Regulation of human airway ciliary beat frequency by intracellular pH

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pH_i affects a number of cellular functions, but the influence of pH_i on mammalian ciliary beat frequency (CBF) is not known. CBF and pH_i of single human tracheobronchial epithelial cells in submerged culture were measured simultaneously using video microscopy (for CBF) and epifluorescence microscopy with the pH-sensitive dye BCECF. Baseline CBF and pH_i values in bicarbonate-free medium were 7.2 ± 0.2 Hz and 7.49 ± 0.02, respectively ($n = 63$). Alkalization by ammonium pre-pulse to pH_i 7.78 ± 0.02 resulted in a 2.2 ± 0.1 Hz CBF increase ($P < 0.05$). Following removal of NH_4Cl, pH_i decreased to 7.24 ± 0.02 and CBF to 5.8 ± 0.1 Hz ($P < 0.05$). Removal of extracellular CO_2 to change pH_i resulted in similar CBF changes. Pre-activation of cAMP-dependent protein kinase (10 μM forskolin), broad inhibition of protein kinases (100 μM H-7), inhibition of PKA (10 μM H-89), nor inhibition of phosphatases (10 μM cyclosporin + 1.5 μM okadaic acid) changed pH_i-mediated changes in CBF, nor were they due to $[Ca^{2+}]_i$ changes. CBF of basolaterally permeabilized human tracheobronchial cells, re-differentiated at the air–liquid interface, was 3.9 ± 0.3, 5.7 ± 0.4, 7.0 ± 0.3 and 7.3 ± 0.3 Hz at basolateral i.e., intracellular pH of 6.8, 7.2, 7.6 and 8.0, respectively ($n = 18$). Thus, intracellular alkalization stimulates, while intracellular acidification attenuates human airway CBF. Since phosphorylation and $[Ca^{2+}]_i$ changes did not seem to mediate pH_i-induced CBF changes, pH_i may directly act on the ciliary motile machinery.

(Resubmitted 14 May 2004; accepted after revision 6 August 2004; first published online 12 August 2004)

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pH_i is an important element of cellular homeostasis and affects a number of cellular functions (for review see Roos & Boron, 1981). Variations in pH_i of airway epithelia may occur *in vivo* in response to shifting luminal CO_2 tension (P_{CO_2}) during a full breathing cycle (Willumsen & Boucher, 1992); however, neither the extent of such a possible pH_i change nor its effect on cellular functions are known. There are only data available on the relationship between extracellular pH and CBF of mammalian airway epithelial cells (van de Donk *et al.* 1980; Luk & Dulfano, 1983; Clary-Meinesz *et al.* 1998): alkaline solutions up to pH 9–10 had no effect on CBF, while acidic solutions with a pH < 7.0 attenuated ciliary beating. Similar results were found when cell cultures were exposed to SO_2, making the bathing solutions extremely acidic (Kienast *et al.* 1994). In another study, pH of the medium between 6.5 and 7.5 did not influence CBF (Ingels *et al.* 1991). It remains unclear, however, by how much extracellular solutions actually changed pH_i in any of these experiments. Changes of mammalian CBF due to pH_i would not only affect cilia during the breathing cycle but also during exacerbations of airway diseases with airway acidification (e.g. asthma).

Surprisingly little information is available on pH-induced changes in ciliary/flagellar beat frequency in non-mammalian systems. Reactivation of isolated newt lung axonemes suggested a bell-shaped reactivation optimum at pH 7.0 and the absence of outer dynein arms, while influencing overall beating frequency, did not affect the bell-shaped pH responsiveness (Hard *et al.* 1992). However, studies on demembranated sperm suggested that mild alkalization increased flagellar beat frequency (Gibbons & Gibbons, 1972; Brokaw & Kamiya, 1987; Keskes *et al.* 1998) with the exception of one study using high Ca^{2+} concentrations (Ho *et al.* 2002). Human spermatozoa lacking outer dynein arms, the arms that mainly determine ciliary frequency (Brokaw & Kamiya, 1987), failed to show higher beat frequency during mild alkalization (Keskes *et al.* 1998), suggesting that,

 DOI: 10.1113/jphysiol.2004.068171

Table 1. Composition of solutions

Solution	1	2	3	4	5	6	7	8	9
Solution name	Standard Hepes	NH_4Cl-Hepes	High K^+ Hepes	Ca^{2+}-free Hepes	Ca^{2+}-free NH_4Cl-Hepes	25 mM gluconate Hepes	25 mM HCO_3^-	Standard internal	Standard internal w/o ATP
NaCl	142	132	17.3	142	132	117	117	10	10
NH_4Cl	—	10	—	—	10	—	—	—	—
KCl	5.3	5.3	130	5.3	5.3	5.3	5.3	—	—
Na-gluconate	—	—	—	—	—	25	—	—	—
$NaHCO_3$	—	—	—	—	—	—	25	—	—
K-gluconate	—	—	—	—	—	—	—	140	150
$CaCl_2$	1.3	1.3	1.3	—	—	1.3	1.3	0.33†	0.1
$MgCl_2$	0.5	0.5	0.5	0.5*	0.5*	0.5	0.5	—	—
$MgSO_4$	0.4	0.4	0.4	0.46*	0.46*	0.4	0.4	—	—
Na_2HPO_4	0.3	0.3	0.3	0.3	0.3	0.3	0.3	—	—
KH_2PO_4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	—	—
Glucose	5.6	5.6	5.6	5.6	5.6	5.6	5.6	—	—
Hepes	10	10	20	10	10	10	—	20	20
Nigericin	—	—	0.015	—	—	—	—	—	—
EGTA	—	—	—	1	1	—	—	—	—
MgATP	—	—	—	—	—	—	—	5	—
CPK	—	—	—	—	—	—	—	50 U ml^{-1}	—
CrP	—	—	—	—	—	—	—	10	—
Equilibrated with	air	air	air	air	air	air	5% CO_2/ 95% O_2	air	air
pH ‡	7.4	7.4	6.8–7.2–7.5–7.8 (adjusted with KOH)	7.4	7.4	7.4	7.4 (adjusted with $NaHCO_3$)	6.8–7.2–7.6–8.0 (adjusted with KOH)	7.2 (adjusted with KOH)

All concentrations are given in mM unless otherwise indicated. *Approximate free [Mg^{2+}] after chelation by EGTA is 0.9 mM: †approximate free [Ca^{2+}] after chelation by ATP is 0.1 mM: ‡pH was adjusted with concentrated NaOH solution unless otherwise indicated. CPK, creatine phosphokinase; CrP, creatine phosphate disodium salt.

in contrast to newt lung cilia (Hard *et al.* 1992), outer dynein arms are involved in the human flagellar response to changing pH_i.

Hypothetically pH changes could have direct effects on the outer dynein arm or influence the activity of axonemal kinases and phosphatases that are sensitive to pH (Cox & Taylor, 1995; Reddy *et al.* 1998). Of particular interest is the cAMP-dependent protein kinase (PKA), an important regulator of mammalian CBF (Wyatt *et al.* 1998; Lieb *et al.* 2002; Zagoory *et al.* 2002), and phosphatases shown to control ciliary beating in protozoa (Klumpp *et al.* 1990; Momayezi *et al.* 1996; Noguchi *et al.* 2003; Deckman & Pennock, 2004). Another important regulator of CBF, $[Ca^{2+}]_i$, was also found to be regulated by pH_i in several cell types (Thomas *et al.* 1979; Browning & Wilkins, 2002).

Thus, the purpose of this study was to define the extent and mode of pH_i action on CBF of human tracheobronchial epithelial cells. Our results suggest that pH_i between 6.8 and 8.0 influences ciliary beating perhaps directly at the axonemal level as pH-mediated CBF changes did not seem to be mediated via kinase/phosphatase systems or $[Ca^{2+}]_i$.

Methods

Chemicals

LHC basal medium, Trace elements 100 ×, Stock 4100 ×, and Stock 11 100 × were purchased from Biosource International (Rockville, MD, USA); Ham's nutrient F-12 and gentamicin from Gibco BRL Laboratories (Grand Island, NY, USA); the acetoxymethyl ester form of the pH-sensitive dye BCECF and fura-2 from Molecular Probes (Eugene, OR, USA); nigericin from Molecular Probes (Eugene, OR, USA) and Calbiochem (La Jolla, CA, USA); thapsigargin and H-89 from Calbiochem (La Jolla, CA, USA); cyclosporin A from Fluka (Buchs, Switzerland); and okadaic acid from Research Biochemicals International (Natick, MA, USA). All other reagents were from Sigma Chemicals (St Louis, MO, USA).

Solutions

Table 1 lists the compositions of solutions used. The free Ca^{2+} and Mg^{2+} concentration of EGTA- and ATP-containing solutions was estimated using

WebMAXC Standard software by Chris Patton from Stanford University, available at http://www.stanford.edu/~cpatton/webmaxcS.htm (constants used: CMC1002. TCM).

Preparation of submerged tracheal epithelial cultures

Primary cultures of tracheal epithelial cells were prepared as previously described (Salathe & Bookman, 1995). Human tissue was obtained from organ donors whose lungs were rejected for transplant through the Life Alliance Organ Recovery Agency of the University of Miami. IRB-approved consents for use of these tissues for research were obtained by the Life Alliance Organ Recovery Agency. Cells from these cultures were used for measurements within 6 days after plating.

Preparation of air liquid interface cultures of tracheal epithelium

Human air–liquid interface (ALI) cultures were prepared according to published methods (Adler *et al.* 1990; Bernacki *et al.* 1999; Nlend *et al.* 2002), except that the cells were plated onto 24 mm diameter, 3 μm pore-sized Transwell collagen-coated inserts (Corning Costar Corporation, Cambridge, MA, USA). The ALI cultures were used for measurements after the cells fully re-differentiated (about 6–8 weeks).

Selective permeabilization of the basolateral membrane of cells grown at the ALI

The basolateral surface of the ALI culture was exposed to 10 000 U ml^{-1} *Staphylococcus aureus* alpha-toxin dissolved in solution 8 (Table 1) for 30 min at room temperature. Solutions 8 and 9 were composed to reflect physiological intracellular K^+, Na^+ and Cl^- concentrations. Similar to the experience of others (Ostedgaard *et al.* 1992), however, we had to use 100 μM Ca^{2+} to avoid cell detachment.

Measurement of CBF

Cells grown on coverslips in submerged culture were mounted at room temperature onto the stage of a Nikon Eclipse E600FN upright water-immersion lens microscope in an open (Warner Instrument RC-25F with a working volume of 150 μl) or closed (Warner Instrument RC-21BR, working volume of 260 μl) perfusion chamber. They were perfused constantly. Ciliated cells were imaged with infrared differential interference contrast (DIC) optics with an optical gain of 600 ×. For online CBF measurements, the light path was directed to a CCD video camera (XC-7500 Sony) and a box of 3 × 3 pixels from the live, digitized, contrast-enhanced video image was selected (where one pixel samples an area of 180 nm × 180 nm). The magnitude spectra from a fast Fourier transform (FFT) of each of the pixel's intensity signals were computed online and displayed on the monitor for immediate adjustments. The intensity signals were recorded and later used for analysis according to published methods (Salathe & Bookman, 1999) using a sliding FFT window approach (128 frames per FFT, sliding the FFT window through the data set by 100 frames at a time), providing a frequency resolution of at least 0.23 Hz and a time resolution of ~3 s. The individual FFT magnitude spectra were peak extracted for graph production (Salathe & Bookman, 1999).

In order to measure CBF in cells grown at the air–liquid interface (but imaged with the apical surface 'submerged'), we used a holding chamber for the Transwell membranes allowing selective perfusion of the apical and basolateral sides of the cells. Data acquisition and processing was identical to the one described for submerged cultures.

Measurement of pH_i

Coverslips without a confluent monolayer (usually 1 day after plating) were preferred for fluorescent measurement of pH_i, because these ciliated cells could be more easily calibrated for pH_i using nigericin (see below). Coverslips were rinsed in solution 1 (Table 1) and loaded with 2.5 μM BCECF-AM in solution 1 at 37°C for 15–30 min and again rinsed 3 times. For fluorescent measurements, a Lambda DG4 excitation system (Sutter, Novato, CA, USA) was used with 10 nm wide excitation filters centred on 495 and 440 nm (Chroma Technology Corp., Brattleboro, VT, USA). 'Ratio-tool' software from Isee Imaging (Raleigh, NC, USA) controlled the output of the lambda DG4. Ratiometric pH estimates were made by capturing the light (535 nm) emitted from the cells through a 60 × water immersion objective (Nikon Inc.) and directing it to a cooled CCD camera (CoolSnap Hq, Photometrics, Tucson, AZ, USA). Individual ciliated cells were identified as regions of interest (ROIs) and the BCECF ratio of emission intensity after excitation at 495 and 440 nm was computed within each ROI every 10–60 s on a pixel-by-pixel basis (after background fluorescence subtraction).

Calibration of pH_i measurements

Preparation of the pH calibration curve. Nigericin was used to calibrate pH_i measurements (Thomas *et al.* 1979). BCECF-loaded cells were perfused with calibration solutions containing 15 μM nigericin and 130 mM KCl at pH 6.8, 7.2, 7.5 and 7.8, respectively (solution 3, Table 1) while the fluorescence ratio was measured. The ratio data were normalized to the ratio at pH 7.2 (normalized fluorescence ratio: NFR; Fig. 1*A*). Each ratio value was accepted when the ratio reached a steady state level at a given calibration pH. Then, the calibration curve was constructed by plotting the average NFR values from 47 cells (5 different organ donors) against the corresponding pH values. The correlation between NFR and pH values

was determined by linear regression analysis ($r^2 > 0.99$, Fig. 1*B*) (Boyarsky *et al.* 1988; Osypiw *et al.* 1994; Paradiso, 1997; Evans *et al.* 2003).

pH_i estimation. A one-point nigericin calibration was performed at the end of each experiment exposing the cells to the calibration solution with a pH of 7.2 (Boyarsky *et al.* 1988). NFR values were calculated by dividing the ratio data from the entire experiment by the ratio obtained in calibration solution at pH 7.2. NFR values were transformed to pH_i by interpolation with the calibration plot.

Nigericin was delivered to the cells via separate tubing. After each experiment with nigericin, the mounting chamber and the manifold were washed with 100% ethanol and distilled water to avoid nigericin-induced changes in pH_i in the following experiments (Richmond & Vaughan-Jones, 1997; Bevensee *et al.* 1999).

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured with the same equipment used for pH_i. Cells grown on coverslips were loaded with 5 μM fura-2 AM in solution 1 (Table 1) for 60 min at room temperature and were washed 3 times with solution 1. Cells were excited through 10 nm wide excitation filters centred on 340 and 380 nm (Chroma Technology Corp., Brattleboro, VT, USA), and the emitted light was captured at 535 nm. Fura-2 ratios were computed every 10–20 s, after background subtraction. Calibration of the calcium signal was done with *in vitro* measurements as described (Salathe & Bookman, 1995) according to Grynkiewicz *et al.* (1985); however, we re-estimated $[Ca^{2+}]_i$ by adjusting fura-2 K_d values with changing pH (Lattanzio & Bartschat, 1991; Browning & Wilkins, 2002) as described below.

Simultaneous measurement of CBF and pH_i or $[Ca^{2+}]_i$

By using a dual-image module and guiding the infrared signal for CBF measurements to the XC-7500 Sony CCD camera while sending all fluorescence signals (< 580 nm) to the cooled CCD camera, we were able to measure recordings of CBF and fluorescence (i.e. pH_i or $[Ca^{2+}]_i$) of the same single cell simultaneously.

Experimental procedures

Three different methods were used to manipulate pH_i: (a) ammonium pre-pulse; (b) removal of CO_2 from the extracellular medium; and (c) permeabilization of the basolateral membrane with the pore-forming agent alpha-toxin (from *Staphylococcus aureus*). For method (a), bicarbonate-free solutions were used to avoid interference with soluble adenylyl cyclase, an enzyme activated by bicarbonate and possibly expressed in these cells (Chen *et al.* 2000; Litvin *et al.* 2003). All coverslips and ALI cultures used in these experiments were washed with bicarbonate-free solution, mounted into an open chamber and exposed to ambient air for at least 30 min before experiments to remove any remaining bicarbonate and CO_2 from the cells. Measurements in open chambers were performed during continuous perfusion with the desired solution at a flow rate of 250 μl min^{-1} using a Harvard pump. After changing from one solution to another, flow rate was increased to 1000 μl min^{-1} for 1 min to accelerate the full exchange of the bathing solution (NH_4Cl pre-pulse was applied for 2 min at 1000 μl min^{-1}). These changes in flow rates did not influence CBF in control experiments, similar to our earlier findings (Lieb *et al.* 2002). For method (b), cells were loaded with BCECF in CO_2/HCO_3^--buffered solution at 5% ambient CO_2 and then mounted into a closed chamber with delivery of all solutions via a closed system. Cells were perfused at a constant flow rate of 1000 μl min^{-1}. Finally for method (c), ALI cultures were mounted into an open chamber with separate perfusion ports for the apical and basolateral side, maintaining perfusion at 500 and 1000 μl min^{-1} for the apical and basolateral side, respectively. Up to six cells per coverslip or ALI filter were measured in each experiment.

Statistics

All experimental data points were compared with date- and culture-matched controls. Two groups were compared using Student's unpaired *t* test, while Tukey-Kramer honestly significant difference test was used for comparison of more than two groups if an analysis of variance indicated a significant difference between groups

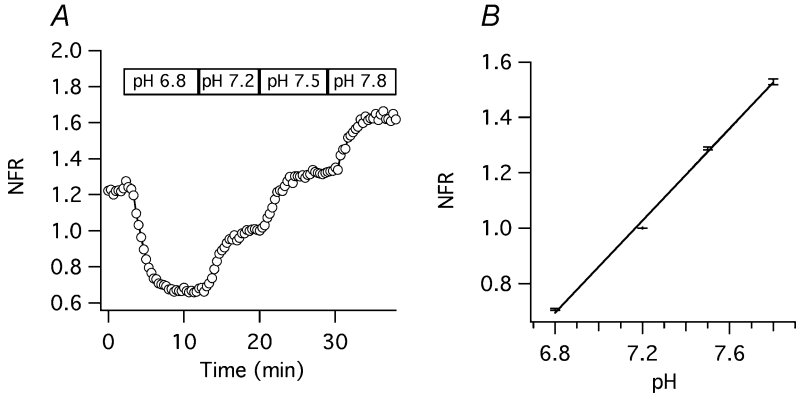

Figure 1. Intracellular calibration of BCECF using nigericin
A, a record of normalized fluorescence ratio (NFR) from a single, submerged, BCECF-loaded ciliated cell. The cell was first perfused with standard Hepes-buffered solution (solution 1, Table 1), which was switched to solutions containing 130 mm K^+ and 15 μm nigericin (solution 3, Table 1), buffered at the indicated pH. Each fluorescence ratio value (I_{495}/I_{440}) was divided by the ratio obtained at pH 7.2 to obtain the NFR. *B*, pH_i dependence of NFR. NFR data were obtained from 47 cells. Linear regression is shown with an $r^2 > 0.99$.

(JMP software, SAS Institute Inc., Cary, NC, USA). A $P < 0.05$ was considered to be significant. Data were expressed as means ± S.E.M.

Results

The effect of changing pH_i on CBF during ammonium pre-pulse

The ammonium pre-pulse technique was used to achieve a change in pH_i of intact cells without changing the pH of the bathing solution (pH_o). Baseline CBF was 7.2 ± 0.2 Hz and baseline pH was 7.49 ± 0.02 ($n = 63$ cells from 7 different organ donors). When the ammonium-free standard Hepes solution (solution 1, Table 1) was rapidly switched to one containing 10 mM NH_4Cl (10 mM NaCl was replaced by 10 mM NH_4Cl, solution 2, Table 1), CBF and pH increased at the same rate, within the time resolution of the measurements (10 s for pH_i in these experiments). CBF reached a maximum of 9.4 ± 0.2 Hz (31 ± 1% above baseline; $P < 0.001$) within 52 ± 7 s, whereas pH reached a maximum of 7.78 ± 0.02 ($P < 0.0001$ compared with baseline) within 48 ± 6 s. Two minutes after the initial change to ammonium chloride, the medium was switched back to the standard Hepes solution. After removing extracellular NH_4^+, a rapid intracellular acidification took place and CBF and pH rapidly decreased to 5.8 ± 0.2 Hz (19 ± 2% below original baseline) and 7.24 ± 0.02, respectively.

Figure 2*A* and *B* shows two representative, simultaneous recordings of CBF and pH_i during and after ammonium chloride exposure. To better visualize the correlation between pH_i and CBF, these two parameters were plotted against each other in panels *C–E*. During the recovery from acid load, CBF either increased together with pH_i (Fig. 2*A* and 35 of 63 cells), or remained at a low level

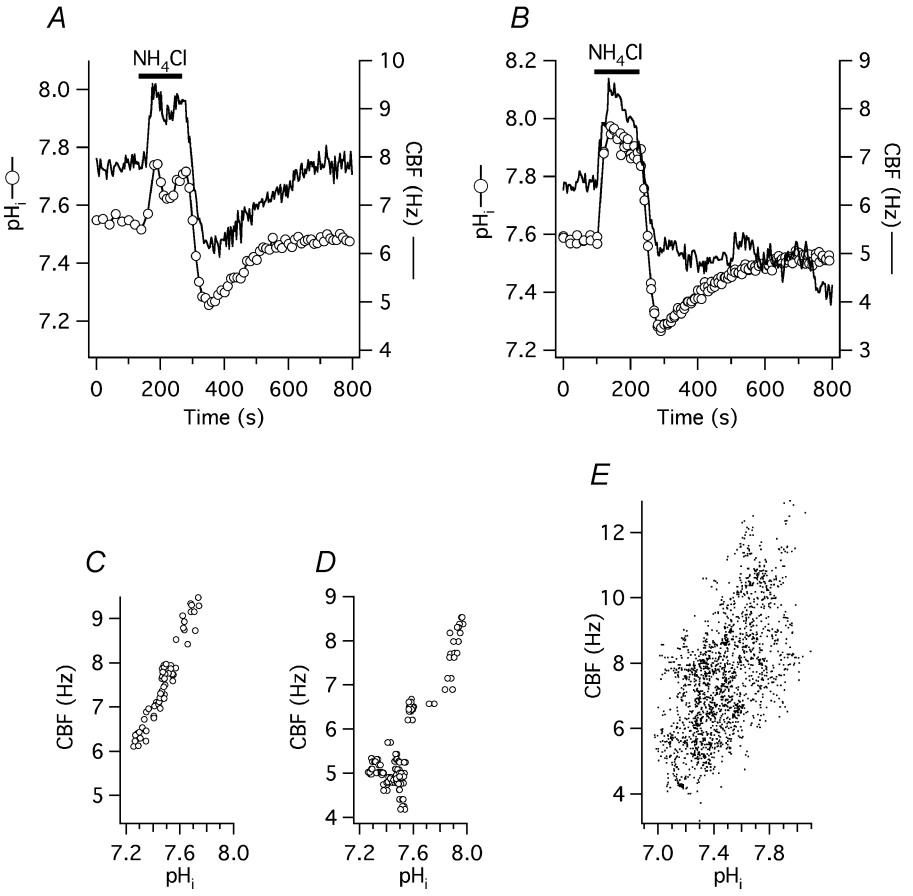

Figure 2. CBF responses to changing pH_i during ammonium pre-pulse
A, simultaneous CBF and pH_i recording from a single, submerged, BCECF-loaded human tracheobronchial epithelial cell perfused with standard Hepes-buffered solution (solution 1, Table 1). Intracellular alkalization and acidification by transient exposure to 10 mM NH_4Cl (solution 2, Table 1) results in pH_i-coupled changes in CBF. During recovery from acid load, CBF again parallels pH_i. *B*, traces from an experiment identical to panel *A*, but recorded from a different cell. Here, CBF fails to follow pH_i during recovery from intracellular acid load. *C*, correlation between CBF and pH_i plotted from the experiment shown in panel *A*. *D*, correlation between CBF and pH_i plotted from the experiment shown in panel *B*. *E*, correlation between CBF and pH_i plotted from 59 cells transiently exposed to 10 mM NH_4Cl.

during the rest of the experiment (Fig. 2*B*). In the 35 cells with recovering CBF, baseline CBF and pH_i were 7.5 ± 0.2 Hz and 7.45 ± 0.03, respectively, not significantly different from the non-recovering 28 cells (6.9 ± 0.2 Hz and 7.53 ± 0.03, both $P > 0.05$). However, the maximum pH_i following ammonium load was significantly higher in the latter group (7.84 ± 0.03) compared with the recovering cells (7.72 ± 0.03). The CBF peaks in both groups after ammonium load were not significantly different (9.7 ± 0.3 Hz in the recovering group *versus* 9.1 ± 0.3 Hz in the non-recovering group). During the following acidification phase, pH_i in non-recovering cells was found to be significantly higher than in cells that recovered their CBF (7.27 ± 0.03 *versus* 7.20 ± 0.02, $P < 0.05$); on the other hand, CBF at pH_i nadir was significantly higher in the recovering group (6.1 ± 0.2 Hz) compared with the non-recovering group (5.4 ± 0.2 Hz). Since the decrease of CBF during intracellular acidification was not significantly different between the two groups, it is possible that a too severe alkalization was responsible for the failure of these cells to recover their CBF after an acid load. This notion was supported by the fact that a lower pH could be reached in many other cells without any ill effects on CBF. However, other factors play a role as in other groups of cells, high pH_i did not seem to influence the ability of CBF to recover after acidification (see inhibitor experiments below).

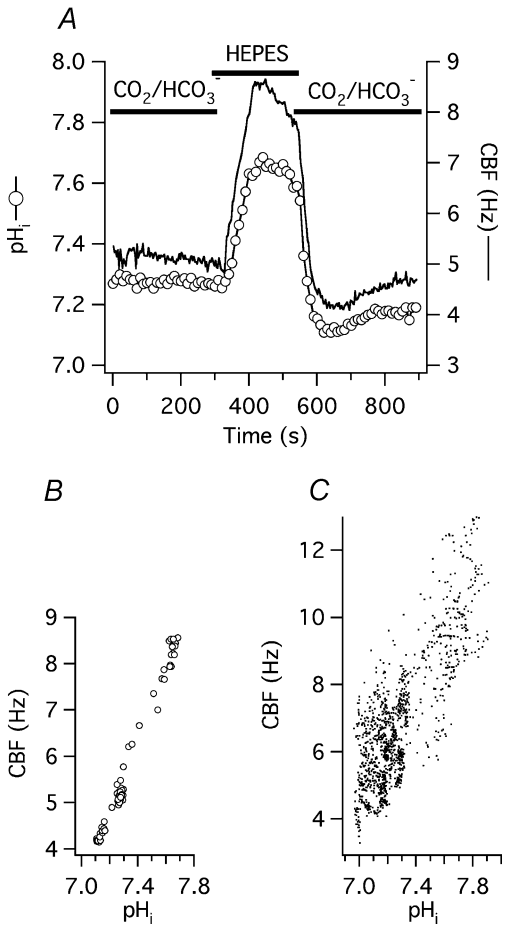

Figure 3. CBF responses to changing pH_i during removal of external CO_2
A, simultaneous CBF and pH_i recording from a single, submerged, BCECF-loaded human tracheobronchial epithelial cell, mounted into a closed chamber and perfused with CO_2/HCO_3^--buffered solution (solution 7, Table 1) in exchange with CO_2/HCO_3^--free, Hepes-buffered solution (solution 6, Table 1). *B,* correlation between CBF and pH_i plotted from the experiment shown in panel *A*. *C*, correlation between CBF and pH_i plotted from 13 cells during and after removal of external CO_2 identical to the experiment shown in panel *A*.

The effect of changing pH_i on CBF during removal of extracellular CO_2

An additional way to change pH_i without changing pH_o was to remove extracellular CO_2 (Thomas, 1984; Willumsen & Boucher, 1992; Paradiso *et al.* 2003). For this purpose, cells in closed chambers were continuously perfused with CO_2/HCO_3^--buffered solution, $pH_o = 7.4$ (25 mM NaCl of the standard Hepes solution was replaced with 25 mM $NaHCO_3$ following equilibration with 5% CO_2–95% O_2, pH 7.4, no Hepes, solution 7, Table 1). Baseline pH_i in this group of cells was 7.24 ± 0.02 ($n = 13$ cells from two donors), significantly lower than in cells bathed in Hepes-buffered, nominally CO_2/HCO_3^--free solution (7.49 ± 0.02, see above; $P < 0.0001$). Similarly, baseline CBF was significantly lower in CO_2/HCO_3^--buffered than in Hepes-buffered solution (6.1 ± 0.3 *versus* 7.2 ± 0.2 Hz, $P < 0.005$). When the CO_2/HCO_3^--buffered solution was switched to a nominally CO_2/HCO_3^--free buffer of the same pH (25 mM $NaHCO_3$ was replaced by equimolar sodium gluconate, buffered with 10 mM Hepes; solution 6, Table 1), a rapid increase in pH_i to 7.75 ± 0.03 was seen ($P < 0.0001$). When the CO_2/HCO_3^--buffered medium was reapplied and extracellular CO_2 entered the cell forming carbonic acid, pH_i fell below the original baseline to 7.04 ± 0.02 ($P < 0.0001$ compared with both baseline and peak pH_i), followed by a slow pH_i recovery. Similar to the ammonium pre-pulse experiments, CBF followed the changes in pH_i, increasing from a baseline of 6.1 ± 0.3 Hz to 10.3 ± 0.5 Hz (or $71 \pm 6\%$ above baseline; $P < 0.0001$) and falling back to 5.7 ± 0.3 Hz ($7 \pm 3\%$ below original baseline; $P < 0.0001$ compared with the peak CBF, but not significantly different from baseline). The time course of the intracellular alkalization and the parallel CBF increase was again within the resolution of the pH_i measurement (10 s): pH_i reached its maximum within 92 ± 6 s and CBF within 106 ± 13 s. Figure 3*A* shows a representative experiment.

Even though removal of external CO_2 both increased CBF and pH_i significantly more than the alkalization phase of ammonium pre-pulse, the ratio of these two parameters ($\Delta CBF/\Delta pH_i$) did not differ between the two methods ($8.4 \pm 0.6\ s^{-1}$ (pH unit)$^{-1}$ *versus* $7.7 \pm 0.4\ s^{-1}$ (pH unit)$^{-1}$

in the CO_2 and NH_4Cl group, respectively; $P > 0.05$). To better visualize the correlation between pH_i and CBF, these two parameters were again plotted against each other (Fig. 3*B* and *C*).

Inhibition and stimulation of PKA does not prevent the effect of pH_i on CBF

Kinases, specifically cAMP-dependent kinase (PKA), are important regulators of mammalian airway ciliary beating. Since the activity of kinase/phosphatase systems has been reported to depend on pH (Cox & Taylor, 1995; Reddy *et al.* 1998), H-7, a broad based serine–threonine kinase inhibitor, was used to inhibit PKA.

Cells were incubated with 100 μM H-7 for at least 20 min (estimated K_i of H-7 for PKA, PKG and PKC is 3.0, 5.8 and 6.0 μM, respectively, Hidaka *et al.* 1984) until a steady state CBF was achieved. Baseline CBF and pH_i in the H-7 group was 7.2 ± 0.3 Hz and 7.45 ± 0.08, respectively ($n = 10$ cells from one donor), not significantly different from date- and culture-matched controls (6.4 ± 0.4 Hz, 7.56 ± 0.08 pH units, $n = 6$, both $P > 0.05$). Following H-7 exposure, CBF invariably decreased but average CBF was not significantly below original baseline after 20 min of H-7 exposure. Cells were exposed to 10 mM NH_4Cl (solution 2, Table 1) in the continuous presence of H-7. During alkalization, pH_i increased significantly to 7.82 ± 0.11 and 7.83 ± 0.07 in the H-7 and control group, respectively ($P > 0.05$). CBF also increased significantly in both groups: to 9.5 ± 0.4 Hz (or $49 \pm 3\%$ above H-7 baseline) in the H-7 group and to 8.7 ± 0.9 Hz (or $36 \pm 6\%$ above baseline) in the control group ($P > 0.05$). After removing external ammonium chloride, pH_i fell in both groups: to 7.30 ± 0.04 in H-7-exposed and to 7.34 ± 0.06 in control cells ($P > 0.05$). Similarly, CBF decreased to the same extent in the H-7 and control groups, falling to 6.1 ± 0.3 Hz (or $96 \pm 2\%$ of the H-7 baseline) and to 5.8 ± 0.4 Hz (or to $91 \pm 2\%$ of the baseline; $P > 0.05$), respectively. A representative experiment is shown in Fig. 4*A*.

The efficacy of PKA inhibition by H-7 was confirmed in experiments using 1 μM forskolin to stimulate CBF, where H-7 (100 μM) significantly attenuated forskolin-induced stimulation of CBF. CBF increased by only 1.6 ± 0.2 Hz (or $31 \pm 3\%$) above baseline in H-7 pre-treated cells compared with 4.8 ± 0.5 Hz (or $86 \pm 11\%$) in control cells (all $n = 6$; $P < 0.001$ for both the absolute and percentage values).

These data were confirmed with a more specific inhibitor of PKA, namely 10 μM H-89 (Davies *et al.* 2000). The reported K_i values for PKA, CaM kinase II and PKC are 48 nM, 29.7 mM, and 31.7 mM, respectively. Cells were

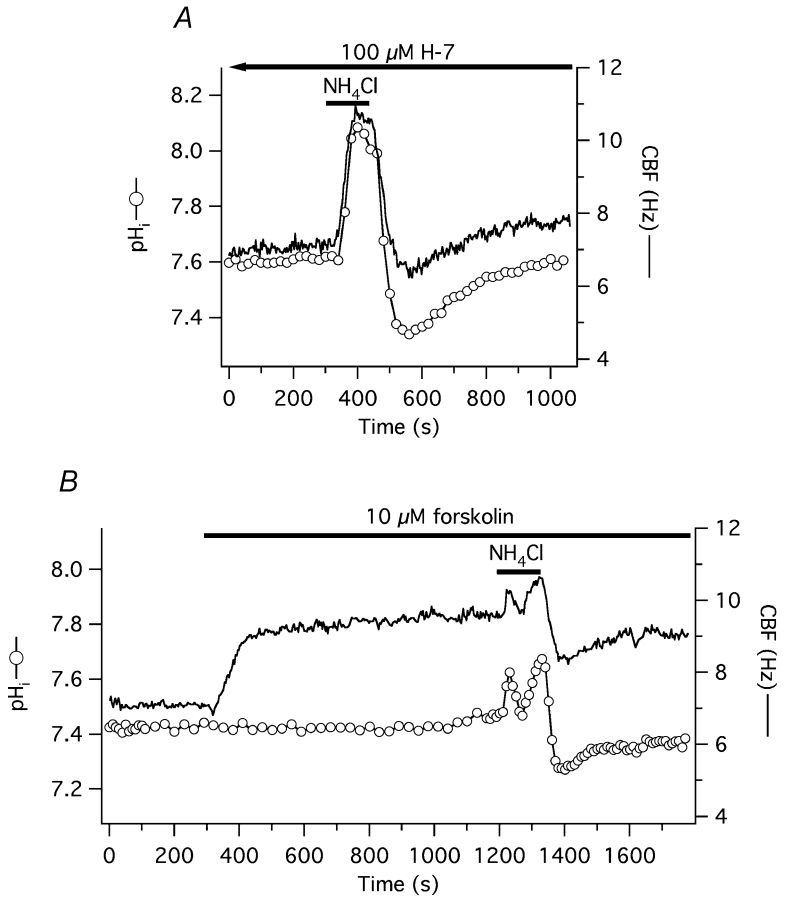

Figure 4. No effect of PKA inhibition or activation on CBF responses to changing pH_i during ammonium pre-pulse
A, simultaneous CBF and pH_i recording from a single, submerged, BCECF-loaded human tracheobronchial epithelial cell pre-treated and continuously perfused with 100 μM H-7 and transiently exposed to 10 mM NH_4Cl. Neither the CBF nor the pH_i responses differed from control cells (see Fig. 2). *B*, simultaneous CBF and pH_i recording from a single, submerged, BCECF-loaded human tracheobronchial epithelial cell continuously exposed to 10 μM forskolin followed by a transient exposure to 10 mM NH_4Cl.

pre-incubated with H-89 for 20 min. As with H-7, H-89 did not influence the pH-mediated changes in CBF during ammonium pre-pulse experiments ($n = 4$) compared with date- and culture-matched control cells ($n = 6$). H-89 also inhibited the CBF increase upon exposure of cells to 10 μM forskolin ($n = 7$ for H-89/forskolin and $n = 8$ for forskolin control).

The possible role of PKA in mediating pH_i-induced changes in CBF was also tested by fully stimulating the enzyme with 10 μM forskolin prior to applying the ammonium pre-pulse. Forskolin was continuously present during and after the ammonium exposure. Baseline CBF and pH_i in the forskolin group before stimulation was 6.9 ± 0.4 Hz and 7.61 ± 0.07, respectively ($n = 15$ cells from 3 donors), not significantly different from controls (6.4 ± 0.5 Hz, 7.52 ± 0.04 pH units, $n = 14$). Following forskolin stimulation, CBF significantly increased by $42 \pm 4\%$ above baseline (up to 9.7 ± 0.5 Hz) and pH_i remained unchanged (7.63 ± 0.08). During the alkalizing phase of the ammonium pre-pulse, pH_i peaked at 7.79 ± 0.10 and 7.78 ± 0.03 in the forskolin and the control group, respectively (not significantly different from each other, but $P < 0.05$ compared with the baseline in both groups). In the forskolin group, peak CBF was $60 \pm 6\%$ above the original baseline (absolute CBF value was 10.9 ± 0.6 Hz) and $13 \pm 4\%$ above the post-forskolin plateau. The percentage change during alkalization of the control group cells was $30 \pm 3\%$ above baseline (absolute CBF value: 8.3 ± 0.3 Hz, significantly lower than peak CBF in the forskolin pre-treated cells, $P < 0.001$). These results show that even if PKA is already stimulated, increasing pH_i still further stimulates ciliary beating. During acidification, pH_i fell to 7.39 ± 0.07 in the forskolin pre-treated cells and to 7.26 ± 0.04 in the control cells (no significant difference between the two groups). As expected, CBF in the forskolin-stimulated cells was still $22 \pm 4\%$ above the original baseline (absolute value: 8.4 ± 0.5 Hz) while the beating frequency in the control group was $32 \pm 4\%$ below baseline (absolute value: 4.3 ± 0.2 Hz; $P < 0.0001$; Fig. 4*B*).

Inhibition of protein phosphatases does not influence the effect of pH_i on CBF

To inhibit protein phosphatases, a combination of inhibitors was used: 10 μM cyclosporin A (inhibitor of calcineurin, a type 2B protein phosphatase, at an $IC_{50} = 5$ nM, Cohen *et al.* 1989) and 1.5 μM okadaic acid (inhibitor of type 1 at an $IC_{50} = 15$–20 nM and type 2A protein phosphatase at an $IC_{50} = 0.1$ nM, Cohen *et al.* 1989). Baseline CBF and pH_i before application of the inhibitors was 6.7 ± 0.2 Hz and 7.46 ± 0.03, respectively ($n = 11$ cells from 2 donors), not significantly different from the date- and culture-matched controls (7.0 ± 0.3 Hz, 7.57 ± 0.06, $n = 10$). Cells in the phosphatase inhibitor group were perfused with the inhibitors for 17 min, resulting in a statistically insignificant CBF decrease (6.0 ± 0.3 Hz) and unchanged pH_i (7.47 ± 0.02). During the alkalization phase of the ammonium pre-pulse, both pH_i and CBF increased to the same level in both groups, significantly above baseline. Maximum pH_i was 7.86 ± 0.01 in inhibitor-treated cells and 7.89 ± 0.06 in control cells ($P > 0.05$), while maximum CBF was 8.3 ± 0.3 Hz ($40 \pm 4\%$ above post-inhibitor plateau frequency) in the inhibitor group and 9.4 ± 0.5 Hz (or $34 \pm 3\%$ above baseline) in the control group ($P > 0.05$ for both percentage and absolute changes). The nadir of pH_i and CBF (as percentage decrease from baseline) during acidification did not differ between the treated and control groups: pH_i was 7.20 ± 0.03 and 7.27 ± 0.06, while CBF fell to $90 \pm 3\%$ of the CBF plateau reached after adding inhibitors and to $89 \pm 3\%$ of the baseline in the two groups, respectively. A representative experiment is shown in Fig. 5.

pH_i does not regulate CBF via changes in $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ plays a crucial role in regulating CBF (e.g. Salathe & Bookman, 1995, 1999; Salathe *et al.* 1997; Ma *et al.* 2002; Zagoory *et al.* 2002). It has been shown in several cell types that intracellular alkalization can increase, while intracellular acidification can decrease $[Ca^{2+}]_i$ via

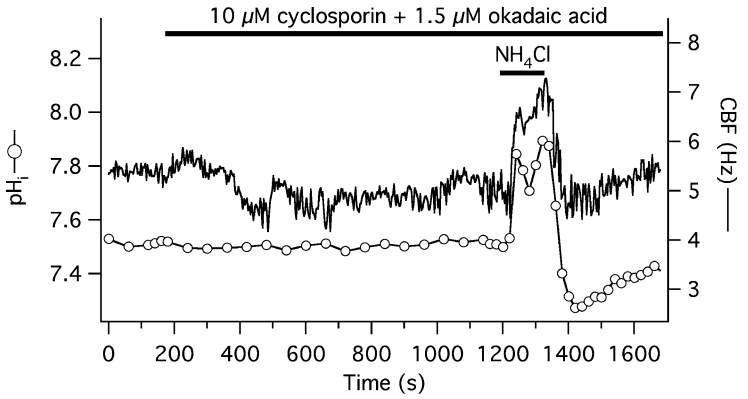

Figure 5. CBF responses to changing pH_i during ammonium pre-pulse – lack of effect by phosphatase inhibition
Simultaneous CBF and pH_i recording from a single, submerged, BCECF-loaded human tracheobronchial epithelial cell perfused with 10 μM cyclosporine A plus 1.5 μM okadaic acid and transiently exposed to 10 mM NH_4Cl.

regulation of Ca^{2+} release from intracellular stores (Thomas *et al.* 1979; Browning & Wilkins, 2002). We investigated whether CBF regulation by pH_i depends on $[Ca^{2+}]_i$.

First, $[Ca^{2+}]_i$ and CBF from the same single cells were measured simultaneously during ammonium pre-pulse (see Methods). The estimated baseline $[Ca^{2+}]_i$ of cells perfused with standard Hepes-buffered solution (solution 1, Table 1) was 47.5 ± 10.3 nM, baseline CBF was 8.0 ± 0.3 Hz ($n = 18$ from 1 donor). Two minutes after starting the ammonium load (solution 2, Table 1), CBF increased as expected and significantly to 10.9 ± 0.4 Hz ($37 \pm 3\%$ above the baseline). Estimated $[Ca^{2+}]_i$, however, did not change significantly (46.1 ± 7.6 nM). After the ammonium pre-pulse was completed and CBF returned to baseline, P2Y receptors were stimulated with 10 μM ATP for 1 min. Exposure to ATP resulted in a significant increase in both estimated $[Ca^{2+}]_i$ (peak: 234.3 ± 36.4 nM) and CBF (peak: 12.6 ± 0.3 Hz or $61 \pm 7\%$ above baseline; see examples in Fig. 6*A* and *B*). To estimate $[Ca^{2+}]_i$, constant K_d (400 nM, Browning & Wilkins, 2002), R_{max}, R_{min} and β-values were used according to Grynkiewicz *et al.* (1985) using *in vitro* calibration procedures as outlined in material and methods. However, the affinity of fura-2 for Ca^{2+} has been reported to increase with increasing pH (i.e. K_d decreases at alkaline pH). In addition, R_{max} as well as β were found to be pH dependent (Lattanzio & Bartschat, 1991; Browning & Wilkins, 2002). Therefore, R_{max}, R_{min} and β were measured *in vitro* in the pH range of 7.0–8.0. The changes in R_{min} and β were negligible. The average maximum intracellular alkalization during ammonium pre-pulse in our hands was ~0.30 pH units (from 7.50 to 7.80, see above). Based on our *in vitro* estimations and an approximate 12.5% decrease in K_d (400 nM to 350 nM) based on the study by Browning & Wilkins (2002), these changes would result in a 17% decrease in estimated $[Ca^{2+}]_i$. Thus, increases in $[Ca^{2+}]_i$ are over-estimated and decreases are under-estimated by the ratio data upon alkalization.

To further show that changes in $[Ca^{2+}]_i$ were not involved in the pH_i-mediated changes in CBF, intracellular Ca^{2+} stores were emptied with 1 μM thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase (Thastrup *et al.* 1990). Upon thapsigargin exposure, baseline CBF of 6.4 ± 0.6 Hz ($n = 8$ cells from 3 donors) quickly increased and then declined until reaching a plateau at 8.2 ± 0.5 Hz or $34.5 \pm 16.3\%$ above baseline ($P > 0.05$; Fig. 6*C*). Three minutes before an ammonium load, extracellular Ca^{2+} was removed (solution 4, Table 1) and CBF decreased to 6.8 ± 0.6 Hz. Longer Ca^{2+}-free medium exposure resulted in cell detachment. After 3 min in calcium-free, EGTA-buffered solution (nominal 0 $[Ca^{2+}]_o$), cells were exposed to 10 mM NH_4Cl (solution 5, Table 1) containing 1 μM thapsigargin, resulting in a $28 \pm 3\%$ acceleration of ciliary beating above pre-NH_4Cl baseline (8.6 ± 0.7 Hz), a value not significantly different from the $39 \pm 5\%$ increase from baseline of 6.3 ± 0.5 Hz to 8.7 ± 0.6 Hz seen in date-matched control cells

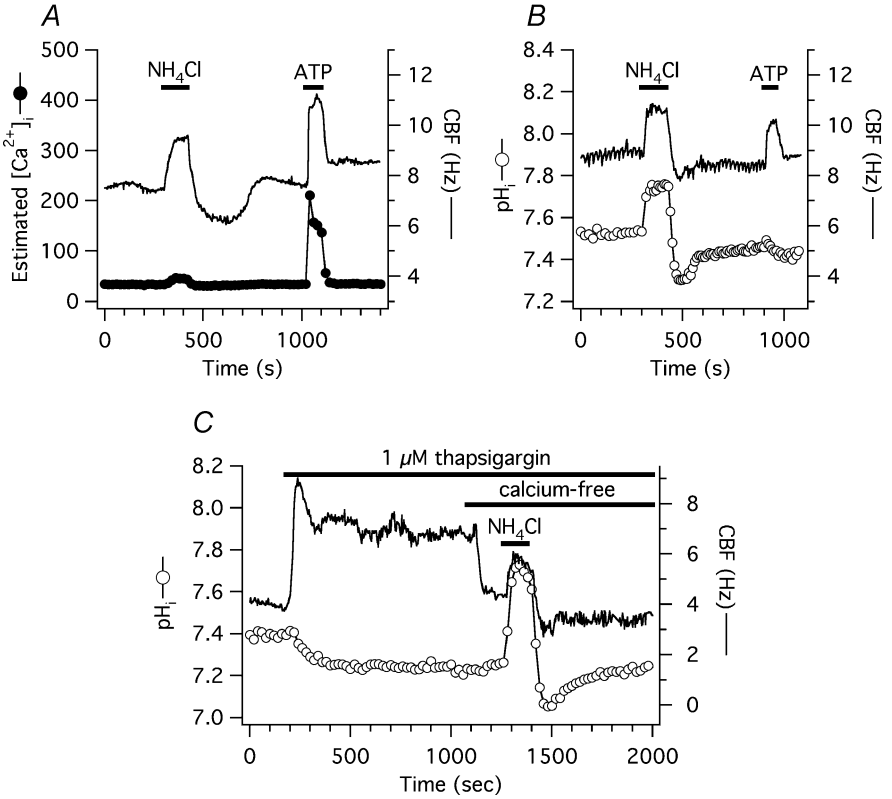

Figure 6. pH_i-mediated changes in CBF are not due to $[Ca^{2+}]_i$ variations
A, simultaneous CBF and pH_i recording from a single, submerged, fura-2-loaded human tracheobronchial epithelial cell, transiently exposed to 10 mM NH_4Cl. Ammonium pre-pulse had a negligible effect on estimated $[Ca^{2+}]_i$, even if corrected for the pH-related changes in K_d after NH_4Cl exposure. Purinergic stimulation with 10 μM ATP, however, resulted in an transient increase of both estimated $[Ca^{2+}]_i$ and CBF. *B,* experiment shown in panel *A* was repeated in cells loaded with BCECF instead of fura-2. NH_4Cl exposure caused a significant change in pH_i, while ATP did not. *C*, simultaneous CBF and pH_i recording from a single, submerged, BCECF-loaded human tracheobronchial epithelial cell, transiently exposed to 1 μM thapsigargin and nominally calcium free solution (solution 4, Table 1) before transient exposure to 10 mM NH_4Cl. pH_i-mediated CBF changes were not different from control cells.

($n = 5$), neither exposed to thapsigargin nor calcium-free extracellular solution. Intracellular acid load after NH_4Cl removal significantly decreased CBF in both groups. Here, however, CBF of cells in nominally free calcium solutions decreased significantly more than CBF of control cells: CBF decreased $22 \pm 3\%$ below pre-ammonium challenge baseline to 5.3 ± 0.5 Hz *versus* $5 \pm 2\%$ to 6.0 ± 0.5 Hz, respectively ($P < 0.05$ for percentage values; $P > 0.05$ for the absolute values). Baseline pH_i was 7.38 ± 0.04 before ammonium exposure in cells bathed in nominally free calcium solutions *versus* 7.48 ± 0.04 baseline in control cells ($P > 0.05$). pH_i peaks were the same in both groups: 7.77 ± 0.04 in nominally free calcium solutions *versus* 7.77 ± 0.05 in controls ($P > 0.05$); pH_i nadir was 7.20 ± 0.04 *versus* 7.27 ± 0.1, respectively ($P > 0.05$).

Basolaterally permeabilized cells grown at the ALI

To control the cytoplasmic environment and to test whether the changes in CBF seen in intact cells were directly related to changes in pH, the basolateral membrane of human tracheobronchial epithelial cells re-differentiated at the ALI was selectively permeabilized with *Staphylococcus aureus* alpha-toxin as described in Methods. *Staphylococcus aureus* alpha-toxin makes the membrane permeable to molecules smaller than 5 kDa, including nucleotides (Bhakdi & Tranum-Jensen, 1991; Ostedgaard *et al.* 1992; Walev *et al.* 1993; Jonas *et al.* 1994; Reddy & Quinton, 1994; Bhakdi *et al.* 1996; Detimary *et al.* 1996; Watanabe & Takano-Ohmuro, 2002). To evaluate the efficacy of membrane permeabilization, the basolateral (i.e. intracellular) solution containing 5 mM MgATP and an ATP regenerating system (ARS, 50 U ml^{-1} creatine phosphokinase, 10 mM creatine phosphate disodium salt, solution 8 at pH 7.2, Table 1, Kakuta *et al.* 1985) was exchanged by a solution without ATP and ARS (solution 9, Table 1). Seven minutes after the removal of this system, CBF fell from a baseline of 6.7 ± 0.5 Hz by 4.2 ± 0.4 Hz (or by $62 \pm 3\%$; $P < 0.0001$ for both absolute and percentage changes, $n = 10$ cells from 2 donors). These results suggested that the basolateral membrane was permeable to ATP, even though the cilia were able to maintain a low beating frequency of 2.5 ± 0.2 Hz (Fig. 7*A*). CBF returned to the original baseline when the ATP–ARS-containing basolateral solution was reapplied. Baseline CBF of non-permeabilized cells was 11.6 ± 0.6 Hz (significantly higher than that of permeabilized cells; $P < 0.0001$) and did not change upon removal of ATP–ARS from the basolateral solution (10.4 ± 0.4 Hz, $P > 0.05$, $n = 4$; Fig. 7*B*).

When alpha-toxin was applied apically (in the same solution used for the basolateral permeabilization; solution 8, Table 1) while the basolateral membrane was perfused with the standard Hepes-buffered solution (solution 1, Table 1), removal of apical ATP–ARS (by changing to solution 9, Table 1) did not change CBF significantly. These results show that alpha-toxin permeabilizes specifically the basolateral membrane as previously reported in an epithelial cell line (Ostedgaard *et al.* 1992).

Correlation between pH_i and CBF in permeabilized cells

After selective permeabilization of the basolateral membrane, the basal compartment of the ALI chamber was perfused with solutions titrated to pH 6.8, 7.2, 7.6 and 8.0 (solution 8, Table 1; see example in Fig. 8*A*). CBF was 3.9 ± 0.3, 5.7 ± 0.4, 7.0 ± 0.3 and 7.3 ± 0.3 Hz at pH 6.8, 7.2, 7.6 and 8.0, respectively ($n = 18$ each from 2 different organ donors; Fig. 8*B*). All these values were significantly different from each other except those measured at pH 7.6 and 8.0, suggesting that intracellular alkalization had a ceiling effect on stimulating CBF in permeabilized cells.

Discussion

Previous studies showed that extracellular alkaline solutions up to pH 9–10.5 had no effect on mammalian airway CBF, while extracellular acidic solutions attenuated

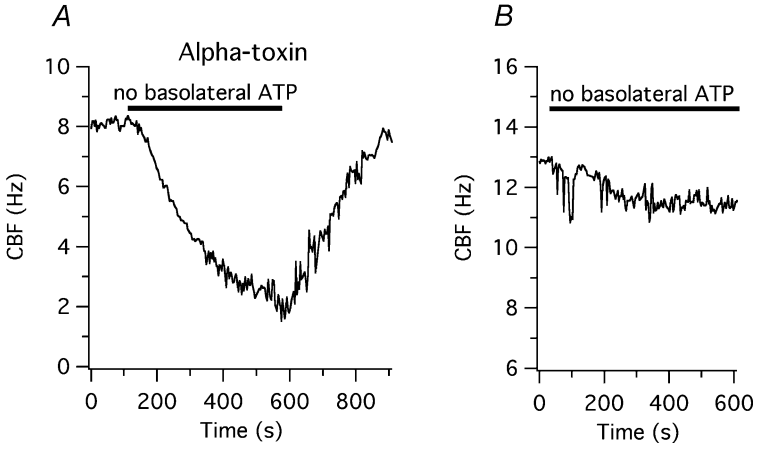

Figure 7. Basolateral permeabilization of human tracheobronchial epithelial cells re-differentiated at the air–liquid interface with alpha-toxin
A, the basolateral surface of human airway epithelial cells grown and re-differentiated on 3 μm pore-sized inserts was permeabilized as described in Methods. Removal of ATP and the ATP-regenerating system (50 U ml^{-1} creatine phosphokinase, 10 mM creatine phosphate) caused a reversible CBF decrease. *B*, when re-differentiated cells were not permeabilized, removal of ATP and the ATP-regenerating system did not change CBF significantly.

ciliary beating (van de Donk *et al.* 1980; Luk & Dulfano, 1983; Clary-Meinesz *et al.* 1998). However, the relationship between *intracellular* pH and CBF has been unclear. Here we demonstrate that relatively small changes in pH_i result in significant changes in ciliary beating of human tracheobronchial epithelial cells. The observed changes in ciliary beating are large enough to alter a more macroscopic physiological parameter, i.e. mucociliary clearance: Seybold *et al.* (1990), for instance, found that a 16% increase in CBF can lead to a 56% increase in mucociliary transport velocity in isolated whole tracheas.

Our experiments suggest that changing pH_i may directly act on the ciliary motile machinery, possibly the outer dynein arm. The exact mechanism of how pH_i changes CBF remains unclear. One possibility is a change in the charge of histidine, possibly affecting dynein ATPase or a dynein light chain that influences dynein ATPase activity. To our knowledge, no data are available from the literature on the former hypothesis. However, a dynein light chain conformation change has been reported to depend on pH-induced changes in histidine ionization (Barbar *et al.* 2001). This dynein light chain, LC8, has been originally found on outer dynein arms (Piperno & Luck, 1979), has been reported to be important for flagellar beating and can be found as a dimer or monomer, depending on pH-induced histidine ionization (Barbar *et al.* 2001). Thus, although completely speculative, histidine charge changes on the outer dynein arm could be important in pH-dependent modulation of CBF.

The baseline pH_i of human airway tracheobronchial ciliated cells submerged in nominally CO_2/HCO_3^--free solutions (pH adjusted to 7.4) at room temperature was surprisingly high at 7.49 ± 0.02. Poulsen & Machen (1996), however, reported a similarly high pH_i (7.41 ± 0.09) in bovine tracheal epithelial cells bathed in CO_2/HCO_3^--free buffers. Similar pH_i values were reported: 7.44 ± 0.01 in rat cardiac myocytes (Evans *et al.* 2003), 7.40 ± 0.02 in rat distal colon (Dagher *et al.* 1994), 7.42 ± 0.02 in rat ileum villus cells (Dagher *et al.* 1997), and 7.67 ± 0.05 in rat ileum crypt cells (Dagher *et al.* 1997). On the other hand, the pH_i of human nasal airway epithelial cells bathed in CO_2/HCO_3^--free medium reported by another group was considerably less at 7.08–7.16 (Willumsen & Boucher, 1992; Paradiso, 1997; Paradiso *et al.* 2003). The reasons for these differences are not clear, but could be due to calibration issues, differences in experimental temperatures (Roos & Boron, 1981), disparate cell types and culturing methods (polarized cell culture). Our results using CO_2/HCO_3^- -buffered solutions, however, agree with the published data: baseline pH_i was 7.24 ± 0.02 in our experiments, 0.24 pH units lower than in nominally CO_2/HCO_3^--free solution (Paradiso *et al.* 2003). The pH_i changes in response to ammonium pre-pulse were similar to those reported by others (Boyarsky *et al.* 1988; Singh *et al.* 1995; Paradiso, 1997; Ramirez *et al.* 2000; Brett *et al.* 2002). Although both the basolateral and apical membrane was available for NH_4Cl in our submerged culture experiments, the shape of the pH_i plot depicted in Fig. 2*A* and *B* suggested that the apical membrane responses were predominant (Willumsen & Boucher, 1992; Boron *et al.* 1994; Singh *et al.* 1995; Paradiso, 1997).

Our experiments in intact human tracheobronchial epithelial cells revealed a close kinetic relationship between pH_i and CBF in the examined pH range of 7.0–7.8 (Figs 2 and 3). The changes occurred coincidentally at the beginning of the alkalization and acidification phase of ammonium pre-pulse or external CO_2 removal, i.e. within the time resolution of the CBF measurements (3 s) and pH recordings (10–20 s).

The mode of pH_i action on CBF is not clear and the possibility with histidine charge changes discussed above remains purely speculative. However, several possibilities could be excluded. Activation of soluble adenylyl cyclase (sAC), an enzyme distinct from the transmembrane adenylyl cyclase class by being insensitive to forskolin but activated by HCO_3^- (Chen *et al.* 2000; Litvin *et al.* 2003), was one possibility that was excluded. Although we have preliminary data from RT-PCR reactions suggesting that sAC is expressed in human tracheobronchial epithelial cells, the exact cellular distribution and localization of sAC in the airway epithelium is not known. However,

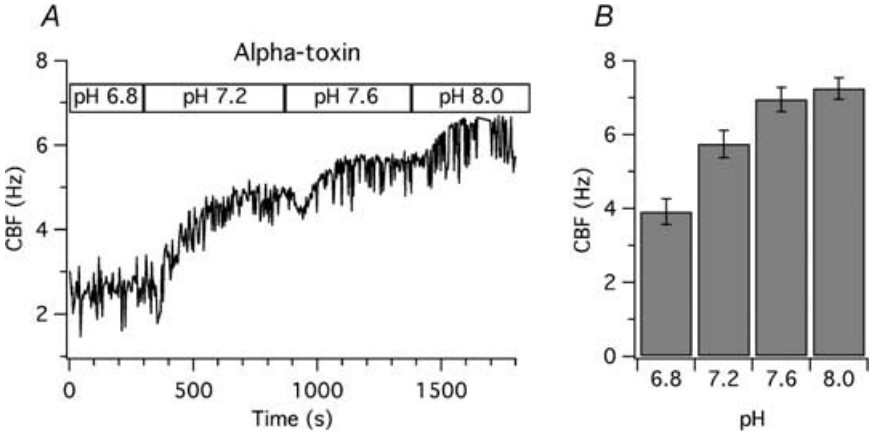

Figure 8. Correlation between pH_i and CBF in basolaterally permeabilized human tracheobronchial epithelial cells
A, representative measurement from a single re-differentiated cell permeabilized with alpha-toxin and basolaterally exposed to different solutions equilibrated at the shown pH. Increasing basolateral (i.e. intracellular) pH resulted in a CBF increase. *B,* data obtained as in panel *A*, but summarized for 18 measured cells. Values are the mean ± S.E.M.

all coverslips and ALI cultures in this study, except those used for the CO_2 removal experiments, were perfused with CO_2/HCO_3^--free solutions and exposed to ambient air for at least 30 min before experimentation. Therefore, the intracellular $[HCO_3^-]$ is expected to approach zero in these cells and changes in pH_i should not have been able to change sAC activity via its HCO_3^- sensitivity.

Protein kinase/phosphatase systems could also be targets of pH_i changes since the catalytic efficiency of PKA is optimal at near neutral pH and is inhibited at acidic pH (Cox & Taylor, 1995). Acidic pH not only inhibits PKA but also activates phosphatases to de-phosphorylate PKA targets, while alkaline pH has the opposite effect on both PKA and phosphatases (Reddy *et al.* 1998). However, neither inhibition of protein kinases with H-7, PKA with H-89 nor pre-stimulation of PKA by the transmembrane adenylyl cyclase activator forskolin could attenuate CBF responses to changing pH_i. The same concentrations of the kinase inhibitors H-7 and H-89 significantly attenuated the CBF-stimulatory effect of forskolin, showing the efficacy of these agents. The use of H-7 as a broad inhibitor also excluded other kinases relevant for CBF (e.g. PKC). Similarly, pre-treatment with a mixture of two phosphatase inhibitors failed to show any significant difference in the coupling of CBF and pH_i (Figs 4 and 5). Although it is more difficult to test the efficacy of phosphatase inhibition, the combination of two inhibitors at the concentrations used (at 100, 3000 and 2000 times the estimated IC_{50} for phosphatase 1, 2A and 2B, respectively) was probably sufficient to inhibit these enzymes.

Another important mediator of CBF regulation, $[Ca^{2+}]_i$, was also reported to be influenced by pH_i in several cell types (Thomas *et al.* 1979; Browning & Wilkins, 2002). However, we could not modify the coupling of pH_i and CBF by emptying internal Ca^{2+} stores with thapsigargin and inhibiting the influx of external Ca^{2+} using nominally Ca^{2+}-free bathing solutions (Fig. 6*C*). Simultaneous measurements of $[Ca^{2+}]_i$ and CBF during ammonium pre-pulse failed to show significant changes in estimated $[Ca^{2+}]_i$ when CBF changed (Fig. 6*A*), even when the estimated $[Ca^{2+}]_i$ was corrected for changes in the K_d of fura-2 due to pH (Lattanzio & Bartschat, 1991; Browning & Wilkins, 2002). Although measurement of the fura-2 fluorescence of the whole cell does not exclude the possibility of a localized rise in $[Ca^{2+}]$ in a subcellular compartment, this possibility is unlikely since the absence of calcium in intracellular stores as well as in the extracellular space did not change the results. The physicochemical effect of increasing pH to decrease the concentration of ionized calcium cannot account for the rise in CBF during alkalization as the opposite, namely a decrease in CBF, would have been expected.

Our experiments on permeabilized cells (Figs 7 and 8) further confirm that CBF is regulated by pH_i and rule out the possibility that CBF changes during ammonium pre-pulse were coupled to changes in extra- or intracellular NH_4^+ and/or NH_3 concentrations rather than to changes in pH_i. That ciliary beating was not completely abolished in our permeabilized cells after 7 min perfusion with ATP and ARS-free bathing solution could be due to the presence of non-diffusible pools of nucleotides (Malaisse & Sener, 1987; Aprille, 1988; Detimary *et al.* 1996) and by residual production of ATP close to cilia.

Together, these data suggest that phosphorylation/dephosphorylation events and changes in $[Ca^{2+}]_i$ are not responsible for the pH_i-mediated changes in CBF and support a direct action of pH_i on axonemal proteins. Studies performed on demembranated mammalian spermatozoa are of special interest in this regard because cilia and sperm flagella share considerable ultrastructural similarities as well as some similarities in their beat regulation (e.g. by PKA, for review see Urner & Sakkas, 2003). Most published experiments on demembranated spermatozoa demonstrate that mild alkalization augments flagellar beat frequency (FBF) of spermatozoa of different species (Gibbons & Gibbons, 1972; Brokaw & Kamiya, 1987; Keskes *et al.* 1998). One of these studies (Keskes *et al.* 1998) also showed that human spermatozoa that lack outer dynein arms failed to increase beat frequency during alkalization suggesting that outer dynein arms, that mainly determine the frequency of ciliary beating (Brokaw & Kamiya, 1987), might be directly involved in the response to changing pH_i. Whether the effects of pH on spermatozoa are mediated through the same mechanisms, remains unclear since some of the pH effects in spermatozoa could be due to the stimulation of sAC.

Our results show that intracellular alkalization results in faster ciliary beating, while intracellular acidification attenuates CBF in human tracheobronchial epithelial cells. Variations in pH_i of airway epithelia may occur *in vivo* in response to shifting luminal CO_2 concentrations from 5 to 0.02% during a full breathing cycle (Willumsen & Boucher, 1992). It is intriguing to speculate that a possible pH_i change during the breathing cycle might influence CBF in the large airways. If it occurs, it would result in a faster ciliary beating during inspiration. Furthermore, airway diseases associated with acidification of the surface liquid and possibly epithelium (e.g. asthma) could depress ciliary activity, possibly contributing to the decrease in mucociliary clearance seen in these diseases.

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Acknowledgements

This work was supported by grants from the American Heart Association (Postdoctoral Fellowship to Z.S.) and the NIH (HL-66125 to G.E.C., and HL-60644 and HL-67206 to M.S.).