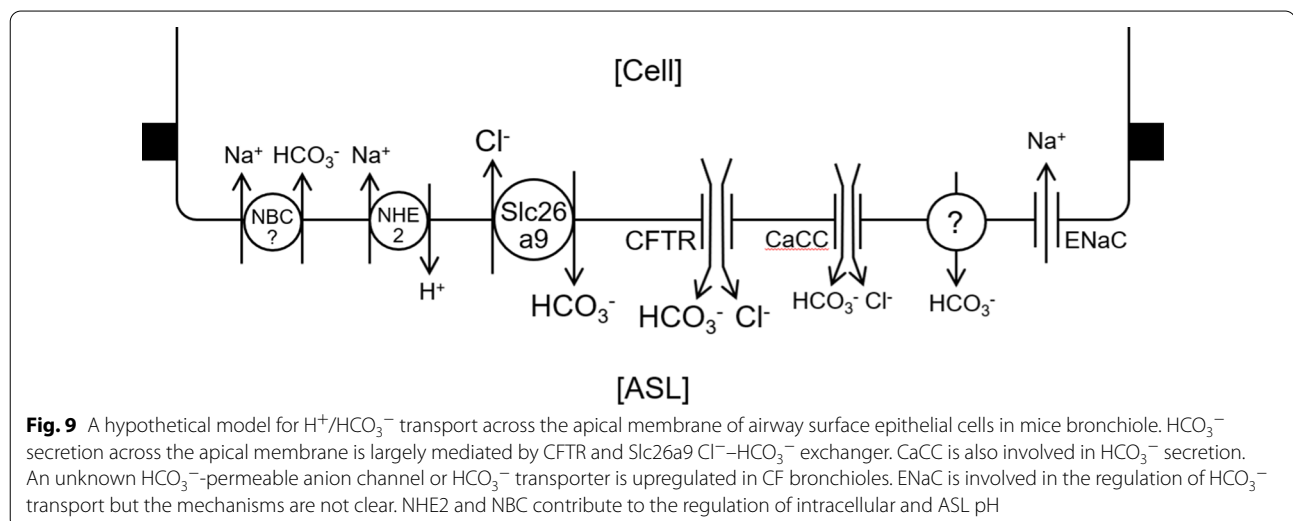
In the present study, functional studies suggested that CFTR and H_2DIDS -sensitive HCO_3^- transporter and/or HCO_3^- -permeable anion channel mediate cAMP-stimulated HCO_3^- secretion and ENaC, H_2DIDS -sensitive Cl^- - HCO_3^- exchangers, NHE, and NBC are involved in $\text{HCO}_3^-/\text{H}^+$ transport across the apical membrane of surface epithelial cells of mice bronchioles (Fig. 9). This is

supported by mRNA expression of Cftr, ENaC subunits, and Slc4, Slc9, and Slc26 families of transporters (Fig. 7).

The activity of H_2DIDS -sensitive Cl^- - HCO_3^- exchanger was detected in the apical membrane (Fig. 4) and probably mediated part of cAMP-stimulated HCO_3^- secretion (Fig. 3c). The candidate molecules are Slc4a2 (Ae2), Slc4a3 (Ae3), Slc26a4 (Pendrin), Slc26a6, and Slc26a9 of which mRNA expression was detected in isolated bronchioles (Fig. 7). In human bronchial epithelia, SLC26A4 (Pendrin) colocalized with CFTR in the apical membrane of ciliated surface cells and mediated most of HCO_3^- secretion when pretreated with IL-4 [24]. SLC26A9 is prominently expressed in brain and on apical membrane of airway epithelial cells and gastric mucosa [3, 31]. A missense variant of SLC26A9 found in a patient of diffuse bronchiectasis failed to activate CFTR in a heterologous expression system [7]. While Slc26a4 (Pendrin) is H_2DIDS -insensitive, Slc26a9 is sensitive to H_2DIDS . Thus, Slc26a9 is likely the major apical Cl^- - HCO_3^- exchanger in mice bronchioles.

Apical H^+ secretion via H^+/K^+ ATPase and vacuolar H^+ -ATPase was reported in airways [52]. Our present study identified the activities of NHE and NBC in the apical membrane of mice bronchioles (Figs. 5, 6) which may contribute to the regulation of intracellular and ASL pH.

NHE activity was detected in the apical membrane and mediated H^+ secretion in tracheal epithelial cells from sheep [1]. The candidate molecules of apical NHE in mice bronchioles are Slc9a1 (Nhe1), Slc9a2 (Nhe2), Slc9a4 (Nhe4), and Slc9a5 (Nhe5) of which mRNA expression was detected (Fig. 7). NHE2 is known to be expressed in the lung, predominantly localized to the apical membrane of epithelial cells [19], and relatively sensitive to



amiloride [51]. Thus, NHE2 is likely the major apical Na^+/H^+ exchanger in mice bronchioles.

While SLC4A4 (NBCe1) and SLC4A5 (NBCe2) were identified in the basolateral membrane of Calu-3 cells [27], NBC isoforms have not been identified in the apical membrane of airway epithelium. Messenger RNA of all NBC isoforms: Slc4a4 (NBCe1), Slc4a5 (NBCe2), Slc4a7 (NBCn1), Slc4a8 (NDCBE), and Slc4a10 (NBCn2) was detected in isolated mice bronchioles (Fig. 7). Our present study cannot identify the membrane localization of the NBC isoforms.

Mechanisms and regulation of HCO_3^- secretion in bronchiole epithelial cells

Surface airway epithelial cells as well as serous cells of the submucosal glands secrete Cl^- and HCO_3^- in response to agents increasing intracellular cAMP (VIP, noradrenaline, etc.) and/or Ca^{2+} (ACh, histamine, etc.) [41]. It is generally accepted that cAMP-mediated secretion involves CFTR and Ca^{2+} -mediated secretion involves CaCC encoded by TMEM16A/ANO1 [15]. Cyclic AMP- and Ca^{2+} -mediated agonists independently and additively increased HCO_3^- secretion in human bronchioles [45]. However, it has been noted that muscarinic responses of fluid secretion are reduced in submucosal glands from patients with cystic fibrosis [42] and a recent study demonstrated a crosstalk of CFTR and TMEM16A in CFBE cells [29].

We assume that continuous decline of pH_i following alkaline load (Figs. 2, 3, 8) demonstrates time course of HCO_3^- secretion into the lumen which is perfused with the HCO_3^- -free solution. Forskolin biphasically stimulated HCO_3^- secretion: transiently accelerated HCO_3^- secretion just after application and increased the rate of steady-state HCO_3^- secretion (Fig. 3a). ACh transiently accelerated HCO_3^- secretion, but did not increase the steady-state HCO_3^- secretion (Fig. 8c). The data indicate that both cAMP-mediated and Ca^{2+} -mediated pathways are involved in HCO_3^- secretion in mice bronchiole epithelial cells.

Luminal CFTR_{inh}-172 and H₂DIDS substantially inhibited both transient and steady-state phases of forskolin-stimulated HCO_3^- secretion (Fig. 3). CFTR was localized not only in serous cells of submucosal glands [17, 23], but also in the apical membrane of surface epithelium of proximal to distal airways in human [26]. GlyH101-sensitive HCO_3^- transport was detected in human bronchioles [45]. Our present data suggest that both CFTR and H₂DIDS-sensitive HCO_3^- transporter (likely SLC26A9 $\text{Cl}^-/\text{HCO}_3^-$ exchanger shown in Fig. 4) and/or HCO_3^- -permeable anion channel are involved in apical HCO_3^- secretion (Fig. 9).

A relatively low concentration of amiloride in the lumen inhibited transient phase of forskolin-stimulated HCO_3^- secretion (Fig. 3e). The data suggest that ENaC is involved in the regulation of HCO_3^- transport, which is consistent with amiloride (1 μM)-induced pH_i increase in the presence of $\text{HCO}_3^-/\text{CO}_2$ (Fig. 6). The cellular mechanisms for the involvement of ENaC in HCO_3^- secretion are not clear.

HCO_3^- secretion in CF bronchiole epithelial cells

ASL pH was more acidic in trachea of CF pigs under basal and methacholine-stimulated conditions [38]. Lower pH of ASL was also observed in nasal epithelium of CF patients [34, 52], while the other study did not find differences in ASL pH of bronchus between CF patients and control [43]. Combination of forskolin and 3-isobutyl-1-methylxanthine alkalinized ASL of cultured bronchial epithelium of normal subjects but acidified CF ASL [12].

In the present study, HCO_3^- secretion was studied in bronchioles isolated from a CF mouse model in which the F508del mutation (most frequent pathogenic variant of CFTR) was introduced (ΔF mouse) (Fig. 8). Although CF mice do not display severe lung disease as observed in humans, an impaired ability to stretch/expand the peripheral lung compartment and increased distances between gas exchange surfaces which are early pulmonary phenotype of human CF were found in young (8–16 weeks old) $\Delta\text{F}/\Delta\text{F}$ mice [14]. Our present study demonstrated higher level of basal pH_i in the presence of $\text{HCO}_3^-/\text{CO}_2$ and larger increase of pH_i by removal of luminal $\text{HCO}_3^-/\text{CO}_2$ in CF bronchioles (Fig. 8), which indicate that basal HCO_3^- secretion is reduced in CF distal airways.

The effects of forskolin and ACh on HCO_3^- secretion in CF bronchioles (Fig. 8) were unexpected. While forskolin stimulation transiently accelerated HCO_3^- secretion in CF bronchioles (comparable to wild-type bronchioles, Fig. 8b), ACh-induced acceleration of HCO_3^- secretion was substantially reduced in CF bronchioles (Fig. 8c). The data are consistent with the presence of a crosstalk of cAMP- and Ca^{2+} -mediated pathways of HCO_3^- secretion. The data also suggest that a cAMP-activated HCO_3^- -permeable anion channel or HCO_3^- transporter was upregulated in CF bronchioles.

The present study has some limitations. (1) The intracellular buffering capacity is not measured and the rate of $\text{H}^+/\text{HCO}_3^-$ flux is not inferred from changes in pH_i . (2) Information of membrane potential is not available and the electrochemical potential gradient for HCO_3^- across the apical membrane is not accurately predicted. (3) RT-PCR of isolated bronchioles does not identify

the cell types (ciliated or nonciliated) and the membrane (apical or basolateral) in which transporters/channels are located.

In summary, we have characterized $\text{HCO}_3^-/\text{H}^+$ transport across the apical membrane of surface epithelial cells of native mice bronchioles. We have demonstrated that cAMP-mediated and Ca^{2+} -mediated pathways are involved in HCO_3^- secretion and that apical HCO_3^- secretion is largely mediated by CFTR and $\text{Cl}^-/\text{HCO}_3^-$ exchange. The impairment of HCO_3^- secretion in CF bronchioles may be related to the pathogenesis of early lung disease in CF.

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by LL, AY, MY, IT, NN, MN, YK, TF, MH, EN, TT, and HI. The first draft of the manuscript was written by LL and HI and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in the manuscript.

Code availability

Not applicable.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethical Committee on Animal Use for Experiment (approval No. M210457-003) and the Recombinant DNA Experiment Safety Committee (approval No. 20-93) of Nagoya University.

Consent for publication

Not applicable.

Competing interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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