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Polarized distribution of HCO_3^- transport in human normal and cystic fibrosis nasal epithelia

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The polarized distribution of HCO_3^- transport was investigated in human nasal epithelial cells from normal and cystic fibrosis (CF) tissues. To test for HCO_3^- transport via conductive *versus* electroneutral Cl^-/HCO_3^- exchange (anion exchange, AE) pathways, nasal cells were loaded with the pH probe 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein and mounted in a bilateral perfusion chamber. In normal, but not CF, epithelia, replacing mucosal Cl^- with gluconate caused intracellular pH (pH_i) to increase, and the initial rates (ΔpH min^{-1}) of this increase were modestly augmented (~26 %) when normal cells were pretreated with forskolin (10 μM). Recovery from this alkaline shift was dependent on mucosal Cl^-, was insensitive to the AE inhibitor 4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonic acid (H_2DIDS; 1.5 mM), but was sensitive to the cystic fibrosis transmembrane conductance regulator (CFTR) channel inhibitor diphenylamine-2-carboxylate (DPC; 100 μM). In contrast, removal of serosal Cl^- caused pH_i to alkalinize in both normal and CF epithelia. Recovery from this alkaline challenge was dependent on serosal Cl^- and blocked by H_2DIDS. Additional studies showed that serosally applied Ba^{2+} (5.0 mM) in normal, but not CF, cells induced influx of HCO_3^- across the apical membrane that was reversibly blocked by mucosal DPC. In a final series of studies, normal and CF cells acutely alkaline loaded by replacing bilateral Krebs bicarbonate Ringer (KBR) with Hepes-buffered Ringer solution exhibited basolateral, but not apical, recovery from an alkaline challenge that was dependent on Cl^-, independent of Na^+ and blocked by H_2DIDS. We conclude that: (1) normal, but not CF, nasal epithelia have a constitutively active DPC-sensitive HCO_3^- influx/efflux pathway across the apical membrane of cells, consistent with the movement of HCO_3^- via CFTR; and (2) both normal and CF nasal epithelia have Na^+-independent, H_2DIDS-sensitive AE at their basolateral domain.

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Studies of airway epithelial salt and water physiology have traditionally centred around measurements of Na^+ and Cl^- transport, and these studies have provided a large body of information about cellular signalling mechanisms important for regulating these ion transport processes and their protective functions in the respiratory tract. In contrast, fewer studies have focused on identifying the membrane elements important for the transport of H^+ and HCO_3^- (Nord *et al.* 1988; Willumsen & Boucher, 1992; Lubman *et al.* 1995; Paradiso, 1997), which are necessary for the maintenance of intracellular pH (pH_i) homeostasis. It is predicted that polarized airway epithelia distribute these mechanisms (apical/basolateral membrane) to establish salt and H^+ gradients between the cytosol and secreted liquids, important for the regulation of airway surface liquid (ASL) ionic composition and pH.

Since the recognition that the cystic fibrosis transmembrane conductance regulator (CFTR) functions as a Cl^- channel (Quinton, 1983), Cl^- transport activity by CFTR-expressing tissues has been well studied (Quinton, 1983, 1990; Willumsen *et al.* 1989; Widdicombe & Wine, 1991; Anderson *et al.* 1991; Linsdell *et al.* 1999). However, the mechanisms underlying HCO_3^- secretion in CFTR-expressing epithelia remain poorly understood, even though HCO_3^- transport activity is impaired in cystic fibrosis (CF; Kaiser & Drack, 1974; Smith & Welsh, 1992). Of relevance to ASL acid–base homeostasis, a role for CFTR as a HCO_3^- transporter has been suggested in CF airway models, based on a variety of technical approaches comparing wild-type CFTR-expressing cells with ΔF508 CFTR-expressing CF cells (Smith & Welsh, 1992; Illek *et al.* 1997) or comparing wild-type mice with CFTR knockout (−/−) mice (Grubb & Gabriel, 1997). These studies suggest the possibility that a defect in HCO_3^- secretion through CFTR may lead to abnormally acidic ASL pH and thus have the potential to contribute to the pathophysiology of CF airway disease.

In addition to conductive CFTR-mediated HCO_3^- secretion, it is well known that a major mechanism for HCO_3^- transport across plasma membranes is Na^+-

independent, electroneutral Cl^-/HCO_3^- exchange (anion exchange; AE). Both Cl^- and HCO_3^- can be transported via AE and the direction of transport is determined by the gradients of both anions across the plasma membrane. Under normal physiological conditions, the ratio of extracellular-to-intracellular Cl^- is greater than the inward-directed HCO_3^- gradient, and AE exchanges extracellular Cl^- for intracellular HCO_3^-. When pH_i is increased, i.e. when cell HCO_3^- increases, the inward-directed HCO_3^- gradient is reduced, thus further favouring efflux of HCO_3^- via electroneutral AE from the cell. Thus, AE functions as an 'acid-loader' by removing cell HCO_3^- during acute cellular alkalosis.

Recent studies have raised the possibility that CFTR and AE may exhibit a complex regulatory interaction when both proteins are co-localized in the same membrane. In this regard, Lee and co-workers (1999*b*) reported regulation of AE by CFTR expressed in NIH 3T3 and HEK 293 cells, as well as regulation of luminal AE by CFTR in mouse submandibular and pancreatic ducts (Lee *et al.* 1999*a*). In contrast to these studies, Mastrocola *et al.* (1998) reported that the transfection of wt CFTR, ΔF508 CFTR or vector did not influence AE activity in C129 cell lines. Whether such a relationship exists in airway epithelia will, in part, depend on determining whether CFTR and AE indeed co-localize at the apical barrier of airway epithelia. Utilizing RT-PCR, Dudeja and co-workers (1999) reported that the message for the AE2 and brain AE3 isoforms, but not AE1 or cardiac AE3 isoforms, was present in proximal human airways. Currently, however, no definitive data are available as to the apical/basolateral membrane distribution of any AE, or on the relative contributions of AE and CFTR on overall HCO_3^- fluxes in human normal and CF nasal epithelia.

Consequently, the aim of the present study was to investigate the polarized distribution (apical/basolateral membrane) of HCO_3^- transport in nasal epithelial cells in the presence and absence of functional CFTR. To this end, polarized normal and CF preparations were loaded with the pH-sensitive probe 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) to measure pH_i, mounted in a bilateral perfusion chamber, and the response of pH_i to ion substitution and inhibitor protocols employed to determine the relative roles of conductive, i.e. CFTR-mediated, *versus* AE-mediated HCO_3^- transport.

METHODS

Subjects

Primary human nasal epithelial tissues were obtained, with written consent, from 12 normal subjects (32 ± 5 years old; seven males, five females) undergoing elective surgery for standard medical indications (e.g. sleep apnoea secondary to nasal obstruction) and nine cystic fibrosis patients (17 ± 5 years old; five males, four females) undergoing polypectomy. All procedures were approved by the University of North Carolina Committee for the Rights of Human Subjects and conformed to the Declaration of Helsinki.

Chemicals and solutions

The acetoxymethyl ester of BCECF (BCECF/AM) and 4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonic acid (H_2DIDS) were purchased from Molecular Probes, Inc. (Eugene, OR, USA). Diphenylamine-2-carboxylate (DPC) was obtained from Research Biochemicals International (Natick, MA, USA). All other chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA).

The standard Krebs bicarbonate Ringer (KBR) solution contained (mM): 125 NaCl, 2.5 K_2HPO_4, 1.3 $CaCl_2$, 1.3 $MgCl_2$, 25 $NaHCO_3$ and 5 D-glucose (5 % CO_2/95 % O_2; pH 7.4). For Cl^--free KBR, NaCl and $MgCl_2$ were replaced mole-for-mole by sodium gluconate and $MgSO_4$, respectively, and $CaCl_2$ was replaced with 2 mM $CaSO_4$ to compensate for Ca^{2+} chelation by gluconate, as previously reported (Clarke & Boucher, 1992). The Hepes-buffered Na^+- and Cl^--free Ringer solution contained (mM): 150 *N*-methyl-D-glucamine (NMG) gluconate, 2.5 K_2HPO_4, 2.0 $CaSO_4$, 1.3 $MgSO_4$, 5 D-glucose and 10 Hepes (pH 7.4). For the Hepes-buffered Na^+-free Ringer solution containing Cl^-, the chemicals were identical to Na^+- and Cl^--free Ringer solution except that NMG gluconate was replaced mole-for-mole by NMGCl (pH 7.4).

Cell culture and perfusion chamber

Human nasal epithelial (HNE) cells were harvested from polyps by enzymatic digestion (Protease XIV (Sigma) for 24–48 h at 4 °C) as previously described (Wu *et al.* 1985). HNE cells were plated on porous Transwell Col filters (pore diameter = 0.40 μm; Costar, Corning, Inc., NY, USA) affixed to O-rings and maintained in serum-free Ham's F-12 medium supplemented with insulin (10 μg ml^{-1}), transferrin (5 μg ml^{-1}), triiodothyronine (3×10^{-8} M), endothelial cell growth supplement (3.75 μg ml^{-1}), hydrocortisone (5×10^{-9} M) and $CaCl_2$ (10^{-3} M). Polarized monolayers were studied 10–12 days after seeding, as previously described (Paradiso *et al.* 2001).

After achieving confluence, polarized monolayers of HNE were loaded with BCECF (5 μM at 37 °C for 25 min) and mounted in a miniature chamber over an objective (Zeiss LD Achroplan × 40, NA 0.6; working distance 1.8 mm) of a Zeiss Axiovert 35 microscope. The cell chamber and the method for independently perfusing the apical and basolateral membranes of cells have been previously described in detail (Paradiso *et al.* 2001).

Fluorimeter and measurements of pH_i

Measurements of pH_i in polarized HNE cells were obtained using a RatioMaster fluorimeter (Photon Technology International, Brunswick, NJ, USA) attached via fibre optics to the microscope. BCECF fluorescence from 30–40 cells (spot diameter ~65 μm) was acquired alternately at 440 and 490 nm (emission ⩾ 520 nm). At a given excitation wavelength (440 or 490 nm), background light levels were measured in non-loaded cells and subtracted from the corresponding signal measured in BCECF-loaded cells prior to taking the ratio (490/440). The corrected ratio was converted to pH_i as previously described (Paradiso, 1997).

Data analysis

Means ± S.E.M. were calculated from the total number of measurements for a given experimental condition. Statistical significance was determined using the Student's paired *t* test, with $P < 0.05$ being considered significant.

RESULTS

Basal pH_i in normal and CF HNE cells bilaterally perfused with nominally CO_2/HCO_3^--free Ringer solution or KBR

Employing H^+-sensitive microelectrodes, an earlier study by Willumsen & Boucher (1992) reported that basal pH_i was 7.1–7.15 in normal and CF HNE cells bilaterally perfused with KBR (5% CO_2/25 mM HCO_3^-, pH 7.4). They also reported that basal steady-state pH_i was not affected by the removal of HCO_3^- from the bathing medium, suggesting that HCO_3^- may not be important for the maintenance of steady-state basal pH_i.

To reassess the role of a CO_2/HCO_3^- buffer system on basal pH_i, we compared the effects of Hepes-buffered NaCl Ringer solution and KBR on basal pH_i in normal and CF HNE cells. As shown in Fig. 1, when normal (Fig. 1*A*, $n = 10$, four individuals) and CF (Fig. 1*B*, $n = 10$, four individuals) HNE cells were bilaterally exposed to Hepes-buffered NaCl Ringer (pH 7.4) solution, basal pH_i was ~7.1 in both cell preparations. When normal and CF cells were subsequently exposed to symmetrical KBR (Fig. 1*A* and *B*), pH_i first rapidly decreased (due to the hydration of CO_2) and then regulated back to a new steady-state basal pH_i, which was lower than the values measured in a Hepes-buffered Ringer solution, in both cell preparations. On average, in Hepes-buffered NaCl Ringer solution, basal pH_i was 7.16 ± 0.03 ($n = 17$; four individuals) and 7.13 ± 0.02 ($n = 17$; four individuals) in normal and CF HNE cells, respectively, consistent with our previous measurements of basal pH_i in Hepes-buffered Ringer solution (also refer to Table 1 of Paradiso, 1997). In contrast, in the presence of bilateral KBR, basal pH_i was 6.95 ± 0.02 ($n = 138$; twelve individuals) and 6.94 ± 0.01 ($n = 102$; nine individuals) in normal and CF HNE cells, respectively.

Our results demonstrate that steady-state basal pH_i in normal and CF HNE cells is ~0.15 pH units more acidic in KBR compared to Hepes-buffered NaCl Ringer solution, clearly suggesting a role for a CO_2/HCO_3^- buffer system for the maintenance of cell pH. This issue is discussed further below.

Effects of amiloride on pH_i in response to an CO_2/HCO_3^- challenge in normal and CF HNE cells

In nominally CO_2/HCO_3^--free NaCl Ringer solution, we have previously identified an amiloride-sensitive Na^+/H^+ exchanger that was restricted to the basolateral membrane in both normal and CF HNE cells (Paradiso, 1997). To extend these observations, we examined whether the Na^+/H^+ exchanger was the major transporter that could account for the recovery of pH_i during an acid challenge in a CO_2/HCO_3^- buffer system (see Fig. 1). As shown in Fig. 2, when normal (Fig. 2*A*, $n = 7$, three individuals) and CF (Fig. 2*B*, $n = 7$, three individuals) HNE cells were symmetrically perfused with Hepes-buffered NaCl Ringer solutions, the addition of amiloride (500 μM) to the serosal compartment elicited no change in basal pH_i. However, in the presence of serosal amiloride, when the perfusate was subsequently changed to bilateral KBR, pH_i rapidly decreased to ~6.6 and remained acidic in both normal and CF cell preparations; pH_i recovered towards a new steady-state basal level in both normal and CF HNE cells only when amiloride was removed from the serosal perfusate (Fig. 2).

The data thus far strongly suggest that the major membrane mechanism for the recovery of pH_i in response to an acid challenge in the presence (Fig. 2) and absence (Paradiso, 1997) of a CO_2/HCO_3^--buffered solution is a basolateral Na^+/H^+ exchanger in normal and CF HNE cells. Because amiloride completely and reversibly blocked

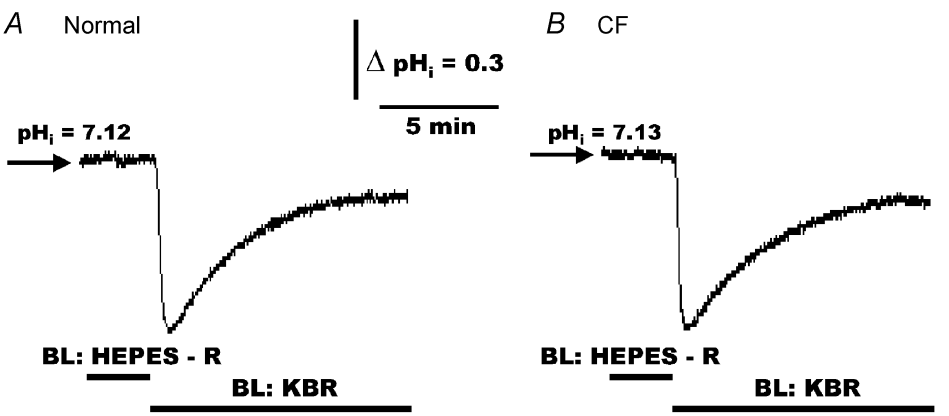

Figure 1. Basal intracellular pH (pH_i) in polarized monolayers of normal and cystic fibrosis (CF) human nasal epithelial (HNE) cells bilaterally perfused with nominally CO_2/HCO_3^--free Ringer solution or Krebs bicarbonate Ringer (KBR)

For both normal (*A*) and CF (*B*) cell preparations, HNE cells were initially bilaterally (BL) perfused with Hepes-buffered NaCl Ringer solution (pH 7.4) before changing to bilateral KBR (pH 7.4) at the times indicated in the tracings. Each trace is representative of ten separate experiments (four different individuals).

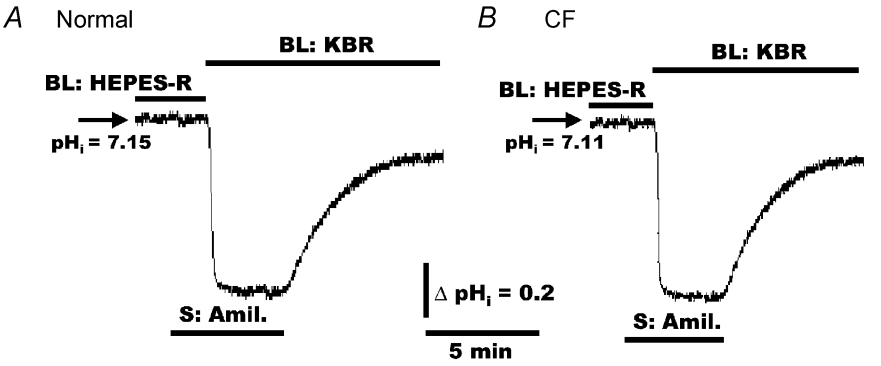

Figure 2. Effects of amiloride on pH$_i$ in response to a CO_2/HCO_3^- challenge in polarized monolayers of normal and CF HNE cells

For both normal (*A*) and CF (*B*) cell preparations, HNE cells were initially bilaterally perfused with Hepes-buffered NaCl Ringer solution (pH 7.4) before adding amiloride (Amil., 500 μM) to the serosal (S) perfusate. In the presence of amiloride, the perfusate was subsequently changed to bilateral KBR (pH 7.4) and amiloride removed from the serosal compartment at the times indicated in the tracings. Each trace is representative of seven separate experiments (three different individuals).

the recovery phase of the acid-load, a role for a Na^+–HCO_3^- cotransporter, i.e. the influx of Na^+ and HCO_3^- across the apical/basolateral membrane, is unlikely. Finally, the observation that amiloride added to the serosal perfusate induced no change of pH$_i$ in nominally CO_2/HCO_3^--free (Hepes-buffered) Ringer solution (Fig. 2) indicates that the basolateral Na^+/H^+ exchanger is inactive, i.e. there is no net efflux of H^+ coupled to influx of Na^+ via the basolateral Na^+/H^+ exchanger at a basal pH$_i$ $\geqslant$ 7.1, as previously reported in normal and CF HNE cells (Paradiso, 1997).

Acute effects of H_2DIDS and amiloride on basal pH$_i$

To identify the membrane transporters that acutely modulate pH$_i$ under basal conditions in a CO_2/HCO_3^- buffer system, we tested the effects of mucosal and serosal H_2DIDS (to block a putative AE) and amiloride (to inhibit the Na^+/H^+ exchanger) in normal and CF HNE cells bilaterally perfused with KBR.

As depicted in Fig. 3, exposing normal (Fig. 3*A*) or CF (Fig. 3*B*) cells to H_2DIDS (1.5 mM) added to the mucosal perfusate caused no changes in basal pH$_i$. In contrast,

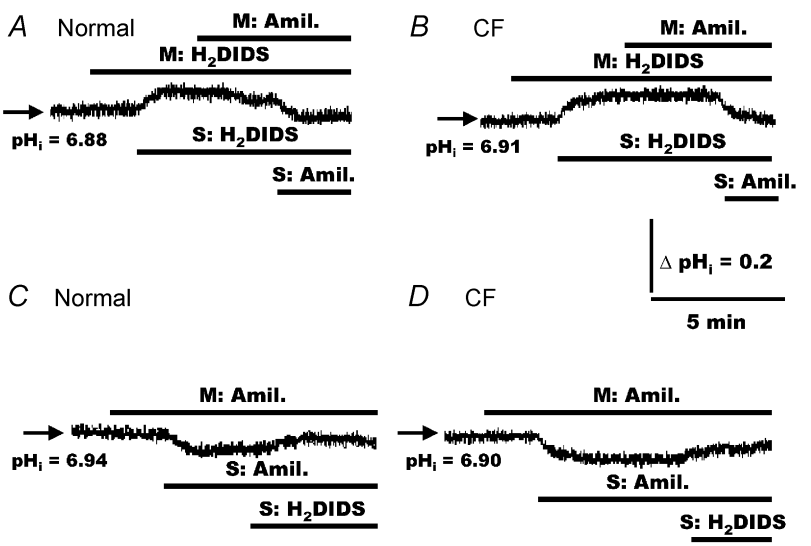

Figure 3. Response of pH$_i$ to mucosal and serosal additions of H_2DIDS and amiloride in polarized monolayers of normal and CF HNE cells

For both normal (*A* and *C*) and CF (*B* and *D*) cell preparations, HNE cells were bilaterally perfused with KBR before adding 4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonic acid (H_2DIDS; 1.5 mM) and amiloride (500 μM) to the mucosal (M) and serosal (S) compartments as indicated in the tracings. Each trace is representative of seven separate experiments (three different individuals).

serosally applied H_2DIDS elicited an alkalinization in both normal (Fig. 3*A*) and CF (Fig. 3*B*) HNE cells. On average, the magnitude of the increase (peak–basal value) of pH_i in response to serosal H_2DIDS was 0.10 ± 0.01 (n = 84, nine individuals) and 0.11 ± 0.01 (n = 68, nine individuals) in normal and CF HNE cells, respectively.

The increase in pH_i in response to H_2DIDS could potentially result from two sources: (i) the activity of a Na^+/H^+ exchanger during inhibition of AE and/or (ii) the cellular accumulation of HCO_3^- during the inhibition of a basolateral AE. To test whether Na^+/H^+ exchange contributed to the alkalinization of cells in response to H_2DIDS added to the serosal perfusate, we examined the response of pH_i to amiloride after H_2DIDS treatment. As shown in Fig. 3, the addition of amiloride (500 μM) to the mucosal perfusate failed to alter pH_i in either H_2DIDS-treated normal (Fig. 3*A*) or CF (Fig. 3*B*) HNE cells. In contrast to mucosal amiloride addition, serosally applied amiloride (500 μM) following H_2DIDS-elicited alkalinization induced a re-acidification of cell pH back to basal values in both normal (Fig. 3*A*) and CF (Fig. 3*B*) cell preparations, consistent with the inhibition of basolateral Na^+/H^+ exchange. Furthermore, under basal conditions, mucosal addition of amiloride again failed to alter pH_i, whereas the addition of amiloride to the serosal perfusate caused cell pH to decrease in both normal (Fig. 3*C*) and CF (Fig. 3*D*) HNE cells. As noted above, these data are consistent with our previous report that the Na^+/H^+ exchanger is restricted to the basolateral membrane in both normal and CF HNE cells (Paradiso, 1997). On average, the magnitude of the decrease of pH_i in response to serosal amiloride was 0.08 ± 0.02 (n = 7, three individuals) and 0.09 ± 0.01 (n = 7, three individuals) in normal and CF HNE cells, respectively.

The absolute change of pH_i in normal and CF cells in response to serosally applied amiloride was not significantly different from the increase in cell pH induced by the serosal addition of H_2DIDS, measured in both cell preparations. Furthermore, serosally applied H_2DIDS following serosal amiloride treatment caused only a small or no change of pH_i in both normal (Fig. 3*C*) and CF (Fig. 3*D*) HNE cells, consistent with little accumulation of HCO_3^- within cells under basal conditions during the short time period employed in these studies. Taken together, these results indicate that constitutively active basolateral, but not apical, H_2DIDS-sensitive AE and Na^+/H^+ exchange contribute to the acute regulation of pH_i in normal and CF airway epithelia under basal conditions in the presence of a CO_2/HCO_3^- buffer system.

Effects of mucosal Cl^- substitution on pH_i in normal and CF HNE cells

The results thus far indicate that normal and CF airway epithelia lack a H_2DIDS-sensitive pathway for modulating pH_i at their apical membrane (see Fig. 3), suggesting the absence of an apically located AE. To test this notion more rigorously, we investigated whether changes in the luminal Cl^- concentration could alter pH_i in normal and CF HNE cells. Our expectation was that if AE were present at the apical membrane of cells, then removal of luminal Cl^-, i.e. establishing a cell-to-lumen Cl^- gradient, should increase pH_i via a lumen-to-cell influx of HCO_3^-. For these studies, normal and CF HNE cells were perfused initially with bilateral KBR and effects of unilateral replacement of mucosal Cl^- with gluconate (constant 5% P_{CO_2}/25 mM HCO_3^-; pH 7.4) examined. As depicted in Fig. 4, removal of luminal Cl^- (serosal bath constant KBR, pH 7.4) induced a transient alkalinization that returned to baseline in normal HNE cells (Fig. 4*A*). The mean maximum change (peak–basal value) of pH_i elicited by this mucosal Cl^--free treatment was 0.18 ± 0.02 (n = 7, four individuals). In contrast, this manoeuvre failed to alter pH_i in CF airway epithelia (Fig. 4*B*; n = 8, three individuals).

Because HNE cells may have at their basolateral border an H_2DIDS-sensitive AE (see Fig. 3), it is possible that any Cl^- gradient imposed across the apical membrane was ultimately also imposed across the basolateral membrane by lowering intracellular Cl^-, resulting in an increase in the serosal-to-cell Cl^- gradient across the basolateral

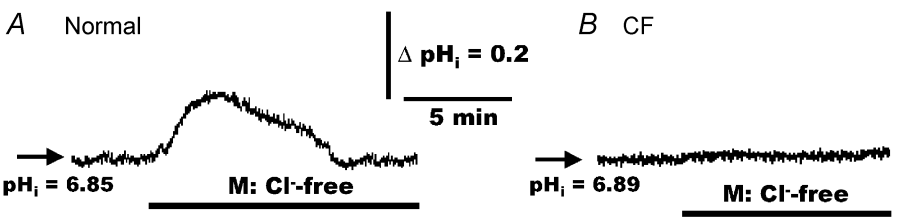

Figure 4. Effects of mucosal Cl^--free Ringer solution on pH_i in polarized monolayers of normal and CF HNE cells

For both normal (*A*) and CF (*B*) cell preparations, HNE cells were initially bilaterally perfused with KBR before changing to mucosal (M) Cl^--free (gluconate replacing Cl^-; constant 5% P_{CO_2}/25 mM HCO_3^-, pH 7.4) Ringer solution at the times indicated in the tracings. The serosal perfusate remained KBR throughout these studies. *A*, representative example of seven separate experiments (four different individuals). *B*, representative example of eight separate experiments (three different individuals).

membrane. For example, a change in pH_i due to an outward-directed (cell-to-lumen) Cl^- gradient coupled to an inward-directed (lumen-to-cell) HCO_3^- influx pathway may be reduced (shunted) if a dominant basolateral AE removes HCO_3^- entering the cell across the apical membrane. Therefore, to 'isolate' the influx of HCO_3^- across the apical membrane from the efflux of HCO_3^- across the basolateral membrane in response to changes in luminal Cl^- concentration, we added H_2DIDS to the serosal perfusate to block any compensatory effects of the putative basolateral AE on HCO_3^--dependent changes of pH_i. As shown in Fig. 5, in the presence of serosal H_2DIDS, replacing luminal Cl^- with gluconate (imposing a cell-to-lumen Cl^- gradient) alkalinized normal cells, consistent with significant lumen-to-cell HCO_3^- uptake in normal HNE cells (Fig. 5*A*). The maximum change of pH_i from steady-state level (induced by H_2DIDS) to peak value (elicited by Cl^- removal) was 0.38 ± 0.02 ($n = 11$, four individuals), which was significantly greater ($P < 0.01$) than the change in pH_i induced by luminal Cl^- removal in non-H_2DIDS-treated normal cells (i.e. 0.18 ± 0.02 pH units; see above). Furthermore, returning Cl^- to the luminal perfusate (increasing the lumen-to-cell Cl^- gradient) stimulated cells to re-acidify back to basal levels, consistent with cell-to-lumen HCO_3^- movement in normal airway epithelia (see Fig. 5*A*). In contrast, removal of luminal Cl^- in the presence of serosal H_2DIDS induced no detectable change of pH_i in CF airway cells (Fig. 5*B*; $n = 8$, three individuals). It should be noted that we have previously shown that these Cl^--linked changes of pH_i in normal (or CF HNE) cells are absent in a nominally CO_2/HCO_3^--free environment (see Fig. 3 of Paradiso, 1992), indicating that the responses of pH_i to altered luminal Cl^- concentrations reflect movement of HCO_3^- across the apical membrane in normal nasal cells.

Although the changes in cell pH shown in Fig. 5*A* in response to altering mucosal Cl^- could be interpreted as being consistent with the activity of AE at the apical membrane in normal HNE cells, Fig. 5*C* shows that these Cl^--dependent changes of pH_i were not blocked by mucosal H_2DIDS (1.5 mM). On average, in the presence of mucosal H_2DIDS, the magnitude of change of pH_i from steady-state level (induced by serosal H_2DIDS) to peak value obtained in luminal Cl^--free was 0.36 ± 0.03 ($n = 11$, four individuals), which was not significantly different from the mean value determined in the absence of mucosal H_2DIDS.

Effects of forskolin and DPC on pH_i in response to mucosal Cl– substitution in normal HNE cells

Three observations reported here and by others suggest that the change in pH_i in response to the removal/re-addition of luminal Cl^- may be mediated by the movement

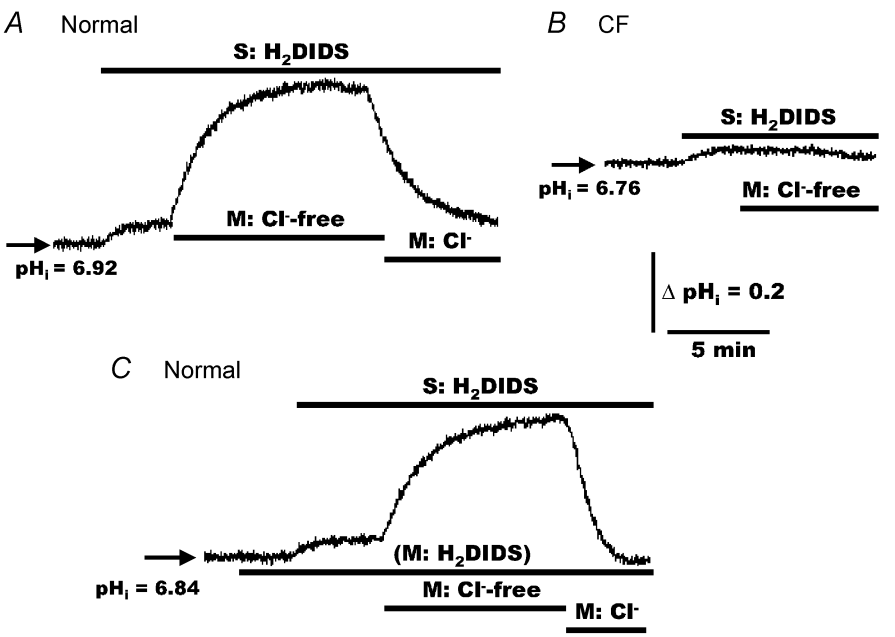

Figure 5. Effects of mucosal Cl^- substitution on pH_i in polarized monolayers of normal and CF HNE cells exposed to serosal and/or mucosal H_2DIDS

For both normal (*A* and *C*) and CF (*B*) HNE cell preparations, cells were initially perfused bilaterally with KBR and H_2DIDS (1.5 mM) added to the serosal and/or mucosal compartment before removing Cl^- (gluconate replacing Cl^-; constant 5% P_{CO_2}/25 mM HCO_3^-, pH 7.4) or re-adding Cl^- to the mucosal perfusate, as shown in the tracings. The serosal perfusate remained KBR throughout the study. *A* and *C*, representative examples of eleven separate experiments (four different individuals). *B*, is representative of eight separate experiments (three different individuals).

of HCO_3^- across the apical membrane via CFTR, which appears to be constitutively active in normal HNE cells. First, unilaterally replacing mucosal Cl^- with gluconate induced an alkalinization of pH_i under basal conditions in normal (Fig. 5*A*), but not CF (Fig. 5*B*), HNE cells. Second, removal of luminal Cl^- under basal conditions causes a major depolarization of the apical membrane electrical potential difference (V_a) in normal, but not CF, HNE cells (Willumsen *et al.* 1989). Depolarization of V_a would favour conductive entry of HCO_3^- into the cell, perhaps via CFTR (Illek *et al.* 1997), which would lead to cell alkalinization. Finally, recent studies by Huang *et al.* (2001) reported substantial CFTR channel activity in Calu-3 cells, a human airway cell line, under basal conditions that was approximately half the activity measured after forskolin addition, suggesting that CFTR Cl^- channels were half-maximally activated under basal conditions. Moreover, they showed that the constitutive activation of the CFTR Cl^- channel reflected release of 5′-adenosine triphosphate (ATP) into the lumen that was metabolically degraded into adenosine. Adenosine activated the apically located adenosine receptors (A_{2B}-subtype) and adenylyl cyclase present in the apical membrane by means of a G protein (i.e. G_s). Sufficient adenosine 3′,5′-cyclic monophosphate (cAMP) was generated to activate protein kinase A, and hence CFTR, in a diffusionally restricted apical microdomain, without increasing cAMP in other cellular compartments. Similar constitutive release of ATP and generation of adenosine have been reported both *in vivo* (Donaldson *et al.* 2000) and *in vitro* (Watt *et al.* 1998) in normal and CF HNE cells, and constitutive activation of CFTR (~half-maximal) has been reported in normal nasal epithelium *in vivo* (Knowles *et al.* 1995) under basal conditions.

To investigate the effects of maximal CFTR activation on pH_i, we examined the effects of forskolin on changes in mucosal Cl^- concentration in normal HNE cells. As shown in Fig. 6, in the presence of serosal H_2DIDS the addition of forskolin (10 μM) to the mucosal/serosal perfusate and subsequently H_2DIDS to the mucosal compartment elicited no change in pH_i in normal HNE cells perfused bilaterally with KBR. In the presence of forskolin and bilateral H_2DIDS, replacing luminal Cl^- with gluconate (imposing a cell-to-lumen Cl^- gradient) again alkalinized cells, consistent with significant lumen-to-cell HCO_3^- uptake in normal HNE cells (Fig. 6*A*). The maximum change of pH_i from steady-state level (induced by H_2DIDS) to peak value (elicited by Cl^- removal) was 0.37 ± 0.02 ($n = 7$, three individuals), which was not significantly different from the change in pH_i induced by luminal Cl^- removal in non-stimulated normal cells treated with bilateral H_2DIDS (i.e. 0.36 ± 0.03 pH units; also see Fig. 5*C*). Furthermore, returning Cl^- to the luminal perfusate (increasing the lumen-to-cell Cl^- gradient) stimulated cells to re-acidify back to basal levels, again consistent with cell-to-lumen HCO_3^- movement in normal airway epithelia (see Fig. 6*A*).

In the absence (see Fig. 5*C*) and presence (see Fig. 6*A*) of forskolin, the initial rate of alkalinization ($\Delta pH_i\ min^{-1}$) in normal HNE cells, measured over the pH_i range 7.05 to 7.20 in response to mucosal Cl^- removal, was 0.14 ± 0.02 ($n = 11$; four individuals) and 0.19 ± 0.02 ($n = 7$; four individuals), respectively. In the presence of forskolin, the initial rate in the change in pH_i (i.e. $\Delta pH_i\ min^{-1} = 0.19$) induced by luminal Cl^- removal in normal cells was marginally greater ($P < 0.05$) than the change in pH_i (i.e. $\Delta pH_i\ min^{-1} = 0.14$) induced by luminal Cl^- removal in non-stimulated normal HNE cells. This difference represents an increase in the rate of alkalinization in forskolin-treated normal cells over non-stimulated cells by ~26 %. Furthermore, in the presence of bilateral H_2DIDS, bilaterally applied forskolin (10 μM) had no effect on

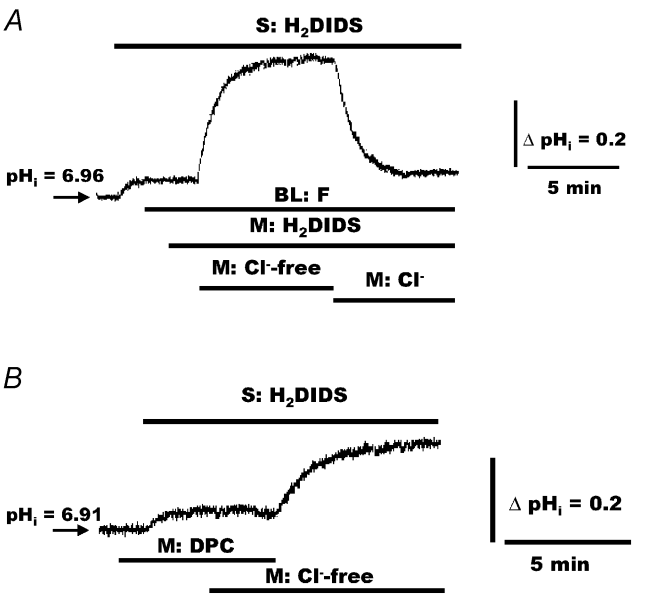

Figure 6. Effects of forskolin and diphenylamine-2-carboxylate (DPC) on pH_i in response to mucosal Cl^- substitution in polarized monolayers of normal HNE cells

A, normal HNE cells were initially perfused with bilateral (BL) KBR. Serosal H_2DIDS (1.5 mM) and bilateral forskolin (F; 10 μM) were added to the perfusate at the times indicated in the tracing, before changing the mucosal perfusate from KBR to Cl^--free (gluconate replacing Cl^-; constant 5 % P_{CO_2}/25 mM HCO_3^-, pH 7.4) KBR. Cl^- was subsequently re-added to the mucosal compartment at the time indicated in the tracing. *B*, normal HNE cells were bilaterally perfused with KBR and mucosal DPC (100 μM) and serosal H_2DIDS (1.5 mM) added to the perfusate at the times indicated in the tracing. In the presence of DPC, changing the mucosal perfusate from KBR to Cl^--free KBR elicited no change in pH_i, whereas removal of DPC induced an alkalinization of the cells. For both traces, the serosal perfusate remained KBR throughout the study. *A*, representative example of seven separate experiments (three different individuals). *B*, representative example of seven separate experiments (four different individuals).

steady-state pH_i in the presence or absence of luminal Cl^- in CF HNE cells ($n = 7$; three individuals; data not shown).

Although there are no available blockers that selectively inhibit the CFTR Cl^- channel activity without potentially affecting other membrane channels/transporters, DPC has been reported (Stutts *et al.* 1990; Zhang *et al.* 2000) to decrease CFTR anion conductance in several cell types, including airway epithelial cells. We utilized DPC to investigate whether the Cl^--induced changes in pH_i in response to unilaterally replacing Cl^- with gluconate in the mucosal bath were mediated via CFTR. As depicted in Fig. 6*B*, in normal HNE cells bilaterally perfused with KBR, the addition of DPC (100 μM) to the mucosal perfusate elicited little or no change in basal pH_i, whereas the subsequent administration of serosal H_2DIDS again caused pH_i to increase. However, mucosal DPC attenuated the increase of pH_i in response to mucosal Cl^--free Ringer solution (compare Fig. 6*B* with Fig. 5*A*). Notably, when DPC was removed from the mucosal compartment, the inhibitory effect of DPC was reversed, and the cells alkalinized. The maximum average change of pH_i from steady-state level (induced by serosal H_2DIDS) to peak value following luminal DPC removal was 0.18 ± 0.02 ($n = 7$, four individuals).

Effects of mucosal DPC on pH_i in response to serosal Ba^{2+} in normal and CF HNE cells

Previous studies on HNE cells have reported that the V_a also reflects, in part, the basolateral K^+ conductance, i.e. cell depolarization in response to the blockade of a basolateral K^+ conductance will be reflected in a depolarization of V_a (Willumsen *et al.* 1989). Based on this information, we designed protocols to test whether CFTR transports HCO_3^- in response to altered V_a by exposing polarized HNE cells to serosal Ba^{2+}, a known K^+ channel blocker (Devor & Frizzell, 1998).

For these studies, we first tested whether Ba^{2+} could alter pH_i in cells perfused with a nominally CO_2/HCO_3^- buffer solution. As illustrated in Fig. 7*A*, when normal HNE cells were bilaterally perfused with Hepes-buffered NaCl Ringer solution, the addition of H_2DIDS (1.5 mM) and subsequently Ba^{2+} (5.0 mM) to the serosal compartment induced no change in basal pH_i. However, when normal (Fig. 7*B* and *C*) and CF (Fig. 7*D*) HNE cells were perfused with bilateral KBR, the addition of H_2DIDS (1.5 mM) to the serosal perfusate again caused pH_i to increase. Moreover, in the presence of bilateral KBR, the subsequent addition of serosally applied Ba^{2+} (5.0 mM) to normal airway epithelia induced a substantial and further increase in pH_i that was completely reversible with the removal of

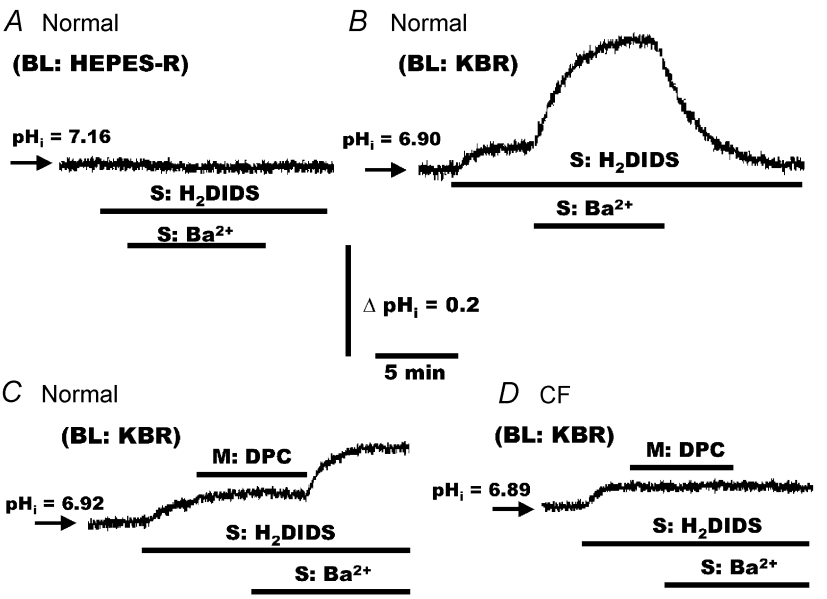

Figure 7. Effects of mucosal DPC on pH_i in response to serosal Ba^{2+} in polarized monolayers of normal and CF HNE cells

A, normal HNE cells were bilaterally perfused with Hepes-buffered NaCl Ringer solution before adding H_2DIDS (1.5 mM) and Ba^{2+} (5.0 mM) to the serosal compartment at the times shown in the trace. For both normal (*B* and *C*) and CF (*D*) HNE cell preparations, cells were perfused bilaterally with KBR and H_2DIDS (1.5 mM) administrated to the serosal perfusate. Following H_2DIDS treatment, DPC (100 μM) and/or Ba^{2+} (5.0 mM) were added to and/or removed from the serosal and/or mucosal compartment in both normal (*B* and *C*) and CF (*D*) cell preparations at the times shown in the tracings. Each trace is representative of six separate experiments (three different individuals).

Ba^{2+} from the serosal compartment (Fig. 7*B*). The magnitude of the change of pH_i from the H_2DIDS-treated to peak values elicited by Ba^{2+} was 0.19 ± 0.03 (n = 6, three individuals). Furthermore, in normal HNE cells, the mucosal addition of DPC (100 μM) reversibly blocked changes of pH_i in response to serosal Ba^{2+} (Fig. 7*C*). Finally, neither mucosal DPC (100 μM) nor serosal Ba^{2+} (5.0 mM) altered pH_i in CF airway cells (Fig. 7*D*). Collectively, these data support a role for CFTR in HCO_3^- transport across the apical membrane in normal HNE cells.

Effects of serosal Cl^- substitution on pH_i in normal and CF HNE cells

For these studies, normal and CF HNE cells were initially perfused in bilateral KBR with H_2DIDS (1.5 mM) added to the serosal perfusate to block the putative basolateral AE. As shown in Fig. 8, in the presence of serosal H_2DIDS, changing the serosal perfusate to Cl^--free (gluconate replacement) KBR (mucosal bath constant KBR, pH 7.4) caused no change in pH_i in either normal (Fig. 8*A*) or CF (Fig. 8*B*) airway cells. However, with serosal Cl^--free Ringer solution, i.e. in the presence of a cell-to-serosal Cl^- gradient, cell pH increased in both normal and CF HNE cells when H_2DIDS was removed from the serosal compartment. The initial rate of alkalinization ($\Delta pH_i\ min^{-1}$) measured over the pH_i range 7.05–7.25 in response to serosal Cl^- substitution was 0.16 ± 0.04 (n = 10; four individuals) and 0.18 ± 0.03 (n = 10; four individuals) in normal and CF HNE cells, respectively. These initial rates were not significantly different. It should be noted that the maximum absolute change of pH_i in response to serosal Cl^- substitution was slightly smaller in normal (ΔpH_i = 0.48 ± 0.02) compared to CF (ΔpH_i = 0.56 ± 0.01) HNE cells. Moreover, unlike CF HNE cells, there was a spontaneous secondary slow re-acidification of pH_i following the peak alkalinization in normal airway cells (Fig. 6*A*). These two observations for normal and CF HNE cells are addressed later in the study. Furthermore, as shown in Fig. 8, the re-addition of serosal Cl^- (establishing a serosal-to-cell Cl^- gradient) caused both normal (Fig. 8*A*) and CF (Fig. 8*B*) HNE cells to re-acidify rapidly back toward basal pH_i values. Taken together, these results are consistent with the activity of a H_2DIDS-sensitive AE at the basolateral membrane in both normal and CF cell preparations.

Effects of mucosal and serosal Cl^- on pH_i in alkalinized normal and CF HNE cells

Because AE can be broadly divided into Na^+-dependent AE, i.e. requiring external Na^+ for the cycling of extracellular Cl^- for intracellular HCO_3^-, or Na^+-independent AE (Boron, 1986), we next tested for Na^+-dependent and Na^+-independent AE activity at the apical and basolateral domains of normal and CF HNE cells. For Na^+-dependent AE, HNE cell preparations were initially perfused bilaterally with KBR, and the perfusate subsequently changed to bilateral Hepes-buffered Cl^--free (sodium gluconate; pH 7.4 (nominally CO_2- and HCO_3^--free)) Ringer solution. This manoeuvre alkalinized cells due to rapid loss of cell CO_2, thereby establishing a cell-to-extracellular HCO_3^- gradient across the apical/basolateral domain of airway cells. Following cell alkalinization, the Cl^- dependency of recovery from an alkaline/HCO_3^- load in the presence of extracellular Na^+ was tested by exposing cells to mucosal or serosal Cl^- (Hepes-buffered NaCl Ringer solution).

As shown in Fig. 9*A*, the pattern in the change of pH_i was markedly different between normal and CF HNE cells when the perfusate was changed from symmetrical KBR to

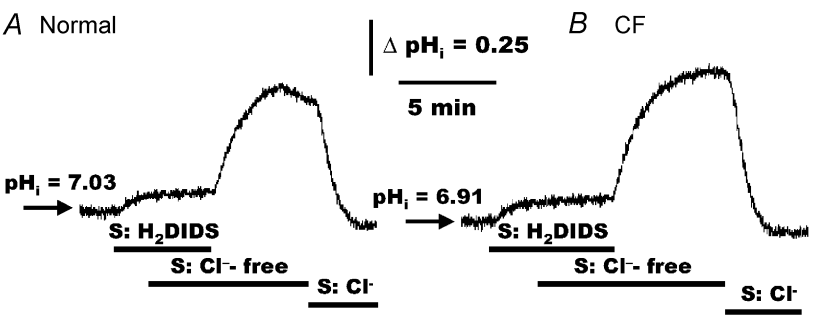

Figure 8. Effects of H_2DIDS and serosal Cl^- substitution on pH_i in polarized monolayers of normal and CF HNE cells

For both normal (*A*) and CF (*B*) HNE cell preparations, cells were initially perfused bilaterally with KBR and H_2DIDS (1.5 mM) added to the serosal compartment before removing Cl^- (gluconate replacing Cl^-; constant 5 % P_{CO_2}/25 mM HCO_3^-, pH 7.4) from the serosal perfusate, as shown in the tracings. In the presence of serosal Cl^--free KBR, removal of serosal H_2DIDS caused pH_i to alkalinize, whereas the re-addition of serosal Cl^- caused cell pH to re-acidify back to basal level. The mucosal perfusate remained KBR throughout the study. Each tracing is representative of ten separate experiments (four different individuals).

symmetrical Hepes-buffered Cl^--free Ringer solution. In normal cells, the initial increase of pH_i following bilateral Cl^--free treatment was followed by a spontaneous secondary re-acidification back towards the basal value. In contrast to normal cells, CF HNE cells rapidly alkalinized and no secondary re-acidification was noted. Moreover, the magnitude of the increase of pH_i following bilateral Cl^--free treatment was significantly ($P < 0.01$) reduced in normal ($\Delta pH_i = 0.54 \pm 0.04$, $n = 9$; three individuals) compared to CF ($\Delta pH_i = 0.79 \pm 0.03$, $n = 9$; three individuals) HNE cells. In the presence of bilateral Na^+, the addition of Cl^- to the mucosal bath (a condition that established a lumen-to-cell gradient for Cl^- and a cell-to-lumen gradient for HCO_3^-) had little effect on the rate of the secondary re-acidification in normal HNE cells and elicited no change in pH_i in CF cells (Fig. 9*A*). However, when Cl^- was added to the serosal perfusate, pH_i rapidly returned towards baseline in both cell preparations (Fig. 9*A*).

To test for the Na^+ dependency of AE activity, HNE cell preparations were initially perfused bilaterally with KBR and the perfusate subsequently changed to bilateral Hepes-buffered Na^+- and Cl^--free (NMG gluconate; pH 7.4 (nominally CO_2- and HCO_3^--free)) Ringer solution to alkalinize cells (Fig. 9*B*). Following cell alkalinization, the Cl^- dependency of recovery from an alkaline load was again tested by exposing cells to mucosal or serosal Cl^- (Hepes-buffered NMGCl Ringer solution). As shown in Fig. 9*B*, in normal HNE cells, the initial increase of pH_i following bilateral Na^+- and Cl^--free treatment was followed by a spontaneous secondary re-acidification back towards baseline. Again in contrast to normal cells, CF HNE cells rapidly alkalinized and no secondary re-acidification was detected. Moreover, the

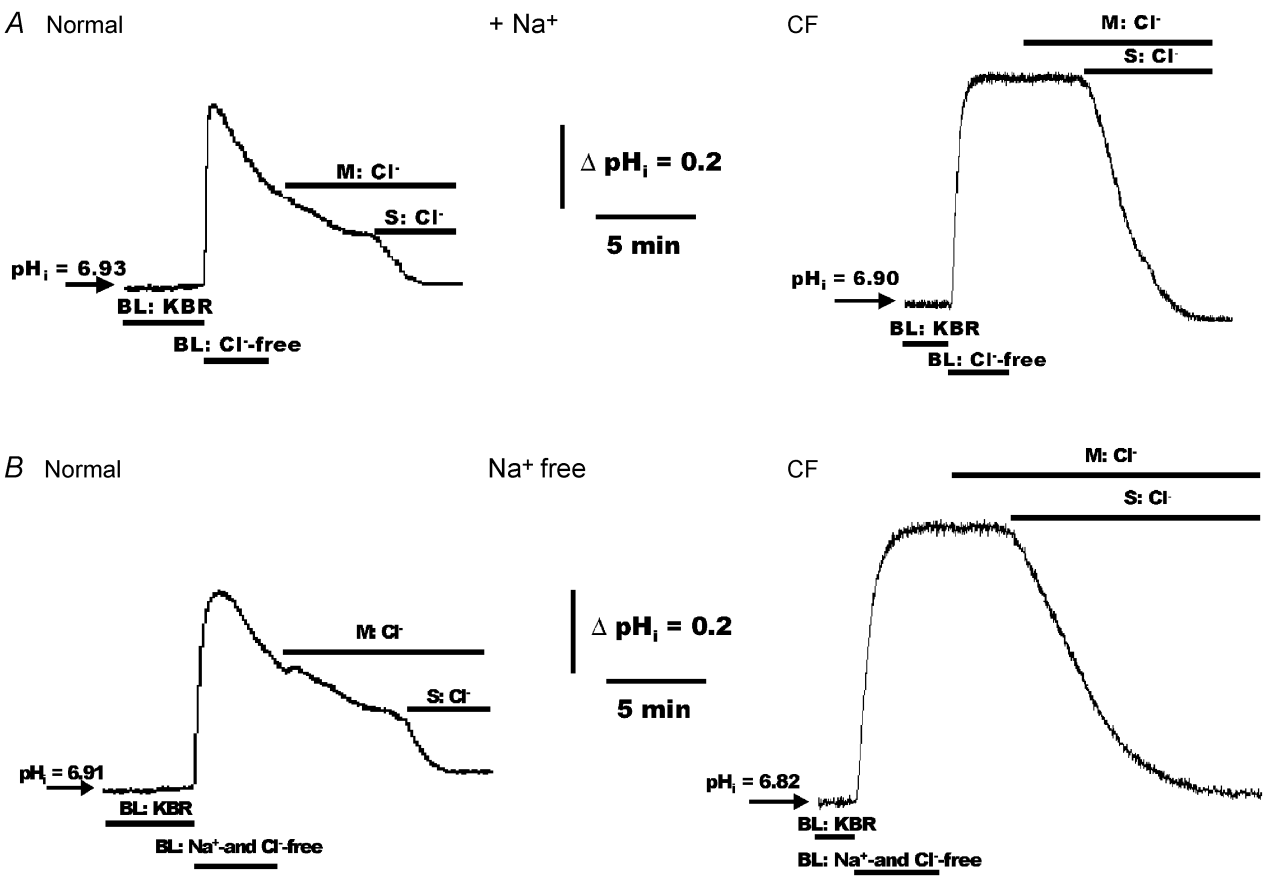

Figure 9. Effects of mucosal and serosal Cl^-(± Na^+) on pH_i in polarized monolayers of alkalinized normal and CF HNE cells

For both normal and CF HNE cell preparations, cells were initially perfused bilaterally with KBR. In one set of experiments shown in panel *A*, HNE (normal and CF) cells were exposed to bilateral Hepes-buffered Cl^--free (sodium gluconate; no CO_2 or HCO_3^-, pH 7.4) Ringer solution to alkalinize the cells (due to the rapid loss of cell CO_2). In a second set of experiments, shown in panel *B*, HNE (normal and CF) cells were exposed to bilateral Hepes-buffered Na^+- and Cl^--free (*N*-methyl-D-glucamine gluconate replacing Na^+ and Cl^-; no CO_2 or HCO_3^-, pH 7.4) Ringer solution. Following alkalinization of the cells, Cl^- was re-added to the mucosal or serosal perfusate at the times depicted in the tracings. Each trace is representative of nine separate experiments (three different individuals).

maximum absolute increase of pH_i following bilateral Na^+- and Cl^--free treatment was again significantly ($P < 0.01$) reduced in normal ($\Delta pH_i = 0.59 \pm 0.03$, $n = 9$; three individuals) compared to CF ($\Delta pH_i = 0.83 \pm 0.02$, $n = 9$; three individuals) HNE cells. The addition of Cl^- to the mucosal bath (again, a condition that established a lumen-to-cell gradient for Cl^- and a cell-to-lumen gradient for HCO_3^-) slightly decreased or had no effect on the rate of the secondary re-acidification in normal HNE cells and elicited no change in pH_i in CF cells (Fig. 9*B*). In contrast to the mucosal addition of Cl^- , when Cl^- was added to the serosal perfusate, pH_i rapidly accelerated towards baseline in both airway cell preparations (Fig. 9*B*). The observation that re-acidification of pH_i in response to serosal addition of Cl^- in alkalinized cells was similar in normal and CF epithelia with (see Fig. 9*A*) or without Na^+ (see Fig. 9*B*) suggests that the predominant AE pathway is Na^+-independent.

Effects of serosal H_2DIDS on pH_i in alkalinized normal and CF HNE cells

We next investigated whether re-acidification of pH_i in response to serosal Cl^- could be blocked by H_2DIDS in airway cells challenged with an alkaline/HCO_3^- load (Fig. 10). In normal (Fig. 10*A*) and CF (Fig. 10*B*) cells bilaterally perfused with KBR, the addition of serosal H_2DIDS (1.5 mM) caused a small increase in pH_i. When the serosal and mucosal compartments were subsequently changed to Hepes-buffered Na^+- and Cl^--free (NMG gluconate; nominally CO_2- and HCO_3^--free) Ringer solution to alkalinize cells, no recovery of pH_i was detected when Cl^- was added to the serosal compartment in the presence of H_2DIDS. However, removal of H_2DIDS from the serosal compartment in the continual presence of Cl^- caused an increase in the rate of pH_i recovery from the alkaline challenge in both normal (Fig. 10*A*) and CF (Fig. 10*B*) HNE cell preparations.

Spontaneous acidification and effects of mucosal DPC on pH_i in alkalinized normal HNE cells

The spontaneous secondary acidification following an alkaline shift in normal HNE cells could potentially result from: (i) the reversal of a Na^+/H^+ exchanger (i.e. a cell-to-extracellular gradient of Na^+ linked to an extracellular-to-cell gradient of H^+), which is known to be present on the basolateral, but not apical, membrane of human nasal cells (Paradiso, 1997); (ii) the reversal of a Na^+–HCO_3^- cotransporter (i.e. loss of both Na^+ and HCO_3^- from cells); and/or (iii) loss of cell HCO_3^- via CFTR.

With regard to Na^+/H^+ exchange, it is possible that during an alkaline shift in pH_i, Na^+ could exit the cell in exchange for extracellular H^+ via the Na^+/H^+ exchanger, and consequently, cells would acidify. However, this possibility

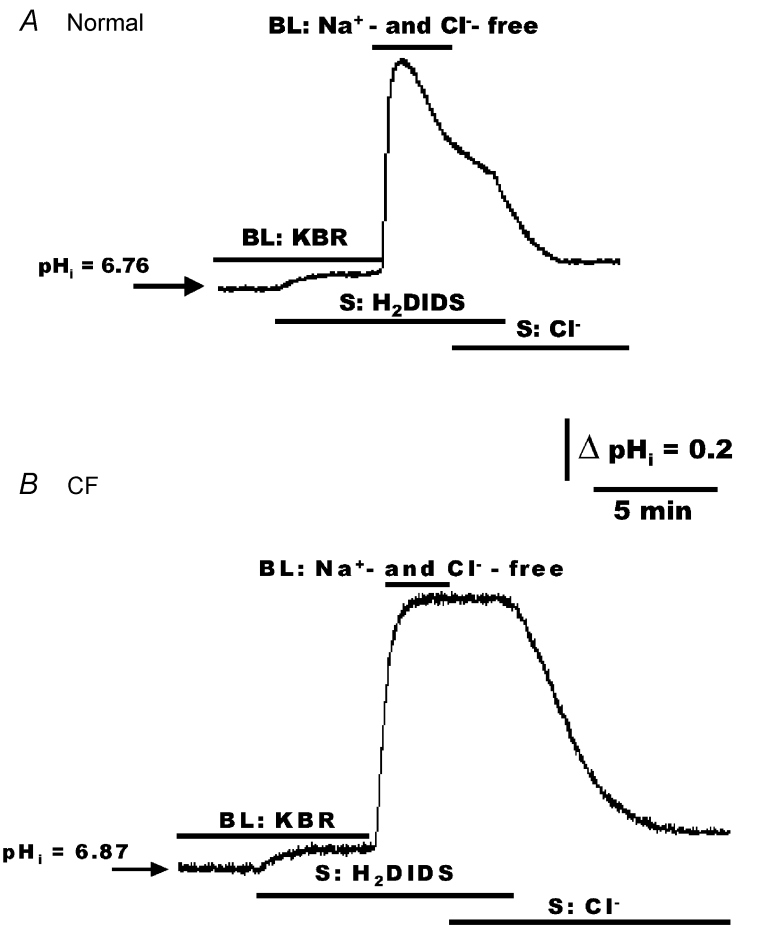

Figure 10. Effects of serosal H_2DIDS (± Cl^-) on changes of pH_i in polarized monolayers of alkalinized normal and CF HNE cells

For both normal (*A*) and CF (*B*) HNE cell preparations, cells were initially perfused bilaterally (BL) with KBR. In the presence of bilateral KBR, H_2DIDS (1.5 mM) was added to the serosal compartment (*A* and *B*) at the times shown in the tracings, before changing from bilateral KBR to bilateral Hepes-buffered Na^+- and Cl^--free (*N*-methyl-D-glucamine gluconate replacing Na^+ and Cl^-; no CO_2 or HCO_3^-, pH 7.4) Ringer solution. Following alkalinization of the cells, serosal H_2DIDS blocked the re-acidification of pH_i when Cl^- was re-added to the serosal perfusate in both normal (*A*) and CF (*B*) HNE cells, as shown in the tracings. Note that in the presence of serosal Cl^-, removal of H_2DIDS from the serosal compartment resulted in a rapid re-acidification of pH_i in both normal and CF HNE cells (*A* and *B*). Each trace is representative of seven separate experiments (three different individuals).

seems unlikely, for three reasons. First, the pattern and rate of the spontaneous secondary re-acidification were similar in the presence and absence of extracellular Na^+ (see Fig. 9). Second, we have repeated these experiments in the absence of extracellular Na^+, pretreating cells with serosal amiloride (500 μM; $n = 6$, three different individuals) to block the activity of the basolateral Na^+/H^+ exchanger (Paradiso, 1997). This manoeuvre did not prevent the secondary re-acidification in normal HNE cells (data not shown). Finally, we have previously reported (Paradiso, 1997) that CF HNE cells also have Na^+/H^+ exchanger activity at their basolateral border that is pharmacologically and kinetically identical to that of the Na^+/H^+ exchanger in normal nasal tissue, yet no spontaneous secondary re-acidification was detected in CF tissue following the initial alkaline shift in pH_i (see Fig. 9).

Although we do not have functional evidence for the presence of a Na^+–HCO_3^- cotransporter at the apical/basolateral aspect of human nasal cells, Devor and co-workers (1999) have functionally identified a basolateral Na^+–HCO^- cotransporter in Calu-3 cells. Thus, it is possible that the spontaneous re-acidification in response to an alkaline shift of pH_i in normal HNE cells resulted from the reversal of the cotransporter. However, like the argument against the involvement of Na^+/H^+ exchange in the re-acidification in response to an alkaline shift in pH_i in normal HNE cells, it again seems unlikely that a cell-to-extracellular movement of Na^+ and HCO_3^- via Na^+–HCO^- cotransport could account for the spontaneous recovery following an alkaline challenge, since, as noted above, the pattern of re-acidification was identical in Na^+-containing (see Fig. 9*A*) and Na^+-free (see Fig. 9*B*) Ringer solutions. Moreover, as noted above in the discussion of Fig. 2, a role for a Na^+–HCO_3^- cotransporter in the regulation of pH_i in surface nasal epithelial cells is not suggested by this study, since basolaterally applied amiloride could completely and reversibly inhibit recovery of pH_i from an acid challenge when airway cells were perfused with KBR.

To elucidate whether the spontaneous secondary re-acidification towards basal values in normal cells challenged by CO_2 removal was mediated via CFTR, we tested whether mucosally applied DPC blocked this process. As shown in Fig. 11*A*, when normal HNE cells were initially perfused with symmetrical KBR, the addition of mucosal DPC (100 μM) elicited no change in basal pH_i. However, changing the mucosal/serosal perfusate to symmetrical Hepes-buffered Na^+- and Cl^--free Ringer solution resulted in a larger alkalinization in cell pH and markedly reduced the secondary re-acidification in DPC-treated compared to non-DPC-treated normal cells (see Fig. 10*A*). On average, the maximum absolute increase of pH_i following bilateral Na^+- and Cl^--free treatment was significantly ($P < 0.01$) increased in DPC-treated cells ($\Delta pH_i = 0.78 \pm 0.02$, $n = 7$; three different individuals) compared to non-treated (i.e. ΔpH_i of 0.59 ± 0.03, see Fig. 10*A*) normal HNE cells. Moreover, the subsequent removal of DPC from the mucosal bath caused pH_i to return rapidly to baseline (Fig. 11*A*). In contrast to normal cells, the mucosal addition or removal of DPC (100 μM) had no effect on the magnitude change of pH_i in CF HNE cells when the perfusate was changed from symmetrical KBR to symmetrical Hepes-buffered Na^+- and Cl^--free Ringer solution (Fig. 11*B*). On average, the maximum

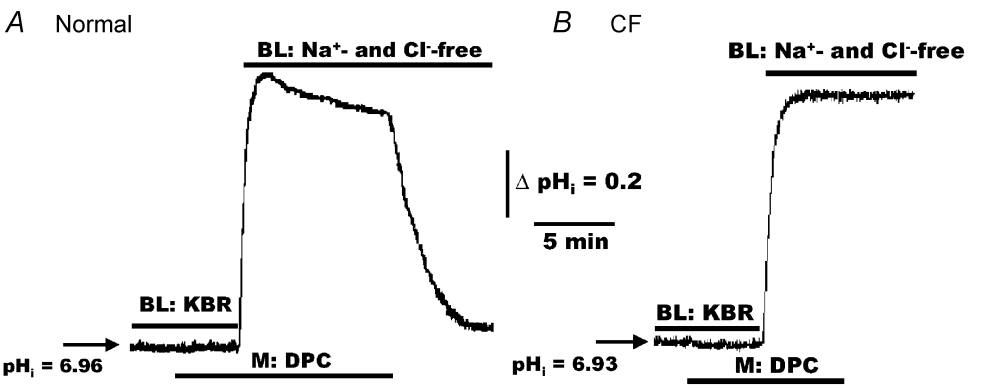

Figure 11. Effects of mucosal DPC on changes of pH_i in polarized monolayers of alkalinized normal and CF HNE cells

For both normal (*A*) and CF (*B*) HNE cell preparations, cells were initially perfused bilaterally (BL) with KBR before adding DPC (100 μM) to the mucosal perfusate at the times shown in each trace. In the presence of DPC, changing from bilateral KBR to bilateral Hepes-buffered Na^+- and Cl^--free (*N*-methyl-D-glucamine gluconate replacing Na^+ and Cl^-; no CO_2 or HCO_3^-, pH 7.4) Ringer solution, resulted in a larger alkalinization of pH_i in normal DPC-treated cells (*A*) compared to normal cells not treated with DPC (see trace *A* of Fig. 10), and that removal of DPC elicited a rapid re-acidification of pH_i back to base level in normal (*A*), but not CF (*B*), HNE cells. Each trace is representative of seven separate experiments (three different individuals).

absolute increase of pH_i following bilateral Na^+- and Cl^--free treatment was not significantly different in CF cells treated with mucosal DPC ($\Delta pH_i = 0.85 \pm 0.03$, $n = 7$; three individuals) compared to non-DPC-treated CF cells ($\Delta pH_i = 0.83 \pm 0.02$; see Fig. 10*B*).

DISCUSSION

Regulation of basal pH_i in normal and CF HNE cells

In a previous study (Paradiso, 1997) using polarized human nasal epithelial cells in primary culture, we reported that basal pH_i was ~7.1 in both normal and CF cells bilaterally perfused with nominally CO_2/HCO_3^--free Hepes-buffered NaCl Ringer solution. Furthermore, we showed in this earlier study that both normal and CF HNE cells expressed at their basolateral membrane a Na^+/H^+ exchanger that was inactive at basal $pH_i \geqslant 7.1$ (Paradiso, 1997).

In the present study, when polarized normal and CF HNE cells were bilaterally perfused in CO_2-containing KBR, basal pH_i was 6.94 and 6.95, respectively (also see Fig. 1). We speculate that the lower basal pH_i in normal and CF HNE cells bilaterally perfused with KBR resulted from the inclusion of CO_2 in the perfusate, which lowered pH_i and led to the activation of both a basolateral H_2DIDS-sensitive AE and a Na^+/H^+ exchanger. This speculation is based on the following observations generated by this study. First, serosally applied H_2DIDS elicited an alkalinization of pH_i in both normal and CF cell preparations (see Fig. 3*A* and *B*), a response predicted for the inhibition of an active basolateral AE. Second, serosally applied amiloride induced an acidification of basal pH_i in both normal and CF cell preparations, a response consistent with the inhibition of an active basolateral Na^+/H^+ exchanger (see Fig. 3*C* and *D*). Third, the parallel activities of AE and a Na^+/H^+ exchanger were revealed by the observation that serosally applied amiloride acidified pH_i following post-H_2DIDS-induced alkalinization of cell pH (Fig. 3*A* and *B*), and that the addition of H_2DIDS subsequent to amiloride caused a small increase in pH_i (Fig. 3*C* and *D*). Taken together, our data strongly suggest that basal pH_i in airway epithelia exposed to physiological P_{CO_2} is maintained via the coordinated activities of a Na^+/H^+ exchanger and AE at the basolateral membrane in normal and CF HNE cells. The coordinated activities of both exchangers may play an important compensatory function in maintaining cell pH within narrow limits during dynamic shifts in CO_2 tension in the airway lumen during the respiratory cycle, i.e. CO_2 falls (~0 Torr) during inspiration and rises (~40 Torr) during expiration.

HCO_3^- transport across the apical membrane in HNE cells

In the present study, ion gradients were manipulated across the apical membrane to investigate whether HCO_3^- influx/efflux pathways were linked to luminal perfusate Cl^- concentrations in normal and CF airway cells. In the presence of serosal H_2DIDS (to block HCO_3^- efflux across the basolateral membrane; see Figs 4 and 5) and symmetrical HCO_3^- (constant 5% P_{CO_2}), removal of luminal Cl^- resulted in cytoplasmic alkalinization in normal HNE cells due to the influx of HCO_3^- across the apical membrane (see Fig. 5*A*; also see Paradiso, 1992). Conversely, when alkalinized normal HNE cells were subsequently exposed to luminal Cl^- , cells rapidly re-acidified towards basal levels (see Fig. 5*A*), consistent with the cellular efflux of HCO_3^- across the apical membrane. Furthermore, as discussed above, these Cl^--dependent changes of pH_i are largely independent of exogenously applied forskolin in normal HNE cells (see Fig. 6*A*), suggesting that the membrane mechanism responsible for the transport of HCO_3^- across the apical membrane is constitutively active under basal conditions.

Although these Cl^--dependent changes of pH_i, in response to changes in luminal Cl^- appeared to be consistent with an apically located AE in normal HNE cells, we argue that the mechanism mediating these changes in pH_i is the constitutively active CFTR Cl^-/HCO_3^- conductance in the apical membrane in normal airway epithelia, based on a series of observations (also see discussion above relevant to Fig. 6*A*). Previous microelectrode studies (Willumsen *et al.* 1989) showed that removal of luminal Cl^- caused V_a to depolarize in normal (but not CF) cells by ~30 mV, whereas the re-addition of luminal Cl^- repolarized V_a. As reported above, normal and CF HNE cells exhibit an average pH_i of ~6.95 when cells are exposed to bilateral KBR. From this mean pH_i value, cell HCO_3^- concentrations, calculated from the Henderson-Hasselbach equation (Roos & Boron, 1981), is ~8 mM. Accordingly, in the presence of luminal KBR, the ratio of HCO_3^- concentration across the apical membrane ($[25\ mM]_{out}/[\sim 8\ mM]_{in}$) is approximately equal to and opposite from the electrical gradient (V_a ~30 mV; Willumsen *et al.* 1989) across this barrier. Therefore, we predict no net movement of HCO_3^- via CFTR under basal conditions. However, removal of luminal Cl^- induces a depolarization of ~30 mV across the apical membrane, i.e. V_a approaches 0 mV, which now favours HCO_3^- influx driven by the lumen-to-cell electrochemical gradient for this anion, and, as seen in Fig. 5*A*, pH_i increases from ~6.97 to ~7.4. The concentration of cellular HCO_3^- at pH_i of ~7.4 will be close to 25 mM, and $[HCO_3^-]_{in} = [HCO_3^-]_{out}$, consistent with the absence of an electrical driving potential across the apical membrane. When Cl^- is restored to the luminal bath (repolarizing V_a by ~30 mV), HCO_3^- now moves from cell-to-lumen, again driven by the electrochemical gradient across the apical barrier, and the cell re-acidifies.

Several other lines of evidence support the notion of electrogenic HCO_3^- transport via CFTR across the apical

membrane in normal HNE cells, rather than electroneutral exchange for Cl^- via an AE. First, removal of luminal Cl^- failed to induce changes of pH_i in CF HNE cells (Fig. 5*B*), clearly suggesting the requirement for functional CFTR to mediate Cl^--associated changes of pH_i in normal airway cells. Second, mucosal H_2DIDS, a known inhibitor of AE (Mastrocola *et al.* 1998), but not CFTR (Paradiso *et al.* 2001), failed to block Cl^--linked changes of pH_i in normal airway cells in the absence (Fig. 5*C*) and presence (Fig. 6*A*) of forskolin. Third, mucosal DPC, an inhibitor of CFTR, reversibly blocked the increase of pH_i in normal airway epithelia in response to mucosal Cl^--free Ringer solution (see Fig. 6*B*). Fourth, both normal and CF cells exposed to bilateral Hepes-buffered Cl^-- and HCO_3^--free Ringer solution acutely alkalinized, whereas only the normal nasal preparations exhibited a secondary re-acidification (see Figs 9 and 10). We propose that this re-acidification resulted from loss of cell HCO_3^- via CFTR, since it was absent in CF airway epithelia (see Figs 9 and 10) and was reversibly inhibited by mucosal DPC in normal airway cells (see Fig. 11). Finally, in normal (but not CF) nasal cells bilaterally perfused with KBR (but not Hepes-buffered Ringer solution), serosally applied Ba^{2+} induced an increase in pH_i that was reversibly blocked by mucosal DPC (see Fig. 7), strongly arguing for the conductive movement of HCO_3^- via CFTR.

Further support for the concept that CFTR mediates apical membrane HCO_3^- translocation is derived from patch clamp studies by Poulsen *et al.* (1994), who reported CFTR-mediated HCO_3^- conductance in NIH 3T3 cells recombinantly expressing wild-type CFTR. Similar findings have been reported by Linsdell *et al.* (1999), Illek *et al.* (1997) and Hogan *et al.* (1997) in a variety of epithelial cell types.

We would note here that some of our findings differ from those of others. Unlike the finding in mouse pancreatic and submandibular ducts, which have at their apical membrane a CFTR-regulated AE system (Lee *et al.* 1999*a*), our findings strongly indicate that CFTR, and not AE, is the primary HCO_3^- transport route in normal airway cells and that AE is restricted to the basolateral membrane in both normal and CF HNE cells. Moreover, recent studies by Wheat and co-workers (2000) reported that CFTR induces the mRNA expression of 'downregulated in adenoma' (DRA), and that DRA is itself an AE at the apical membrane in a tracheal epithelial cell line (CFT-1) derived from a CF patient. They suggested that the tracheal HCO_3^- secretion defect in patients with CF is partly a result of the downregulation of the apically located AE activity mediated by DRA with loss of CFTR function. The apparent discrepancy between their data and ours may be in the selection of tissues used for the studies, i.e. a transformed cell line *versus* epithelial cells in primary culture.

The concept that CFTR mediates HCO_3^- translocation across the apical membrane has implications for regulation of ASL pH as well as pH_i. For example, HCO_3^- secretion via CFTR should have an impact on altering pH and HCO_3^- content of ASL. Consistent with this notion, there is strong evidence that lack of HCO_3^- secretion renders the pH of liquids lining epithelial surfaces abnormally acidic in CF patients. For example, Kaplan and co-workers reported that CF ejaculate is acidic (pH ~6.6) relative to normal semen (pH > 8.0), consistent with the absence of HCO_3^- in seminal fluid (Kaplan *et al.* 1968). Furthermore, studies by Durie (1989) reported that reduced HCO_3^- and Cl^- transport within pancreatic ducts could account for deficient fluid secretion in the pancreas of CF subjects. In addition, Kaiser & Drack (1974) reported diminished excretion of HCO_3^- from single sweat glands of patients with CF. With relevance to human airways, studies by Smith & Welsh (1992) have reported that cAMP stimulates HCO_3^- secretion across normal but not CF nasal epithelia. Importantly, recent studies (Coakley *et al.* 2000) on well differentiated bronchial epithelial cells have shown that ASL pH is more acidic and exhibits a lower HCO_3^- concentration in CF compared to normal airway cultures.

HCO_3^- transport across the basolateral border in HNE cells

Utilizing two experimental approaches to assess for AE, our data demonstrate that normal and CF HNE cells express an AE that is restricted to the basolateral domain of cells. In the first approach, changes in pH_i were detected in response to imposed Cl^- gradients across the basolateral membrane, and the changes of pH_i were reversibly blocked by serosal H_2DIDS in both normal and CF HNE cells (see Fig. 8). In the second approach, recovery from an alkaline challenge was independent of extracellular Na^+, accelerated by serosal, but not mucosal Cl^-, and reversibly inhibited by serosal H_2DIDS in normal HNE cells (see Figs 9 and 10). Like normal airway cells, the recovery from an alkaline load in CF cells was again independent of extracellular Na^+, absolutely dependent on serosal, but not mucosal addition of Cl^-, and reversibly blocked by serosal H_2DIDS (see Figs 9 and 10).

The biological role of the basolateral AE provides several important functions that are interrelated. For example, this exchanger serves as an important mechanism for Cl^- entry and HCO_3^- exit from the cell and thus functions as an acid-loader during acute cellular alkalosis. In addition to the metabolic role for AE, AE coupled to a Na^+/H^+ exchanger will have important influences on the distribution of ions and water between the extracellular space and the cell during volume regulation (Grinstein *et al.* 1985). Finally, for Cl^--driven secretion in airways, the basolateral Na^+/H^+ exchanger and Cl^-/HCO_3^- exchanger, operating in parallel, are functionally equivalent to the

basolateral Na^+–K^+–$2Cl^-$ cotransporter and thus can provide an alternative mechanism for the serosal-to-cell uptake of Cl^-.

Conclusion

We have identified pathways for HCO_3^- translocation across the apical and basolateral domains of polarized airway epithelia. Across the apical membrane of normal airway epithelia, HCO_3^- appears to translocate via a CFTR-mediated electrodiffusive pathway, with no evidence for AE. In contrast, a H_2DIDS-sensitive AE pathway exists for HCO_3^- translocation across the basolateral barrier. The basolateral AE, in concert with the basolateral Na^+/H^+ exchanger, appears to control responses to local environmental changes (e.g. P_{CO_2}) that can acutely alter pH_i. We speculate that in normal airway tissues, the transport of HCO_3^- via CFTR is central to maintaining ASL pH within physiological levels. Moreover, the secretion of HCO_3^- into ASL appears to be balanced by the secretion of H^+ via a recently reported (Paradiso *et al.* 2000) non-gastric form of the H^+,K^+-ATPase, which is localized at the apical membrane in both normal and CF airway epithelia. Furthermore, it is reasonable to suggest that it is the activity of the H^+,K^+-ATPase that, in part, maintains the electrochemical driving force for HCO_3^- secretion via CFTR in normal airway cells by lowering ASL pH. In addition, in CF airways, ASL pH is predicted to be more acidic than normal airway epithelia with loss of CFTR-mediated HCO_3^- secretion. In support of this later notion, our recent *in vitro* studies (Coakley *et al.* 2000) showing that ASL pH is lower (pH 6.13) in CF airway epithelial cells relative to normal cells (pH 6.44) was linked to a reduction in ASL HCO_3^- content in CF compared to normal airway cells. We speculate that the lack of CFTR-mediated HCO_3^- secretion, coupled to a constitutively active H^+,K^+-ATPase, rather than the absence of a regulatory interaction between apical CFTR and AE, accounts for this difference in luminal pH between CF and normal airway epithelia.

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