Decreased Soluble Adenylyl Cyclase Activity in Cystic Fibrosis Is Related to Defective Apical Bicarbonate Exchange and Affects Ciliary Beat Frequency Regulation

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Human airway cilia contain soluble adenylyl cyclase (sAC) that produces cAMP upon HCO₃⁻/CO₂ stimulation to increase ciliary beat frequency (CBF). Because apical HCO₃⁻ exchange depends on ciliary fibrosis transmembrane conductance regulator (CFTR), malfunctioning CFTR might impair sAC-mediated CBF regulation in cells from patients with cystic fibrosis (CF). By Western blot, sAC isoforms are equally expressed in normal and CF airway epithelial cells, but CBF decreased more in CF than normal cells upon increased apical HCO₃⁻/CO₂ exposure in part because of greater intracellular acidification from unbalanced CO₂ influx (estimated by 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) fluorescence). Importantly, ciliated cell-specific cAMP production (estimated by FRET fluorescence ratio changes of tagged CAMP-dependent protein kinase (PKA) subunits expressed under a ciliated cell-specific promoter) in response to increased apical HCO₃⁻/CO₂ perfusion was higher in normal compared with CF cells. Inhibition of bicarbonate influx via CFTR (CFTRinh172) and inhibition of sAC (KH7) and PKA activation (H89) led to larger CBF declines in normal cells, now comparable with changes seen in CF cells. These inhibitors also reduced FRET changes in normal cells to the level of CF cells with the expected exception of H89, which does not prevent dissociation of the fluorescently tagged PKA subunits. Basolateral permeabilization and subsequent perfusion with HCO₃⁻/CO₂ rescued CBF and FRET changes in CF cells to the level of normal cells. These results suggest that CBF regulation by sAC-produced CAMP could be impaired in CF, thereby possibly contributing to mucociliary dysfunction in this disease, at least during disease exacerbations when airway acidification is common.

Adenylyl cyclases are generally thought to be transmembrane-, G-protein-, and forskolin-responsive proteins, but a nontransmembrane, soluble adenylyl cyclase (sAC)² has been identified (1). Mammalian sAC is not activated by G-proteins or forskolin (1) but by HCO₃⁻/CO₂ in a pH-independent manner (2, 3) and by Ca²⁺, which synergizes with HCO₃⁻ (4). sAC expression has been described in many human tissues (5). We have shown that it is expressed in the airway epithelium, where it represents the only known adenylyl cyclase localized to cilia (6). sAC is important for flagellar beating in sperm (7–9), and we have shown its importance for regulating ciliary beating in human airway epithelia via cAMP production upon stimulation with HCO₃⁻/CO₂ (6).

There are different sources of luminal bicarbonate in the airways: it can be secreted from submucosal glands and ciliated cells (10) and can also be produced de novo from CO₂ and H₂O by locally secreted carbonic anhydrase (11). Two proteins responsible for transporting HCO₃⁻ into cells, Slc26a9 (12, 13) and CFTR (14), have been described in the apical membrane of airway epithelial cells. Slc26a9 is mainly a chloride channel with very low bicarbonate permeability (12); thus, the major apical HCO₃⁻ exchange in human airways occurs likely through CFTR (14) or is dependent on it.

In 1989, the CFTR gene was linked to cystic fibrosis (15). Initial attempts to localize the protein in the airways were based on mRNA in situ hybridization and pinpointed expression mainly to submucosal gland acini (16). Significant expression of CFTR was more recently also shown in the apical membrane of ciliated cells from healthy human beings, whereas CFTR was absent in the apical membrane of cells from CF patients homozygous for the ΔF508 mutation (17). Expression of human CFTR (driven by the ciliated cell-specific promoter foxj1) in the trachea of a CF mouse model confirmed targeting of CFTR to the apical membrane of ciliated cells while restoring forskolin-stimulated chloride secretion (18). Electrolyte conductance through CFTR is usually associated with chloride transport, but bidirectional conductance of bicarbonate through CFTR has also been demonstrated and is thought to be important in the airway and in pancreatic ducts (14, 19–21). Regulation of HCO₃⁻ transport through CFTR has been related to sAC in Calu3 cells, an airway epithelial cell line (22), and in corneal endothelium (23), but there are no reports of the influence of diminished bicarbonate transport through CFTR on the activity of sAC in primary airway epithelial cells, especially as it.

References:
1. Schmid, A.; Sutto, Z.; Schmid, N.; Novak, L.; Ivonnet, P.; Horvath, M.; Conner, G.; Fregien, N.; Salathe, M. From the Division of Pulmonary and Critical Care and the Department of Cell Biology and Anatomy, University of Miami, Miami, Florida 33136 and the Department of Respiratory Medicine, Semmelweis University, Budapest 1125, Hungary.
2. The abbreviations used are: sAC, soluble adenylyl cyclase; CBF, ciliary beat frequency; CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; PKA, cAMP-dependent protein kinase; BCECF, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; ALI, air-liquid interface; CFP, cyan fluorescenc; protein; RII, regulatory unit 2; CAT, catalytic unit; YFP, yellow fluorescent protein; tmAC, transmembranous adenylyl cyclase(s).
relates to components of mucociliary transport, especially CBF. Here we investigate the role of CFTR-dependent HCO$_3^-$ transport on the activity of sAC and its influence on CBF regulation in airway epithelial cells. We also examine the consequences of aberrant CFTR function on this process and discuss these findings in the pathophysiological context of cystic fibrosis.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Solutions**—Unless stated otherwise, all of the materials were purchased from Sigma-Aldrich. The compositions of solutions used for apical and basolateral perfusions are shown in Table 1. In bicarbonate-free solutions, sodium or potassium bicarbonate was iso-osmotically replaced by sodium or potassium gluconate (see Table 1).

**Air-Liquid Interface (ALI) Cell Culture**—Normal human airways were obtained from organ donors whose lungs were rejected for transplant. Institutional Review Board approved consent for research with these tissues was obtained by the Life Alliance Organ Recovery Agency of the University of Miami and confirmed to the declaration of Helsinki. Lungs from CF patients were obtained with IRB-approved consent at the time of transplant. All CF patients had an abnormal sweat test and the following genotypes: homozygous for ΔF508 ($n=3$), G524X/ΔF508 ($n=1$), or 621(G→T)/ΔF508 ($n=1$). For the patient with 621(G→T)/ΔF508 mutation, Ussing chamber experiments showed chloride conductance abnormalities typical for CF. The patient with the G524X/ΔF508 mutation received a lung transplant at age 25. Based on these findings, all cells used from CF patients had, as expected from the mutations, severe defects in apical chloride conductance caused by either a lack of CFTR or malfunctioning CFTR and were used interchangeably. Airway epithelial cells were isolated and dedifferentiated through expansion. Passage 1 cells were re differentiated at an ALI on collagen-coated 24-mm T-clear or 12-mm Snapwell filters (Costar Corning) as described previously (24–27).

**Deciliation**—Fully differentiated epithelial cells, grown at the ALI, were washed with Dulbecco’s modified phosphate-buffered saline. Then 500 μl of deciliation buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM CaCl$_2$, 1 mM EDTA, 7 mM 2-mercaptoethanol, and 0.1% Triton X-100) was applied to the apical side of the culture (6, 28). The cultures were gently shaken for 1 min before the apical fluid was collected and centrifuged at low speed (1,500 × g) to pellet cells. The supernatant was recentrifuged at high speed (16,000 × g), and the resulting pellet containing axonemes was resuspended and stored at −80°C until use.

**Western Blot**—ALI cultured cells were lysed (1% SDS in 10 mM Tris, pH 8.5, and 0.1 mM EDTA) in the presence of protease inhibitors and cleared from debris by centrifugation. Ten μg of protein (cilia, whole, and deciliated cells) of normal and CF cell lysates and 20 μg of purchased protein from testis (BD Biosciences, San Jose, CA) per lane were separated using SDS-PAGE on 12% gels (Bio-Rad) and electroblotted onto Immobilon P membranes (Millipore, Billerica, MA). The membranes were blocked with 10% nonfat dry milk and 0.05% Tween 20 in PBS and incubated with rabbit anti-sAC serum 1:10,000 (gift from W. Geng, Southwestern Medical Center, Dallas, TX). After incubating with a secondary horseradish peroxidase-labeled antibody (KPL Inc., Gaithersburg, MD), chemiluminescence (Pierce) was used for detection and quantification on a ChemiDoc XRS system (Bio-Rad). The membranes were stripped with Restore Western blot stripping buffer (Pierce) and reprobed with rabbit anti-β-actin (1:100) (Sigma-Aldrich) or anti-acetylated tubulin (1:1,000) (Sigma-Aldrich). Human testis protein medley was used as a positive control. The signals were quantified using ImageJ. Densities from all sAC bands were added and divided by densities of acetylated tubulin (whole cells and cilia) bands. The ratio from CF cells was divided by the ratio from normal cells giving the relative sAC expression in whole cells and cilia in CF compared with normal cells. The same procedure was performed with the 50-kDa sAC band in whole cell and cilia preparations.

**Pseudotyped Lentivirus Vectors and Infection of Airway Epithelial Cells**—Third generation, propagation-deficient, HIV-pseudotyped lentiviruses encoding fluorescently tagged PKA subunits (RII-CFP and CAT-YFP) under transcriptional control of the ciliated cell-specific foxj1 promoter were used as described previously (25) for FRET. Briefly, recombinant lentiviruses were constructed using the pRRLsinPPT.CMV.MCS.Wpre vector (29). For the initial constructs, genes encoding the catalytic PKA subunit and the regulatory PKA subunit RII, fused to the fluorescent proteins YFP and CFP, respectively (30), were cloned into the multiple cloning site downstream of the ciliated cell-specific foxj1 promoter for better expression in ciliated cells (31). Lentiviruses were prepared by co-transfecting HEK 293T cells with lentivirus and packaging plasmids using calcium phosphate co-precipitation. Virus-containing medium was collected, and undifferentiated airway epithelial cells, plated onto collagen-coated T-col filters in bronchial epithelial growth medium, were incubated with both viral constructs overnight at 37 °C in 5% CO$_2$. The following day, virus was discarded, and medium was changed to ALI medium top and bottom until cells reached confluence, when apical media were removed to establish the ALI. Expression of the fluorescently tagged proteins was monitored and captured using an inverted fluorescence microscope. Confocal images were obtained on a Zeiss LSM 510/UV confocal microscope.

**Measurement of CBF and FRET in Airway Epithelial Cells**—All cultures were placed in a customized closed chamber allowing independent perfusion of the apical and basolateral compartments, which were fully enclosed. The chamber was mounted at room temperature on the stage of an upright Nikon E600fm microscope, and water was added on top of the closed chamber for use of a 63× water immersion objective with a numerical aperture of 1.0. If required, the cells were permeabilized apically or basolaterally. For basolateral permeabilization, we used *Staphylococcus aureus* α-toxin (10,000 units/ml) (25, 32), and for apical permeabilization, we used nystatin (180 μg/ml) as described previously (33). Solutions used for perfusion of permeabilized and nonpermeabilized cells are described in Table 1 and were used as follows. For experiments without permeabilization, solution 1 was used for apical and basolateral perfusion, whereas solution 2 was used apically for bicarbonate exposure after obtaining a base-line CBF with solution 1. For experiments with basolateral permeabilization, the cells were apically perfused with solution 1 and basolaterally with solution...
Chroma). The Metafluor™ FRET module (Molecular Devices) cell at the two different emission wavelengths of CFP and YFP allowing simultaneous visualization and recording of the same reflects the two images side-by-side onto the CCD chip, thereby the microscope using a Dual-View (Optical Insights, Tucson, AZ) cooled CCD camera (Roper Scientific, Tucson, AZ) attached to Novato, CA). The cells were imaged with a CoolSnap Hq-via a DG4 rapid wavelength switcher (Sutter Instruments, Brattleboro, VT). Ratio tool software from Isee Imaging (Raleigh, NC) controlled the output of the Lambda DG4. Ratiorometric pH was estimated by capturing the light (535 nm) emitted from the cells through a 63× water immersion objective (Nikon Instruments Inc. Melville, NY) and directed to a cooled CCD camera (CoolSnap Hq, Photometrics, Tucson, AZ). Individual ciliated cells were identified as regions of interest, and the BCECF ratio of emission intensity after excitation at 495 and 440 nm was computed within each region of interest every 10–60 s on a pixel-by-pixel basis (after background fluorescence subtraction). The system was calibrated with 15 μM of the ionophore nigerin in 130 mM KCl-containing solutions at different pH levels between 6.8 and 7.8 (32).

**Ussing Chamber Experiments**—Fully differentiated cells cultured on Snapwell inserts were mounted into Ussing chambers. CF and normal epithelial cells were bathed with Krebs Henseleit buffer at 37 °C and studied under short circuit conditions (voltage clamped at 0 mV) with transepithelial resistance (Rₑ) determined by the application of intermittent (2 s) 1-mV bipolar pulses. After cultures were equilibrated, amiloride (10 μM, luminal) was added to block electrogenic Na⁺ absorption. cAMP-dependent Cl⁻ secretion was measured at the plateau phase after the addition of 10 μM forskolin. After establishing a new base-line current, 10 μM ATP was added for measuring calcium-dependent chloride channel activation over PᵥYᵥ₃ activation.

**Statistics**—The results were compared by one-way analysis of variance and, if a significant difference was found, by the Tukey-Kramer honestly significant difference test using JMP software (SAS). p < 0.05 was accepted as significant.

**RESULTS**

Chloride and Bicarbonate Conductance of CFTR—The lack of chloride conductance through functional CFTR was confirmed for all CF lungs used. Fig. 1 illustrates Ussing chamber experiments with cells from patients with mutations for 621(G→T)/ΔF508 (Fig. 1A) and homozygous ΔF508 (Fig. 1B). No significant chloride conductance was found upon apical stimulation with 10 μM forskolin (tmAC agonist) in any of the tested CF cultures. In contrast, normal control cultures showed significant increases in Iₑc upon forskolin stimulation. The expected Iₑc response to ATP could be documented in both CF and normal cells (Fig. 1).
bicarbonate and subsequently produces cAMP to activate PKA (6). The response of ciliary beating to apical bicarbonate is complicated by the fact that HCO$_3^-$ equilibrates with H$_2$O and CO$_2$ in the presence of H$^+$ and that CO$_2$ can freely cross the cell membrane and acidify the cytoplasm. This cytoplasmic acidification leads to a decrease of CBF (32) that is partially rescued in normal cells by the bicarbonate stimulation of sAC. Because bicarbonate is mainly conducted through CFTR at the apex of human airway epithelial cells (14, 19–21), we hypothesized that cells from cystic fibrosis patients cannot rescue the acidification-induced CBF decrease upon increased apical HCO$_3^–$/CO$_2$ exposure because of a defective apical HCO$_3^–$ exchange caused by absent or dysfunctional CFTR.

To investigate the effect of apical bicarbonate changes on CBF in normal and CF cells, exposure of the apical membrane was alternated from 0 to 25 mM sodium bicarbonate, whereas the basolateral perfusate did not contain bicarbonate. The bicarbonate-containing solution was equilibrated with CO$_2$ by bubbling the solutions with 5% CO$_2$ before filling into gas-impermeable syringes and tubing. Using a similar experimental setup, we showed previously that apical perfusion with bicarbonate leads to a decrease of CBF because the pH effect of CO$_2$ on CBF is stronger than the sAC-mediated CBF increase after stimulation by bicarbonate (6). Mean CBF base lines in normal cells (6.6 $\pm$ 0.2 Hz; $n = 42$ from six different lung donors) and in CF cells (7.1 $\pm$ 0.2 Hz; $n = 53$ from three different CF patients) were the same ($p > 0.05$). On the other hand, $\Delta$CBF from base line upon switching to apical bicarbonate-containing solutions was $-23.6 \pm 1.3%$ in normal cells, significantly less than in CF cells where $\Delta$CBF was $-30.3 \pm 1.7%$ ($p < 0.05$) (Fig. 3A). The absolute decrease in CBF was also significantly larger in CF cells, 2.14 $\pm$ 0.2 Hz, versus normal cells, 1.57 $\pm$ 0.2 ($p < 0.05$). These results (Fig. 3, A and B) suggest that an apical HCO$_3^–$ exchange defect in CF could affect regulation of CBF in these cells, possibly because of a lack of sAC activation. Alternatively, the differences could be solely related to a difference in overall pH$_i$, again caused by a defect in apical HCO$_3^–$ exchange.

To examine the latter possibility, we measured the changes in pH$_i$ using BCECF and fluorescence microscopy (Fig. 4). In agreement with the hypothesis that bicarbonate is transported into the cells through CFTR in normal but not so in CF cells, we found greater acidification in CF cells (pH decrease by $-0.49 \pm 0.04$; $n = 10$, two different donors) than in normal cells (pH decrease by $-0.34 \pm 0.02$; $n = 10$, two different donors; $p < 0.05$).

To determine whether or not this pH difference explained the differential CBF regulation in normal versus CF cells, we tested the effects of HCO$_3^–$ on normal and CF cells after permeabilizing the basolateral or apical membrane. Permeabilization of the membranes allows free, CFTR-independent exchange of HCO$_3^–$, equalizing the intracellular pH and bicarbonate concentration in normal cells and in cells from a patient with CF. Basolateral permeabilization was accomplished with $\alpha$-toxin and confirmed by a significant CBF decrease upon basolateral perfusion with ATP-free solutions as described previously and demonstrated in Fig. 5B (6). While changing the bicarbonate concentration over the basolaterally permeabilized membrane, the apical perfusate was kept without bicarbonate in these

Expression of Soluble Adenylyl Cyclase Protein in Airway Epithelial Cells—To examine the expression and localization of sAC in normal and CF airway epithelial cells, Western blots were performed using protein lysates from whole cells, deciliated cells, isolated cilia/axonemes, and human testis (positive control). All of the samples (except testis) were prepared from fully differentiated ALI cell cultures from donors without underlying lung disease (normal) and from the lungs of patients with cystic fibrosis. To probe the Western blots, a polyclonal rabbit antibody against a peptide (SLSEGDALLA) (5) near the N terminus of human sAC proteins was used as described previously (6). For quality and quantity controls, the same blots were stripped and reprobed with antibodies against $\beta$-actin (deciliated cells) and against acetylated tubulin (whole cells, cilia, and testis). Protein loading was equal in CF versus normal cells: 10 $\mu$g for all samples except 20 $\mu$g for testis.

Ciliary Beat Frequency—We have previously shown that apical and basolateral changes in HCO$_3^–$/CO$_2$ exposure influence CBF in human airway epithelial cells. sAC is stimulated by

FIGURE 2. Multiple sAC protein isoforms are expressed in human airway epithelial cells. Western blots of human airway epithelial cells and human testis extract (positive control) using a polyclonal sAC antibody against the sequence SLSEGDALLA near the N terminus show three distinct bands at 190, 80, and 50 kDa. Testes and whole human airway epithelial cells show all three bands. Cilia/axonemes contain only one band at 50 kDa, which is reduced/missing in deciliated cells, suggesting that this 50-kDa form is localized to the axoneme. For quality control of loading, the blots were stripped and reprobed with antibodies against $\beta$-actin (deciliated cells) and against acetylated tubulin (whole cells, cilia, and testis). Protein loading was equal in CF versus normal cells: 10 $\mu$g for all samples except 20 $\mu$g for testis.
the changes were not different between normal and CF cells.

where CBF was stimulated upon increases in bicarbonate, and these results were confirmed in apically permeabilized cells, compared with normal cells.**

between normal cells upon apical HCO3- perfusion with 25 mM HCO3-/CO2.**

CBF was significantly lower than that of CF cells (**0.4 Hz (**18.9 **2.8% from a base line of 10 **36, six different donors), and in CF cells, whereas **22, three different donors) in cells from CF donors; **A** apical perfusion with 25 mM HCO3-/CO2 perfusion in nonpermeabilized cells.

percentage of the decrease of CBF from base line to a new level upon apical perfusion with 25 mM HCO3-/CO2.**

CBF upon apical HCO3- perfusion was **−4.3 ± 3.9% in normal cells (**n = 15, three different donors), whereas ΔCBF was **−8.3 ± 2.8% (**n = 22, three different donors) in cells from patients with CF (**p > 0.05** (Fig. 6A). The base-line CBF of the normal cells). Inhibiting PKA with 10 μM H89 (Fig. 7C) revealed a ΔΔCBF of **−5.6 ± 1.5% in normal cells (**n = 36, four lungs), whereas ΔΔCBF in CF cells (**n = 24, three different lung donors) was **4.5 ± 2.0% (**p < 0.05 compared with normal cells).

Baseline CBF was not different between all pairs of treated and untreated normal and CF cells, except that H89 decreased CBF base line in CF but not normal cells.

100 μM 4,4-dinitrostilbene-2,2-disulfonic acid (DNDS), an inhibitor of chloride channels other than CFTR (in intact cells) and anion exchangers, had no influence on the observed CBF changes: ΔΔCBF (presence and absence of DNDS) was **0.9 ± 1.4% for normal cells (**n = 12, two different lungs) and **−0.9 ± 1.1% for CF cells (**n = 7, one lung donor) (**p > 0.05 for comparison of ΔΔCBF). Base-line CBF was not different between normal and CF cells.

To ensure that the effects observed under perfusion of the apical membrane were not related to osmotic differences, all of experiments. CBF responses to changes in HCO3- in basolaterally permeabilized cells were statistically not significantly different between normal versus cystic fibrosis cells (Fig. 5A). In normal cells, ΔCBF was **−15.4 ± 1.2% from a base line of 8.0 ± 0.4 Hz (**n = 36, six different donors), and in CF cells, ΔCBF was **−18.9 ± 2.1% from a base line of 10 ± 0.45 Hz (**n = 29, two different donors). Although the base-line CBF of normal cells was significantly lower than that of CF cells (**p < 0.05) during basolateral permeabilization, ΔCBF was not (**p > 0.05). The etiology of the different base-line frequencies remains unclear. These results were confirmed in apically permeabilized cells, where CBF was stimulated upon increases in bicarbonate, and these changes were not different between normal and CF cells. ΔCBF upon apical HCO3- perfusion was **−4.3 ± 3.9% in normal cells (**n = 15, three different donors), whereas ΔCBF was **−8.3 ± 2.8% (**n = 22, three different donors) in cells from patients with CF (**p > 0.05** (Fig. 6A). The base-line CBF of the two groups was not significantly different (**p > 0.05). The finding of equal CBF responses in normal and CF cells in permeabilized cells suggests similar mechanisms of CBF regulation by bicarbonate (if it can enter the cells in equal amounts) in CF and normal cells.

To further characterize the influence of sAC on CBF, we used inhibitors to block the sAC pathway at different levels; CFTR was blocked with inhibitor 172 (CFTRinh172), the activation of sAC with the specific sAC inhibitor KH7 (35, 36), and the effect of cAMP on ciliary beating by inhibiting PKA with H89 (37). To evaluate the effects of these inhibitors on CBF, we used ΔΔCBF as a measurement. ΔΔCBF is the difference between ΔCBF with and without the use of these different inhibitors upon apical HCO3-/CO2 perfusion in normal or CF cells. Inhibition of CFTR with 20 μM CFTRinh172 resulted in a ΔΔCBF of **−2.9 ± 0.9% in normal cells (**n = 44, four different lung donors); in contrast, it did not exaggerate the CBF response in CF cells (**n = 20, two different lung donors) with a ΔΔCBF of **2.4 ± 1% (**p < 0.05 compared with normal cells; Fig. 7A). Inhibition of sAC with 25 μM KH7 (Fig. 7B) resulted in a ΔΔCBF of **−4.8 ± 0.8% in normal cells (**n = 31, three different donors), whereas KH7 resulted in no further CBF decrease in CF cells with a ΔΔCBF of **2.3 ± 1.6% (**n = 10, two different CF donors; **p < 0.05 compared with untreated normal and CF cells, except that H89 decreased CBF base line in CF but not normal cells.

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To ensure that the effects observed under perfusion of the apical membrane were not related to osmotic differences, all of
the solutions were osmotically balanced as shown in Table 1. For all experiments, the solutions contained either 25 mM of sodium gluconate or 25 mM sodium bicarbonate. To make certain that the CBF changes were not nonspecific because of changes of different anions but rather specific to bicarbonate, we also performed a series of experiments exchanging the solutions between 25 mM sodium chloride and sodium gluconate (n = 10, three different lungs); these experiments did not reveal a significant change in CBF upon the changes in salt solution perfusion. The solvent MeSO (1%) did not affect the CBF response.

Thus, inhibiting bicarbonate transport through CFTR (CF cells, CFTRinh-172, but not DNDS), inhibiting sAC by KH7, and inhibiting PKA with H89 made normal cells behave like CF cells with respect to CBF decreases upon apical increases in HCO\textsubscript{3}\textsuperscript{-}/CO\textsubscript{2}. The fact that CFTRinh-172, KH7, and H89 change the CBF response to apical HCO\textsubscript{3}/CO\textsubscript{2} in normal but not in CF cells (Fig. 7, A–C), together with the previous data, suggests that apical HCO\textsubscript{3}/CO\textsubscript{2} exposure not only decreases pH\textsubscript{i}, but also activates sAC via HCO\textsubscript{3} influx in normal cells to produce cAMP, thereby stimulating CBF via PKA activation. In CF cells, however, this mechanism of sAC activation is defective, most likely because of the inability of luminal HCO\textsubscript{3} to enter the cell apically because of defective CFTR.

Real Time Measurement of cAMP Production in Single Airway Epithelial Cells with FRET—To confirm that apical HCO\textsubscript{3}/CO\textsubscript{2} exposure increases intracellular cAMP levels upon sAC stimulation more in normal cells compared with CF cells, we used our previously developed method of measuring changes in intracellular cAMP. By measuring the cAMP-induced dissociation of fluorescently tagged subunits of PKA by FRET (25), we can estimate intracellular cAMP levels in real time. Undifferentiated normal and CF human airway epithelial cells were co-infected with two lentiviruses, each encoding a fusion protein of a PKA subunit (RII-CFP or CAT-YFP), and both constructs were driven by the ciliated cell-specific foxj promoter (Fig. 8). Cells showing FRET capability by emitting at 535 nm under excitation at 435 nm were chosen for FRET measurements. Apical perfusion with 25 mM bicarbonate increased the FRET ratio (ΔFRET-RATIO) by 0.0076 ± 0.0007 arbitrary units in normal cells (n = 32, three donors) but only by 0.0045 ± 0.0007 arbitrary units (n = 25, three different donors) in CF cells (p < 0.05) (Fig. 3C).

These changes correspond to cAMP levels of ~100 μM in normal and 50 μM in CF cells (25). However, during basolateral perfusion with 25 mM bicarbonate in basolaterally permeabilized cells, the ΔFRET-RATIO increase was similar in normal cells (n = 17, two different donors) and CF cells (n = 13, two different donors) (Fig. 5C). These data support the notion that sAC can be activated by apical bicarbonate exposure in normal but less so in CF cells, suggesting an important role for CFTR-mediated bicarbon-
**CBF, sAC, CF, and Apical HCO$_3^-$ Exchange**

...ate entry under these conditions in normal cells, thereby helping to regulate CBF.

As described above for CBF, we used CFTR$_{inh172}$, KH7, and H89 to dissect the effect of bicarbonate on cAMP production as measured by FRET. To evaluate the effects of these inhibitors on intracellular cAMP concentrations, we used the $\Delta$FRET-RATIO as a measurement. $\Delta$FRET-RATIO is the difference between FRET-RATIO with and without the use of the different inhibitors upon apical HCO$_3^-$/CO$_2$ perfusion in normal or CF cells. Inhibition of CFTR with 20 $\mu$m CFTR$_{inh172}$ resulted in a $\Delta$FRET-RATIO of $-0.0061 \pm 0.0012$ arbitrary units in normal cells ($n = 9$, two different lungs); in contrast, it did not exaggerate the FRET-RATIO in CF cells ($n = 6$, two different lungs) with a $\Delta$FRET-RATIO of $-0.0011 \pm 0.0007$ arbitrary units ($p < 0.05$ compared with normal cells; Fig. 7D). Inhibition of sAC with 25 $\mu$m KH7 (Fig. 7E) resulted in a $\Delta$FRET-RATIO of $-0.0022 \pm 0.0007$ arbitrary units in normal cells ($n = 9$, two different donors), and KH7 resulted in a $\Delta$FRET-RATIO of 0.0001 $\pm$ 0.0002 arbitrary units in CF cells ($n = 8$, two different lungs; $p < 0.05$ compared with normal cells). As expected, inhibiting PKA with 10 $\mu$m H89 (Fig. 7F) did not change the FRET-RATIO. The $\Delta$FRET-RATIO was 0.0006 $\pm$ 0.0009 arbitrary units in normal cells ($n = 14$, two different lungs) and 0.0005 $\pm$ 0.0007 arbitrary units in CF cells ($n = 11$, two different lungs; $p > 0.05$ compared with normal cells). H89 inhibits PKA phosphorylation, but not the dissociation of the catalytic and regulatory unit of PKA, thereby still allowing FRET to occur (37).

As with the CBF measurements, we repeated experiments for evaluating FRET changes with solutions containing different anions to assure that the bicarbonate effects were specific. Exchanging the solutions between 25 mM sodium chloride and sodium gluconate ($n = 14$, two different lungs) did not reveal a significant change in FRET ratio upon the changes in salt solution perfusion. The solvent Me$_2$SO (1‰) did not

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**TABLE 1**

Solution components

Concentrations of solution components are given in mM. Solutions 1 and 2 are for non-permeabilized cells, and solutions 3, 4, and 5 are for permeabilized cells. pH adjustment to an extracellular pH of 7.4 for nonpermeabilized cells were done with NaOH for solution 1 and with NaHCO$_3$ for solution 2. pH adjustment to an intracellular pH of 7.2 for permeabilized cells were done with KOH for solutions 3 and 5 and with KHCO$_3$ for 4. Solutions containing HCO$_3^-$ were equilibrated with 5%CO$_2$, 95%O$_2$; all others were with air. CrP, creatine phosphate disodium.

| Solution 1 | Solution 2 | Solution 3 | Solution 4 | Solution 5 |
|------------|------------|------------|------------|------------|
| NaCl       | 117        | 117        | 10         | 25         |
| KCl        | 5.3        | 5.3        | 10         | 10         |
| Sodium gluconate | 25       |            |            |            |
| NaHCO$_3$  | 25         |            |            |            |
| KHCO$_3$   |            |            |            |            |
| Potassium gluconate | 140    | 25         | 115        | 140        |
| CaCl$_2$   | 1.3        | 1.3        | 0.45$^a$   | 0.45$^a$   |
| MgCl$_2$   | 0.5        | 0.5        |            | 0.1        |
| MgSO$_4$   | 0.4        | 0.4        |            |            |
| Na$_2$HPO$_4$ | 0.3      | 0.3        |            |            |
| KH$_2$PO$_4$ | 0.4      | 0.4        |            |            |
| Glucose    | 5.6        | 5.6        |            |            |
| Heps       | 20         | 20         | 20         | 20         |
| Mg-ATP     | 10         | 10         |            |            |
| CrP        | 10         | 10         |            |            |

$^a$ Approximate concentration of free Ca$^{2+}$ after chelation by ATP is 0.1 mM.
Cyclic AMP is one of the major regulators of CBF (38). Until recently, cAMP in airway epithelial cells was mainly thought to be produced by tmAC. Apically localized tmACs are G-protein-responsive producers of cAMP. Despite microdomains of cAMP signaling, this cAMP is likely available to cilia; exogenous β-adrenergic agonists increase cAMP concentrations over stimulation of β2-receptors and endogenous, and apically released ATP is hydrolyzed to adenosine, stimulating A2b receptors to produce cAMP (39), all via G-protein coupling to tmAC. ATP can also release intracellular calcium by stimulation of P2Y2 receptors and thereby activate calcium-sensitive apical tmACs to produce cAMP. The intracellular production of cAMP is even more important because calcium-mediated CBF increase is also dependent on the presence of at least small amounts of cAMP (38). The ciliary target for cAMP-associated CBF stimulation is PKA (38), which phosphorylates a dynein light chain, making the cilia beat faster. Calcium is mainly released from intracellular stores upon different stimulation, e.g. via G-protein-coupled receptors (GP) such as the purinergic receptor P2Y2. Calcium diffuses to the cilia, where it has a direct activating effect on the axoneme. There are interactions between calcium, sAC, and cAMP. sAC is stimulated by calcium, and calcium requires small amounts of cAMP for its action at the cilium. Intracellular pH influences CBF, probably over a direct action on the axoneme. Additionally, a decrease in pH increases the conductance of CFTR, which could in turn further stimulate sAC and increase CBF if more HCO3-/CO2- is available.

Intracellular Ca2+, ATP

HCO3-/CO2

PKA

sAC

CFTR

Ca2+

Figure 9. Model of CBF regulation via the major effectors in the human airways, including cAMP, calcium, and pH. CBF, sAC, CF, and Apical HCO3-/CO2- Exchange

DISCUSSION

Our data demonstrate that apical bicarbonate influx requires functional CFTR and that this influx can be important for the regulation of cilia beating in airway epithelial cells. This finding may shed new light on the discussion about the importance of CBF regulation in the development of mucociliary dysfunction in patients with cystic fibrosis, a topic usually dominated by the discussion of periciliary fluid depletion.

Cyclic AMP is one of the major regulators of CBF (38). Until recently, cAMP in airway epithelial cells was mainly thought to be produced by tmAC. Apically localized tmACs are G-protein-responsive producers of cAMP. Despite microdomains of cAMP signaling, this cAMP is likely available to cilia; exogenous β-adrenergic agonists increase cAMP concentrations over stimulation of β2-receptors and endogenous, and apically released ATP is hydrolyzed to adenosine, stimulating A2b receptors to produce cAMP (39), all via G-protein coupling to tmAC. ATP can also release intracellular calcium by stimulation of P2Y2 receptors and thereby activate calcium-sensitive apical tmACs to produce cAMP. The intracellular production of cAMP is even more important because calcium-mediated CBF increase is also dependent on the presence of at least small amounts of cAMP (38). The ciliary target for cAMP-associated CBF stimulation is PKA (38), which phosphorylates an outer dynein light chain in the axoneme (28). In contrast to tmACs located at the apical membrane (but not on the ciliary membrane), sAC is localized to human cilia (6), where it produces cAMP in close proximity to the dynein arms (Fig. 9). The activation of sAC by HCO3-/CO2- is well established (2–4, 40), and the sAC-mediated production of cAMP with its subsequent CBF effect has been shown using FRET-based estimation of intracellular cAMP (6). The observed changes in CBF and FRET upon HCO3-/CO2-exposure were blocked by the sAC-specific inhibitors KH7 (35, 36) and 2-HD-17β estradiol (41), whereas the tmAC-specific inhibitor SQ 22536 had no effect (6). Here we dissect the proposed sAC activation pathway by using inhibitors at different steps of the pathway. CFTRinh172 inhibits the transport of bicarbonate into normal cells as shown in Ussing chamber experiments (Fig. 1). KH7 (sAC inhibitor) blocks HCO3-/CO2-mediated changes in CBF and FRET, and H89, a catalytic PKA inhibitor, blocks HCO3-/CO2-mediated changes in CBF but not FRET, because the dissociation of the catalytic and regulatory unit of PKA is not inhibited, thereby allowing FRET to occur (37). Furthermore, CFTRinh172 changes the CBF and FRET response of normal cells to apical HCO3-/CO2-stimulation into a CF-like pattern. Together, these results support our hypothesis of disturbed CBF response upon apical HCO3-/CO2-perfusion in CF cells, based on missing bicarbonate transport mediators.
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ated by CFTR (either directly or indirectly). The importance of sAC for CBF and possibly other cellular functions is highlighted by the recent finding that chloride and bicarbonate conductance through CFTR is increased during states of low intracellular pH (42), allowing sAC activation to counteract the negative CBF effect of low pH even more. The fact that CFTR conductance is also increased by sAC itself, at least in airway epithelial cell lines (22), makes this rescue system a powerful, self-enhancing circuit.

Bicarbonate comes from two major sources in the airways. Submucosal glands secrete bicarbonate as shown in pig airways (10, 43). The secretion of bicarbonate is increased by IL-17A (44), a T-cell product. Production of IL-17A in the airways is increased by infections with Pseudomonas aeruginosa (45) and Klebsiella pneumonia (46), suggesting increased airway content of bicarbonate during airway disease exacerbations. The second source of airway bicarbonate comes from the reaction of H₂O and CO₂. The airway concentration of CO₂ can be significantly increased secondary to hypoventilation in airway diseases, because it occurs during exacerbations of asthma, chronic obstructive lung disease (COPD), CF, and pulmonary infections. The spontaneous balance between H₂O/CO₂ and HCO₃⁻ is shifted to the bicarbonate side by secretion of carbonic anhydrase from serous acinar and ductal cells, as has been shown in human bronchial and tracheal submucosal glands (11) in the airways of rats (47) and guinea pigs (48). Altogether, these mechanisms indicate that there is increased availability of bicarbonate in the airway lumen during exacerbation of airway diseases. CFTR is a channel that allows bidirectional chloride and bicarbonate flux, the direction of which will depend on the driving force. Different conductance directions for chloride in sweat glands and in the airways exemplify this principle. The above mentioned increased luminal bicarbonate content during airway disease exacerbations may be a driving force for its conductance into airway epithelial cells. Submucosal glands can produce 20 meq/liter bicarbonate (10, 43), which may even be increased by intraluminal production as mentioned above. Intracellular bicarbonate concentrations are described between 4 and 12 mM in different organs, depending on the predominant acid-base status (49–51). Based on these concentrations, our hypothesis is valid, at least during disease exacerbations, when the intracellular bicarbonate concentration is lower, and the extracellular concentration is higher. Once in the cell, bicarbonate stimulates sAC, thereby producing cAMP with a subsequent increase in CBF and mucociliary clearance during non-CF airway disease exacerbations. In patients with cystic fibrosis, however, this system is disturbed at two levels based on malfunctioning CFTR: at the secretion site of bicarbonate in submucosal glands and at the absorption site of the apical membrane of ciliated cells.

Based only on one in vitro study examining solely base-line CBF, the function of cilia has been thought to be normal in CF until depletion of periciliary fluid impedes ciliary beating (52). Nourished by the development of the beta epithelial sodium channel (β-ENaC) overexpressing mouse as a model for cystic fibrosis (53), depletion of airway surface liquid is thought to be the major reason for airway disease in cystic fibrosis (54), and this fact is not disputed by our results. Our data, however, add a ciliary mechanism by which mucociliary dysfunction is worsened at least during disease exacerbation. The fact that bicarbonate is not secreted from submucosal glands and cannot enter the ciliated cells at times when ciliary beating may need it most puts a break on an important rescue mechanism of mucociliary clearance. This is not only a problem once the airways are battling recurrent infections but also affects newborn patients with cystic fibrosis. During the initial infections of the airways, the ciliary machinery cannot counteract the slowing effect of the airway acidification. This, in addition to the altered airway surface liquid, leads to impaired mucociliary clearance and consecutive worsening of the airway inflammation.

One of the difficulties of the performed experiments is the fact that CBF is influenced by bicarbonate via sAC-mediated cAMP (6) and by changes in intracellular pH (32) that in return are also influenced by bicarbonate. In a previous study, we approached this problem by saturating the intracellular compartment with CO₂ by apical HCO₃⁻/CO₂ exposure before perfusing the permeabilized basolateral membrane with HCO₃⁻/CO₂ to increase the intracellular bicarbonate content (6). Here, we measure intracellular pH with BCECF, using ratiometric fluorescence microscopy, to compare the influence of apical HCO₃⁻/CO₂ perfusion on the intracellular pH in CF and normal airway epithelial cells. These experiments show that the pH in CF cells in fact drops significantly more than in regular cells, confirming our hypothesis that bicarbonate entry is dysfunctional in CF cells secondary to abnormal CFTR, whereas transmembranos CO₂ diffusion is not impaired. The fact that the inhibition of PKA with H89 does significantly decrease CBF in normal cells upon exposure to HCO₃⁻/CO₂ without affecting CF cells supports the hypothesis that the observed pH change is not the only factor influencing CBF under these conditions.

In summary, bicarbonate-stimulated cAMP production by sAC increases ciliary beat frequency and may serve as an important rescue mechanism of mucociliary clearance during exacerbations of airway diseases. The dependence of this pathway on functioning CFTR suggests that this compensation mechanism is defective in patients with CF, thereby possiblycontributing to mucociliary dysfunction in this disease.

Acknowledgments—We thank Weidong Geng and Orson Moe for making the sAC antibodies for the Western blots available to us. We also want to thank Jochen Buck and Lonny R. Levin for helpful advice and George McNamara from the analytic imaging core facility of the University of Miami for help with the confocal microscope.

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