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# Cystic Fibrosis Transmembrane Conductance Regulator Regulates Luminal $Cl^-/HCO_3^-$ Exchange in Mouse Submandibular and Pancreatic Ducts*

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**We have demonstrated previously the regulation of $Cl^-/HCO_3^-$ exchange activity by the cystic fibrosis transmembrane conductance regulator (CFTR) in model systems of cells stably or transiently transfected with CFTR (Lee, M. G., Wigley, W. C., Zeng, W., Noel, L. E., Marino, C. R., Thomas, P. J., and Muallem, S. (1999) *J. Biol. Chem.* 274, 3414–3421). In the present work we examine the significance of this regulation in cells naturally expressing CFTR. These include the human colonic T84 cell line and the mouse submandibular gland and pancreatic ducts, tissues that express high levels of CFTR in the luminal membrane. As in heterologous expression systems, stimulation of T84 cells with forskolin increased the $Cl^-/HCO_3^-$ exchange activity independently of CFTR $Cl^-$ channel activity. Freshly isolated submandibular gland ducts from wild type mice showed variable $Cl^-/HCO_3^-$ exchange activity. Measurement of $[Cl^-]_i$ revealed that this was largely the result of variable steady-state $[Cl^-]_i$. Membrane depolarization with 5 mM $Ba^{2+}$ or 100 mM $K^+$ increased and stabilized $[Cl^-]_i$. Under depolarized conditions wild type and ΔF/ΔF mice had comparable basal $Cl^-/HCO_3^-$ exchange activity. Notably, stimulation with forskolin increased $Cl^-/HCO_3^-$ exchange activity in submandibular gland ducts from wild type but not ΔF/ΔF mice. Microperfusion of the main pancreatic duct showed $Cl^-/HCO_3^-$ exchange activity in both the basolateral and luminal membranes. Stimulation of ducts from wild type animals with forskolin had no effect on basolateral but markedly stimulated luminal $Cl^-/HCO_3^-$ exchange activity. By contrast, forskolin had no effect on either basolateral or luminal $Cl^-/HCO_3^-$ exchange activity of ducts from ΔF/ΔF animals. We conclude that CFTR regulates luminal $Cl^-/HCO_3^-$ exchange activity in CFTR-expressing cells, and we discuss the possible physiological significance of these findings regarding cystic fibrosis.**

$HCO_3^-$ secretion is a primary function of many CFTR[1]-expressing cells (1–3). Most of the $HCO_3^-$ is secreted by duct or duct-like cells to the lumen and thus requires transductal $HCO_3^-$ transport. Little is known about the pathways mediating $HCO_3^-$ entry in the basolateral membrane (BLM). The best studies available to date are in the pancreatic duct, in which Case and co-workers (4–6) provided strong evidence that $HCO_3^-$ influx is mediated largely by a BLM $Na^+$-$HCO_3^-$ cotransport. $HCO_3^-$ efflux across the luminal membrane (LM) and its regulation are equally poorly understood. Most models assume that the electroneutral portion of $HCO_3^-$ secretion is mediated by a luminal $Cl^-/HCO_3^-$ exchanger (AE, anion exchanger) (1–3, 6). This function is also believed to mediate part of $Cl^-$ absorption by the duct. $Cl^-$ is supplied to the duct lumen in the plasma-like primary fluid secreted by acinar cells (1–3, 7). The pathophysiology of cystic fibrosis indicates that CFTR plays a critical, but poorly defined, role in $HCO_3^-$ secretion and $Cl^-$ absorption. In tissues such as the salivary glands, in which acinar cells secrete the bulk of the fluid, CFTR is assumed to mediate the electrogenic part of $Cl^-$ absorption (2). The same role is attributed to CFTR in sweat glands (7) and intestinal epithelia (8, 9). Recent work suggests that airway epithelia absorb $Na^+$ and $Cl^-$ to produce a hypotonic airway surface fluid (Ref. 10 and references within, but see Ref. 11 and references within). Because $Na^+$ and $Cl^-$ concentrations in airway surface liquid produced by cystic fibrosis airway epithelium are isotonic (10, 11), CFTR may mediate electrogenic $Cl^-$ absorption in airway epithelia (12, 13). In glands like the pancreas, fluid secretion by acinar cells is limited, and the duct secretes the bulk of the fluid in pancreatic juice (1). In this type of gland the limited supply of $Cl^-$ secreted by acinar cells led to the proposal that CFTR mediates $Cl^-$ secretion to the lumen of duct cells to fuel the $Cl^-/HCO_3^-$ exchanger (1). However, a recent work showed that agonist- and cAMP-stimulated $HCO_3^-$ secretion in guinea pig pancreatic duct is independent of luminal $Cl^-$ (6). Hence, the role of CFTR in ion transport by these tissues remains obscure.

To date direct evidence in support of the two models is meager indeed. Localization of CFTR in the luminal membrane of all CFTR-expressing epithelia is well documented (14–16). $Cl^-/HCO_3^-$ exchange activity was found in the BLM and LM of pancreatic (17) and submandibular gland (SMG) ducts (18). In SMG ducts the AE isoform 2 (AE2) was localized in the BLM (19). The isoform(s) expressed in the LM is not known.

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[1] The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; BLM, basolateral membrane; LM, luminal membrane; AE, $Cl^-/HCO_3^-$ (anion) exchanger; SMG, submandibular gland; WT, wild type; ΔF, deletion mutant of Phe-508 from WT CFTR; PSA, pancreatic solution A; BCECF-AM, 2′7′-bis(2-carboxyethyl)-5-(6)-carboxyfluorescein acetoxymethyl ester; MQAE, *N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate.

A relationship between $HCO_3^-$ secretion and CFTR was documented in two cell lines and intestinal epithelia. In a human airway epithelial cell line CFTR-dependent $HCO_3^-$ conductance (20, 21) was proposed to be mediated by CFTR itself. By contrast, similar studies in a human pancreatic duct cell line concluded that electrogenic $Cl^-$ and $HCO_3^-$ secretions are mediated by independent proteins (22). In duodenal epithelium basal and acid-stimulated $HCO_3^-$ secretions were reduced or absent in CFTR −/− mice (23). Surprisingly, in a recent study Seidler *et al.* (24) showed that all forms of $HCO_3^-$ secretion stimulated by agonists or agents that elevate cAMP, cGMP, and, in particular, $[Ca^{2+}]_i$ were impaired in the intestinal epithelia of CFTR −/− mice. These studies suggest the likely involvement of CFTR in the electrogenic component of $HCO_3^-$ secretion, which is particularly prominent in the intestine (25, 26). However, a large fraction of $HCO_3^-$ secretion in tissues such as salivary glands (2) and the rat and mouse pancreas (1) is mediated by an electroneutral $HCO_3^-$ transport mechanism. The role of CFTR in this critical component of $HCO_3^-$ secretion is unknown.

The intimate relationship between CFTR expression and $HCO_3^-$ secretion seen in intestinal (23, 24) and airway epithelia (27) raises the question of whether and how CFTR modulates $HCO_3^-$ secretion in other CFTR-expressing tissues. In addition to its possible function as a regulator of a $HCO_3^-$ conductive channel, CFTR may also regulate luminal $Cl^-/HCO_3^-$ exchange activity. In a recent study (28) we used cells stably transfected with CFTR and transient transfection of WT CFTR and several CFTR mutants to demonstrate regulation of AE activity by CFTR. To evaluate the physiological relevance of these findings, in the present work we report the regulation of the luminal AE activity by CFTR in the mouse SMG and pancreatic ducts.

The SMG and pancreatic ducts were selected as model systems for several reasons. The fraction of electroneutral $HCO_3^-$ secretion in these tissues is relatively high (1, 2). Among all CFTR-expressing tissues, the mechanism of fluid and electrolyte secretion is understood best in the SMG (2). The SMG and pancreatic ducts express high levels of CFTR (15), and the abundance of ducts in the SMG (2, 29) facilitates experimentation. Although the mouse is not the ideal species to study $HCO_3^-$ secretion (1, 6, 30), it was selected for the present work because of the availability of ΔF/ΔF mice (31). To supplement the studies in the native ducts we also used the human colonic cell line T84 because these cells have been used extensively to study the properties of naturally occurring CFTR (32, 33).

We show here that stimulation of CFTR with cAMP increased $Cl^-/HCO_3^-$ exchange activity in T84 cells. More importantly, stimulation of CFTR by cAMP resulted in selective activation of luminal $Cl^-/HCO_3^-$ exchange activity in SMG and pancreatic ducts of WT mice, which was absent in ducts prepared from ΔF/ΔF mice. We conclude that CFTR regulates luminal $Cl^-/HCO_3^-$ exchange activity of SMG and pancreatic ducts and probably other CFTR-expressing cells.

## EXPERIMENTAL PROCEDURES

*Solutions*—The standard perfusate was termed solution A and contained (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 Hepes (pH 7.4 with NaOH), and 10 glucose. The $HCO_3^-$-buffered NaCl solution B contained (in mM) 120 NaCl, 25 $NaHCO_3$, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 Hepes (pH 7.4 with NaOH), and 10 glucose. The $HCO_3^-$-buffered $Cl^-$-free solution C contained (in mM) 120 $Na^+$-gluconate, 25 $NaHCO_3$, 5 $K^+$-gluconate, 1 $MgSO_4$, 9.3 hemicalcium cyclamate, 5 Hepes (pH 7.4 with NaOH), and 10 glucose. To prepare $HCO_3^-$-buffered high KCl (100 mM $K^+$) solution D, 95 mM NaCl in solution B was replaced with 95 mM KCl. To prepare $HCO_3^-$-buffered, high $K^+$ (100 mM $K^+$), $Cl^-$-free solution E, 95 mM $Na^+$-gluconate was replaced with 95 mM $K^+$-gluconate in solution C. For calibration of intracellular $Cl^-$, solution E was supplemented with 5 μM nigericin and 10 μM tributyltin cyanide. The KSCN solution contained (in mM) 127 KSCN, 25 choline- $HCO_3^-$, and 5 Hepes (pH 7.4 with 2 M Tris). The osmolarity of all solutions was adjusted to 310 mosM with the major salt prior to use.

*Culture of T84 Cells*—T84 cells were purchased from American Type Culture Collection (ATCC CCL 248, Rockville, MD) and maintained in a 1:1 mixture of Ham's F-12 medium and Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. The cells were plated on a sterile 22 × 40-mm coverslip at a density of $2.5 \times 10^5$ cells/$cm^2$ for intracellular pH measurements.

*Animals and Preparation of Ducts*—A cystic fibrosis mouse model in which the ΔF508 mutation was introduced in the mouse CFTR by gene targeting in ES cells (31) was obtained from Dr. Kirk R. Thomas (Eccles Institute of Human Genetics, HHMI, University of Utah School of Medicine, Salt Lake City). The mice were maintained on a standard diet, and genotyping was carried out on day 14 postpartum as described previously (16).

Duct fragments from the mouse SMG were prepared by a slight modification of our published procedure (16). Mice were sacrificed by exposure to a methoxyflurane-saturated atmosphere and subsequent cervical dislocation, and the SMGs were removed to a cold pancreatic solution A (PSA). The composition of PSA was (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 Hepes (pH 7.4 with NaOH), 10 glucose, 10 pyruvate, 0.1% bovine serum albumin, and 0.02% soybean trypsin inhibitor. Each gland was cleaned by injection of 5 ml of PSA and minced. The minced tissue was transferred to 8 ml of PSA containing 2.5 mg of collagenase (CLS4, 254 units/mg; Worthington Biochemicals) and digested for 8–10 min at 37 °C. The dissociated cells were then washed twice with PSA, resuspended in 2 ml of PSA, and kept on ice until use.

Microperfusion experiments were performed with microdissected pancreatic ducts from WT and ΔF/ΔF mice. The procedure for preparation and perfusion of the main pancreatic duct was identical to that used for perfusion of the rat pancreatic duct (17). The ducts were dissected in PSA, cannulated, and perfused through the lumen and the bath with solution A. After completion of BCECF loading the ducts were perfused with $HCO_3^-$-buffered solution B for at least 10 min prior to manipulation of $Cl^-$ gradients.

*Intracellular pH ($pH_i$) Measurements*—The procedure of $pH_i$ measurement in T84 cells was identical to that described in detail in our recent work (28). In the case of SMG cells, the dissociated cells were loaded with BCECF by a 10-min incubation at room temperature in PSA containing 1 μM BCECF-AM. The cells were then washed with PSA and plated on a polylysine-coated coverslip that was assembled into a perfusion chamber. The chamber was placed on an inverted microscope, and intralobular ducts were identified by morphology. The BCECF fluorescence of 10–16 cells of a duct fragment was recorded at excitation wavelengths of 440 and 490 nm. Fluorescence ratios of 490/440 were calibrated using the procedures described previously (28). In the case of the perfused pancreatic duct BCECF loading was accomplished by including 2.5 μM BCECF-AM in the luminal perfusate for 10 min.

Changes in $Cl^-/HCO_3^-$ exchange activity were estimated from the initial rate of $pH_i$ changes (T84 cells and pancreatic ducts) or from the extent of $pH_i$ changes (SMG ducts). Initial rates of $pH_i$ changes were obtained from the first derivative of the traces using a single exponential fit. The extent of $pH_i$ changes was estimated by averaging the $pH_i$ changes measured as a result of $Cl^-$ removal and addition. All results are given as mean ± S.E. of the indicated number of experiments.

*Intracellular $Cl^-$ Measurement*—$[Cl^-]_i$ was measured with the aid of the $Cl^-$-sensitive dye MQAE using the procedure described before for 6-methoxy-*N*-(3-sulfopropyl)quinolinium (SPQ; 18) with minor modifications. SMG cells were suspended in PSA containing 10 mM MQAE and incubated for 20 min at room temperature and 40 min at 0 °C before plating on coverslips. About 2 min after plating, unattached cells and external MQAE were washed by starting the perfusion with solution A. MQAE fluorescence was measured at an excitation wavelength of 360 nm with the dichroic mirror and emission cut-off filter set normally used to monitor Fura-2 fluorescence. At the end of each experiment a two-point calibration procedure was performed. To obtain the maximal fluorescence the cells were perfused with high $K^+$, $Cl^-$-free solution containing 5 μM nigericin and 10 μM tributyltin cyanide. Incubation in a $Cl^-$-free solution without ionophores did not result in complete depletion of intracellular $Cl^-$. To obtain the minimal fluorescence the cells were then exposed to a solution containing 127 mM KSCN. Significant dye leak, in particular after exposure to tributyltin cyanide, precluded a more extensive *in vivo* calibration. A Stern-Volmer constant of 12.4 $M^{-1}$ reported before for rabbit SMG ducts (34) was used to calculate $[Cl^-]_i$.

The results of multiple experiments with each cell type and under the

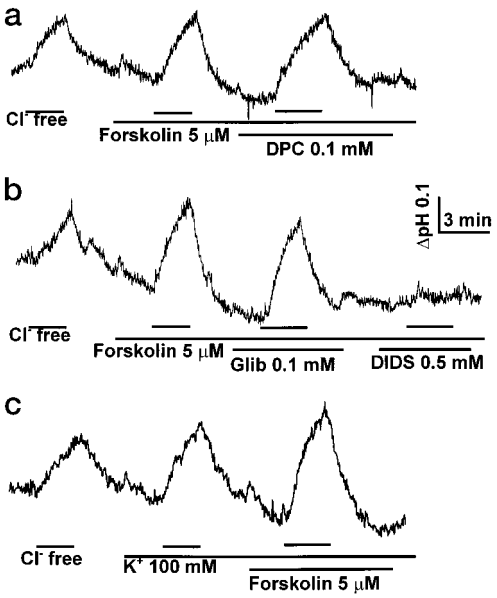


FIG. 1. **AE activity in T84 cells.** T84 cells attached to coverslips and loaded with BCECF were bathed in a $HCO_3^-$-buffered solution. As indicated by the *bars*, $Cl^-$ was removed and added to the perfusing medium before and after stimulation with 5 $\mu$M forskolin. The cells were also incubated with 0.1 mM *N*-phenylanthranilic acid (*DPC*) (*panel a*), 0.1 mM glibenclamide (*Glib*), and then 0.5 mM DIDS (*panel b*), or high $K^+$-containing medium (*panel c*) before and during removal and addition of $Cl^-$. *Upper deflection* in all traces indicates an increase in $pH_i$. The results of multiple experiments are summarized in Fig. 2.

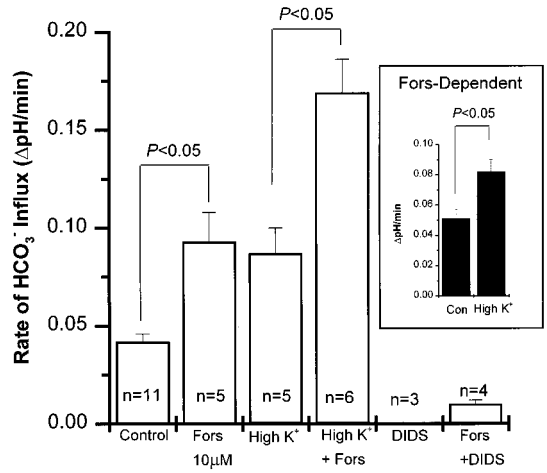


FIG. 2. **Properties of AE activity in T84 cells.** The protocols of Fig. 1 were used to evaluate the effect of forskolin on AE activity in the presence of normal or high $K^+$ media and the effect of DIDS on AE activity before and after stimulation with forskolin. The *inset* plots the forskolin-stimulated AE activity under normal and high $K^+$ conditions. The figure shows the mean ± S.E. of the indicated number of experiments.

different conditions were analyzed using paired or nonpaired Student's *t* test, as appropriate.

## RESULTS

*Regulation of AE Activity in T84 Cells*—We have described previously the regulation of AE activity by CFTR in cells stably or transiently transfected with CFTR (28). The purpose of the present work was to determine whether such regulation exists in a cell line and in native cells naturally expressing CFTR. The first set of experiments was performed with the human colonic cell line T84, which can serve as a suitable model system in future studies. This cell line has been used in the past in several studies as a model system to characterize natively expressed CFTR (32, 33).

Fig. 1 shows representative experiments, and Fig. 2 summarizes the results under each experimental condition. DIDS-sensitive, $Cl^-$- and $HCO_3^-$-dependent changes in $pH_i$ indicate the expression of relatively modest AE activity in T84 cells. Stimulation of the cells with 5 $\mu$M forskolin caused a reproducible reduction in $pH_i$. This reduction in $pH_i$ was less pronounced than that observed in NIH 3T3 and HEK 293 cells expressing high levels of CFTR. Removal and addition of $Cl^-$ to the incubation medium showed that forskolin increased the rate of AE activity by about 2.2-fold or 0.051 ΔpH unit/min. As was found in NIH 3T3 and HEK 293 cells expressing CFTR (28), the AE activity stimulated by forskolin was not affected by inhibition of CFTR-mediated $Cl^-$ current with 0.1 mM *N*-phenylanthranilic acid (*DPC* in Fig. 1*a*) or 0.1 mM glibenclamide (*Glib* in Fig. 1*b*). On the other hand, the AE activity was nearly abolished by 0.5 mM DIDS (Fig. 1*b*), as was found in 293 cells expressing modest levels of CFTR (see Fig. 7*b* of Ref. 28).

The potential regulation or function of CFTR as a $HCO_3^-$ channel (20, 35–37) raised the possibility that the rate and extent of $HCO_3^-$ influx during $Cl^-$ removal are underestimated because of the CFTR-dependent efflux of $HCO_3^-$ which entered the cells through the anion exchanger. To test this possibility we measured the effect of membrane depolarization on $HCO_3^-$ fluxes. In Figs. 1*c* and 2, T84 cells were depolarized by raising the external $K^+$ concentration from 5 to 100 mM. This had a minor effect on $pH_i$. Membrane depolarization nearly doubled the initial rate of $HCO_3^-$ influx observed upon $Cl^-$ removal (Figs. 1*c* and Fig. 2, *first* and *third bars* from *left*). Stimulation with forskolin of cells bathed in high $K^+$ medium increased AE activity by about 1.9-fold (compare *third* and *fourth bars* of Fig. 2), similar to the stimulation found in the presence of 5 mM external $K^+$ (*first* and *second bars* in Fig. 2). However, in the presence of high external $K^+$, forskolin stimulation increased the absolute rate of $HCO_3^-$ influx by 0.082 ΔpH/min, which was approximately 1.6-fold higher than that found in normal $K^+$ medium (Fig. 2, *inset*). The simplest interpretation of these results is that membrane depolarization increased the steady-state level of intracellular $Cl^-$ (see below). If the internal $Cl^-$ site of the AE was not saturated with $Cl^-$ present in the cells under normal conditions, the increased $[Cl^-]_i$ caused by membrane depolarization will increase the rate of $Cl^-_{in}/HCO_3^-{}_{out}$ exchange. Another contributing factor can be reduction in a potential $HCO_3^-$ permeability under depolarized conditions. An additional implication of the findings in Fig. 1*c* is that most of the $HCO_3^-$ fluxes induced by changes in transcellular $Cl^-$ gradients are caused by the electroneutral AE activity.

*AE Activity in the SMG of WT and ΔF/ΔF Mice*—A critical aspect of the regulation of AE activity by CFTR is to determine whether it occurs in native CFTR-expressing cells. We elected to study the relationship between the two proteins in the mouse SMG and pancreatic ducts because of the availability of the ΔF/ΔF mouse strain. The rat and mouse SMG and pancreatic ducts express a high level of CFTR protein in the luminal membrane (16) and $Cl^-/HCO_3^-$ exchange activity in the basolateral and luminal membranes (17, 18). However, a previous study in the perfused main duct of the mouse SMG suggested very low, if any, $Cl^-/HCO_3^-$ exchange activity in either membrane of this duct (30). We reevaluated these findings by measuring $Cl^-/HCO_3^-$ exchange activity in the intralobular duct of the mouse SMG. As illustrated in Fig. 3, *a* and *b*, we found high variability in $Cl^-/HCO_3^-$ exchange activity in isolated SMG ducts. The traces in Fig. 3, *a* and *b*, represent the range of high and low AE activity in SMD ducts from WT mice, respectively. Fig. 3*c* shows AE activity in a SMG duct fragment from a ΔF/ΔF mouse. The AE activity of ducts from mutant mice was less variable, as reflected in the averaged results (Fig. 3*d*). In a

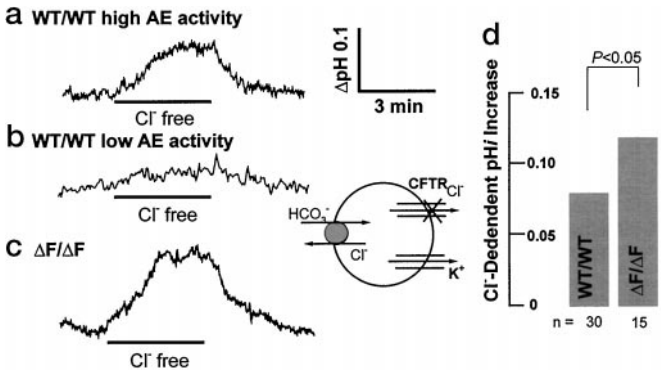


FIG. 3. **AE activity in the SMG duct of WT and ΔF/ΔF mice.** Ducts were isolated from the SMG of WT (*panels a* and *b*) or ΔF/ΔF (*panel c*) mice and used to measure $pH_i$ as described under "Experimental Procedures." All solutions were buffered with $HCO_3^-$. Where indicated by *bars*, $Cl^-$ was removed and added to the incubation medium. *Panel d* shows the summary of the results of multiple experiments performed with ducts from 30 WT and 15 ΔF/ΔF mice. The model in the figure shows the possible relationship between the illustrated transporters to explain the results in *panel d*.

significant number of experiments it was difficult to estimate accurately the initial rate of $HCO_3^-$ efflux. Therefore in these experiments we elected to evaluate AE activity from the extent of $pH_i$ changes caused by $Cl^-$ removal, which gave more reproducible results and allowed us to include all of the experiments performed in the statistical analysis.

Fig. 3*d* shows that removal of $Cl^-$ increased $pH_i$ by about 0.078 ± 0.006 pH unit in ducts from WT animals. The same protocol was used to evaluate AE activity in SMG ducts from ΔF/ΔF mice. Because of the regulation of AE activity by CFTR demonstrated before (28), we expected to find the same or lower AE activity in the SMG duct of ΔF/ΔF mice. Surprisingly, AE activity in ducts isolated from SMG of ΔF/ΔF mice was significantly higher than in ducts isolated from the SMG of WT mice (Fig. 3, *c* and *d*).

*$[Cl^-]_i$ in SMG Ducts of WT Mice*—A potential explanation for the paradoxical findings above is illustrated in the model in Fig. 3. If CFTR was at least partially active in unstimulated cells from WT mice, and resting membrane potential was similar in ducts from WT and ΔF/ΔF mice, the steady-state $[Cl^-]_i$ is expected to be variable and lower in SMG from WT mice. To test this possibility we measured $[Cl^-]_i$ in SMG ducts with the aid of the $Cl^-$-sensitive dye MQAE. The results are summarized in Table I. In 17 ducts from 7 mice $[Cl^-]_i$ in unstimulated cells averaged about 24 mM. After a 5–10 min stimulation with forskolin there was a slight increase in steady-state $[Cl^-]_i$ by about 1.5 mM, which did not reach statistical significance. On the other hand, depolarizing the cells with 100 mM external $K^+$ or 5 mM $Ba^{2+}$ significantly increased steady-state $[Cl^-]_i$ by about 5 mM. Also under depolarized conditions forskolin had no effect on steady-state $[Cl^-]_i$. The overall increase in $[Cl^-]_i$ by membrane depolarization with high $K^+$ and $Ba^{2+}$ averaged 4.8 ± 0.3 mM. This can account for most (but not all) of the increase in $Cl^-_{in}/HCO_{3\ out}^-$ exchange activity under depolarized conditions shown below. Thus, depolarization increased $pH_i$ by an additional 0.088 ± 0.007 pH unit. With a buffer capacity at $pH_i$ 7.3 and in the presence of $HCO_3^-$ of about 72 mM $H^+$ or $HCO_3^-$/pH unit (38), this amounts to 6.3 ± 0.5 mM base equivalents. Hence, the increase in steady-state $[Cl^-]_i$ can account for about 75% (4.8/6.3) of the increased $HCO_3^-$ influx. The remaining portion can be explained by reduced $HCO_3^-$ leakage under the depolarized conditions. The possible contribution of a $HCO_3^-$ leak pathway is consistent with our previous reports of a $Ba^{2+}$-sensitive and agonist-regulated $H^+/HCO_3^-$ leak pathway in the luminal membrane of the SMG duct (18, 29, 38). Not

TABLE I

*$[Cl^-]_i$ in control and depolarized submandibular duct cells*

SMG ducts were loaded with MQAE and perfused with $HCO_3^-$-buffered solution B for at least 10 min before exposure to high $K^+$ medium and/or stimulation with 5 μM forskolin. At the end of each experiment fluorescence was calibrated to estimate $[Cl^-]_i$ as detailed under "Experimental Procedures."

| | $[Cl^-]_i$ | $\Delta[Cl^-]_i$ |
|---|---|---|
| | *mM* | *mM* |
| Control ($n$ = 17) | 23.7 ± 1.9 | — |
| Forskolin ($n$ = 4) | 25.3 ± 2.1 | 1.6 |
| High $K^+$ ($n$ = 7) | 28.9 ± 1.2* | 5.2 |
| High $K^+$ + forskolin ($n$ = 5) | 28.6 ± 1.1* | 4.9 |
| $Ba^{2+}$ ($n$ = 4) | 28.0 ± 0.7* | 4.3 |

* $p < 0.05$ relative to control.

only did membrane depolarization increase $[Cl^-]_i$, but it also reduced the variability of $[Cl^-]_i$ in unstimulated cells (Table I), which facilitated evaluation of $Cl^-/HCO_3^-$ exchange activity in cells from WT and ΔF/ΔF mice. Hence, in most subsequent experiments $Cl^-/HCO_3^-$ exchange activity was measured under depolarized conditions.

*AE Activity under Depolarized Conditions*—The effect of membrane depolarization with $Ba^{2+}$ on $Cl^-/HCO_3^-$ exchange is illustrated in Fig. 4. In SMG ducts from WT mice, inhibition of $K^+$ conductance is expected to depolarize the membrane and reduce $Cl^-$ efflux, increasing and stabilizing steady-state $[Cl^-]_i$. As a consequence, the maximal $Cl^-/HCO_3^-$ exchange activity of these cells could be measured. In SMG ducts from ΔF/ΔF mice membrane depolarization is expected to have smaller, if any, effect on the $Cl^-$-dependent changes on $pH_i$. Importantly, membrane depolarization minimizes the contribution of all $Cl^-$ channels expressed in these cells (39) to allow a better comparison between the electroneutral $Cl^-/HCO_3^-$ exchange activity of ducts from WT and ΔF/ΔF mice. The traces of an individual experiment (Fig. 4, *a* and *b*) and the averages obtained from multiple experiments (Fig. 4*c*) show that this was indeed the case.

In these experiments Ba-Hepes (0.5 M, pH 7.4) was added to $HCO_3^-$-buffered solutions in which $Cl^-$ was replaced by gluconate. These solutions were clear for about 30 min, after which precipitates, probably of Ba-gluconate, started to form. At this time old solutions were replaced with fresh $Ba^{2+}$-containing, $Cl^-$-free solutions. Exposing SMG ducts bathed in a $HCO_3^-$-buffered medium to 5 mM $Ba^{2+}$ caused a small and reproducible reduction in $pH_i$, the cause of which was not investigated in the present work. Membrane depolarization with $Ba^{2+}$ increased the apparent AE activity by more than 2-fold in SMG ducts from WT mice. By contrast, $Ba^{2+}$ had a small, statistically insignificant effect on AE activity of SMG ducts from ΔF/ΔF mice. Furthermore, in the presence of $Ba^{2+}$, the AE activity of SMG duct from WT mice tended to be higher than that of ducts from ΔF/ΔF mice, although it did not reach statistical significance ($p$ = 0.083). Finally, the $Cl^-$-dependent changes in $pH_i$ were blocked completely by DIDS (Fig. 4, *a* and *b*).

*Regulation of AE Activity by Forskolin*—The effect of forskolin stimulation on AE activity of SMG duct from WT and ΔF/ΔF mice is shown in Fig. 5. Fold stimulation was determined from the extent of $pH_i$ changes caused by $Cl^-$ removal and addition before and after forskolin stimulation of the same duct fragments. Even in the absence of $Ba^{2+}$, stimulation of SMG duct from WT mice with forskolin increased AE activity by about 1.7-fold. By contrast, and as expected, forskolin had no effect on the AE activity of ducts isolated from the SMG of ΔF/ΔF mice. We noticed that multiple removals and additions of $Cl^-$ to the incubation medium resulted in a slightly reduced AE activity in each successive round. Thus, the small, frequently observed

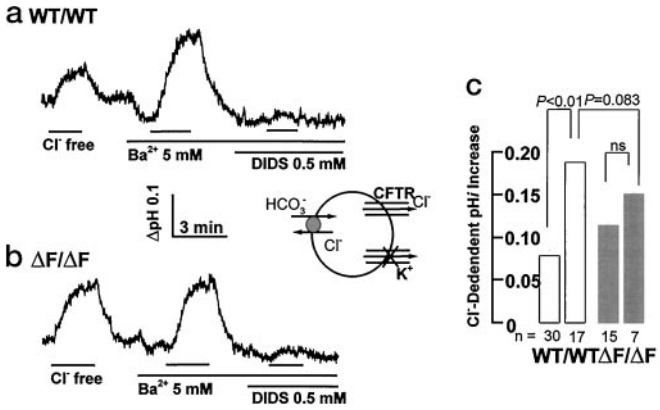


FIG. 4. **Effect of membrane depolarization on AE activity in SMG ducts from WT and ΔF/ΔF mice.** As indicated by the *bars*, ducts from WT (*panel a*) or ΔF/ΔF (*panel b*) mice were exposed to $Cl^-$-free medium in the absence or presence of 5 mM $Ba^{2+}$. All solutions were buffered with 5 mM Hepes and 25 mM $CO_2/HCO_3^-$. *Panel c* shows the mean ± S.E. of the number of experiments performed under each condition.

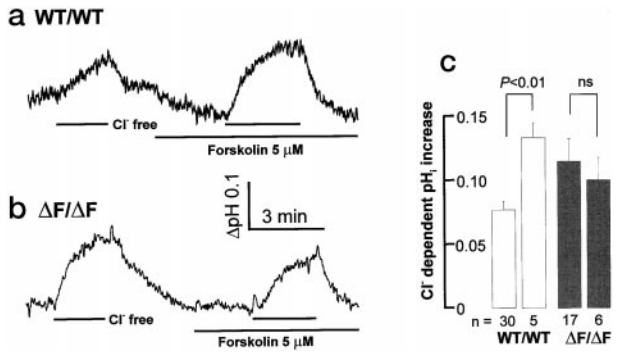


FIG. 5. **Effect of forskolin on AE activity in SMG duct from WT and ΔF/ΔF mice.** The AE activity of SMG ducts from WT (*panel a*) or ΔF/ΔF mice (*panel b*) was measured before and after stimulation with 5 μM forskolin. *Panel c* shows the mean ± S.E. of all experiments performed. Note that forskolin stimulated AE activity of SMG ducts from WT but not ΔF/ΔF mice.

reduction in AE activity in forskolin-stimulated ducts from ΔF/ΔF mice is probably the result of this artifact. Because in most experiments a control test preceded an experimental test, the effect of forskolin on AE activity in SMG duct from WT mice is probably underestimated. Nevertheless, it was sufficiently large to be highly statistically significant (Fig. 5*c*).

To determine the actual stimulation of AE by CFTR in SMG duct we measured the effect of forskolin in ducts treated with 5 mM $Ba^{2+}$. Fig. 6 shows that forskolin stimulation of SMG ducts from WT mice, but not from ΔF/ΔF mice, incubated in $Ba^{2+}$-containing solutions increased AE activity. In SMG ducts from WT mice, forskolin increased $pH_i$ caused by $Cl^-$ removal by 0.06 pH unit above that measured in the same unstimulated ducts, which consists of an approximately 1.3-fold stimulation. However, when $pH_i$ changes caused by AE activity are compared in forskolin-stimulated ducts from WT and ΔF/ΔF mice, $pH_i$ changes in WT ducts are higher by about 0.12 pH unit, which is 1.9-fold above that measured in ducts from ΔF/ΔF mice. This stimulation is comparable to that found in the absence of $Ba^{2+}$ as illustrated in Fig. 5.

To avoid the possibility of a nonspecific effect of $Ba^{2+}$, we tested the effect of high $K^+$ on AE activity in SMG ducts from WT mice. Fig. 7 shows that depolarizing the membrane potential with 100 mM $K^+$ was as effective as 5 mM $Ba^{2+}$ in unmasking the maximal AE activity. As expected, the $Cl^-$-dependent $pH_i$ changes were inhibited by DIDS (Fig. 7*a*). Stimulation of the ducts with forskolin increased the AE activity of these

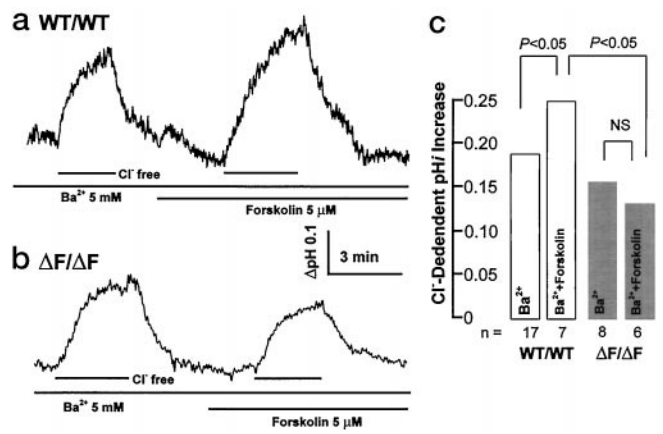


FIG. 6. **Stimulation of AE activity by forskolin in the presence of $Ba^{2+}$.** SMG ducts from WT (*panel a*) or ΔF/ΔF (*panel b*) mice were incubated in $HCO_3^-$-buffered solutions containing 5 mM $Ba^{2+}$ throughout the experiment. As indicated by the *bars*, the ducts were exposed to $Cl^-$-free solutions before and after stimulation with 5 μM forskolin. *Panel c* shows the summary of all experiments performed in terms of mean ± S.E. Note that forskolin-stimulated ducts from WT mice showed higher AE activity than nonstimulated ducts from WT mice and forskolin-stimulated ducts from ΔF/ΔF mice.

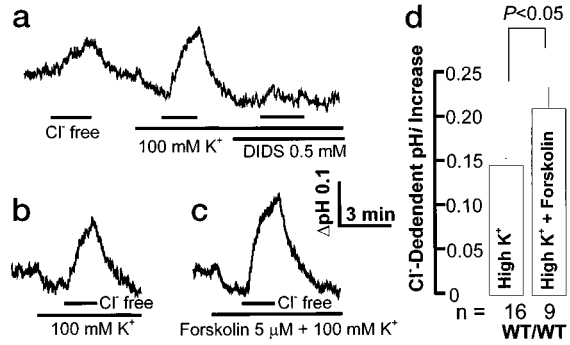


FIG. 7. **Effect of high $K^+$ on $pH_i$ changes in SMG duct from WT mice.** SMG ducts from WT mice were incubated in $HCO_3^-$-buffered solutions. As indicated by the *bars*, the ducts were exposed to $Cl^-$-free solutions in the presence of 5 or 100 mM $K^+$ before (*panels a* and *b*) or after stimulation with 5 μM forskolin (*panel c*). *Panel d* illustrates the mean ± S.E. of the indicated number of experiments.

ducts. In these experiments we compared the AE activity measured in the first exposure of all ducts to $Cl^-$-free medium (Fig. 7, *b* and *c*). The forskolin-stimulated increases in $pH_i$ in the presence of 100 mM $K^+$ or 5 mM $Ba^{2+}$ were similar.

*Membrane-specific Regulation of $Cl^-/HCO_3^-$ Exchange by CFTR in Pancreatic Ducts*—CFTR-expressing cells are likely to express more than one anion exchanger, the housekeeping AE2 in the basolateral membrane and as yet unidentified AE isoform or other exchanger protein involved in transcellular $HCO_3^-$ transport in the luminal membrane. Indeed, AE activity was measured previously in both membranes of the SMG (18) and pancreatic ducts (17). To extend our findings to another native CFTR-expressing tissue and determine the membrane localization of the AE activity regulated by CFTR, we measured luminal and basolateral AE activity in the microperfused pancreatic ducts of WT and ΔF/ΔF mice. Fig. 8 summarizes the results of multiple experiments. It was satisfying to find that stimulation with forskolin exclusively increased the activity of the luminal AE without affecting the basolateral AE in pancreatic ducts from WT mice. Furthermore, such regulation was absent in ducts from ΔF/ΔF animals. In the first protocol we measured AE activity under polarized conditions (Fig. 8*a*). As we reported before for the rat duct, luminal AE activity was higher than basolateral AE activity in the mouse pancreatic

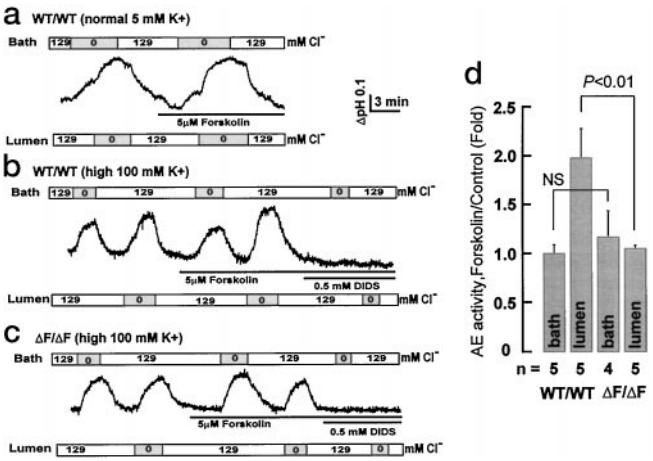


FIG. 8. **CFTR stimulates the luminal but not the basolateral AE activity in the pancreatic ducts.** The main pancreatic ducts of WT (*panels a* and *b*) and ΔF/ΔF (*panel c*) mice were cannulated, dissected, and perfused through the luminal and basolateral sides with the $HCO_3^-$-buffered solution B. Where indicated by the *bars*, the luminal and basolateral AE activities were measured by perfusing the bath and the lumen with a $HCO_3^-$-buffered, $Cl^-$-free solution C (*panel a*). In *panels b* and *c* the ducts were perfused with the high $K^+$, depolarizing solution D before exposure to high $K^+$, $Cl^-$-free solution E. In all experiments, as indicated by the *bars*, the ducts were stimulated with 5 $\mu$M forskolin. Forskolin was included in the luminal solution. *Panel d* summarizes the results of multiple experiments with ducts from WT and ΔF/ΔF mice.

duct (compare the rate and extent of $pH_i$ changes caused by the $Cl^-$ removal from the bath and lumen in *panels a* and *b* before forskolin stimulation). Stimulation with forskolin caused the typical initial acidification, had no effect on basolateral AE activity, and prominently increased luminal AE activity in ducts from WT mice. Forskolin had no effect on luminal or basolateral AE activity of ducts from ΔF/ΔF mice (data not shown).

To obtain a better estimate of the AE activity in each membrane and under resting and stimulated conditions, we measured separately the basolateral and luminal AE activity under depolarized conditions. The *left part* of Fig. 8*b* shows the higher rate (and extent) of $pH_i$ changes caused by $Cl^-$ removal and addition to the lumen (and thus the higher AE activity) of pancreatic duct from WT mice. The *right part* of the trace shows that stimulation with forskolin had no effect on the basolateral AE activity but increased the initial rate (and extent) of $pH_i$ changes because of removal of luminal $[Cl^-]_{out}$ in the same duct. Both activities were inhibited completely by 0.5 mM DIDS. By contrast, forskolin had no effect on basolateral and luminal AE activity of ducts from ΔF/ΔF mice (Fig. 8*c*). Hence, we can conclude that CFTR regulates the luminal but not the basolateral AE activity.

## DISCUSSION

In the present work we used the SMG and pancreatic ducts of WT and ΔF/ΔF mice to study regulation of $Cl^-/HCO_3^-$ exchange activity by CFTR. Along with our experience with these cells, the SMG and pancreatic ducts offer other advantages as model systems. Both ducts express high levels of CFTR (16) and basolateral and luminal AE activity (17, 18). In addition, the SMG offers the availability of several experimental preparations from microperfused ducts (2, 18) to isolated single cells (2, 16); and most importantly, among all CFTR-expressing tissues, the mechanisms of fluid and electrolyte transport are understood best in the SMG (2).

In agreement with our previous work in the rat SMG duct (18), in the present work we were able to demonstrate AE activity in the mouse SMG duct. The evidence includes a DIDS-sensitive, $Na^+_{out}$-independent, electroneutral $Cl^-_{in}$-dependent $HCO_3^-$ influx and $Cl^-_{out}$-dependent $HCO_3^-$ efflux. By contrast, a work with the main SMG duct concluded low or no AE activity in the mouse duct (30). This discrepancy may be the result of sufficiently high $Cl^-$ and/or $HCO_3^-$ conductance in the previous study (30) which masked the AE activity.

We had a particular interest in evaluating $HCO_3^-$ conductance in native CFTR-expressing cells and its possible regulation by CFTR. In a recent work, Ishguro *et al.* (6) reported the intriguing finding that luminal AE mediated $HCO_3^-$ transport in resting but not cAMP-stimulated guinea pig pancreatic ducts. In addition, CFTR-dependent $HCO_3^-$ conductance was reported in pancreatic duct cells (35) and in an airway epithelial cell line expressing CFTR (20). This would imply that stimulation of CFTR should dramatically increase the $HCO_3^-$ conductance of the luminal membrane. In the present work we were unable to obtain evidence in support of such a conclusion. Our results in the mouse are in agreement with the finding that $HCO_3^-$ conductance is at least 6-fold lower than that of $Cl^-$ (20, 35) and the absence of $HCO_3^-$ conductance in sweat duct (40). Hence, in the presence of physiological $Cl^-$ and $HCO_3^-$ gradients it is not likely that CFTR mediates or modulates the $HCO_3^-$ conductance in the luminal membrane of the rat and mouse secretory epithelia. Accordingly, the electrogenic component of $HCO_3^-$ transport in T84 cells (Fig. 1) and SMG ducts (Figs. 4 and 7) was rather small and not affected by inhibitors of CFTR $Cl^-$ channel activity (Fig. 1 and not shown). Furthermore, stable and transient transfection of CFTR in NIH 3T3 and HEK 293 cells, respectively, did not increase $HCO_3^-$ conductance even when the cells were stimulated with forskolin (compare the effect of high $K^+$ in Fig. 10 of Ref. 28 and Figs. 1*c* and 7 of this paper).

Low luminal $HCO_3^-$ conductance in the mouse SMG and pancreatic duct cannot be extended to other species and tissues. This is because, most likely, different mechanisms mediate $HCO_3^-$ transport in different tissues and species. For example, the electrogenic component of $HCO_3^-$ transport is much higher in the duodenum than in the colon (3) or the SMG (2). The pancreatic juice of the guinea pig contains a much higher $HCO_3^-$ concentration than that of the rat (1, 6). However, despite this variability we can safely conclude that in tissues secreting $HCO_3^-$ in a mechanism similar to that of the mouse SMG or the pancreatic ducts (*a*) $HCO_3^-$ conductance is of secondary importance in $HCO_3^-$ secretion, and (*b*) regulation of luminal AE activity by CFTR may be the major mechanism by which CFTR regulates $HCO_3^-$ secretion. In this respect it would be of particular significance to test the effect of CFTR stimulation on luminal AE activity and $HCO_3^-$ conductance in guinea pig SMG and pancreatic ducts.

The major finding of the present work was extending the finding of regulation of AE activity by CFTR to native CFTR-expressing tissues such as the SMG and pancreas. Even though the increase in AE activity after forskolin stimulation of SMG duct was not as prominent as that observed in the transfected cell lines (28), it could be clearly demonstrated even when the cells were not depolarized (Fig. 5). It is important to note that the extent of stimulation of AE activity by CFTR in SMG ducts may be significantly underestimated. Studies on the rat SMG duct showed the presence of AE activity in both the BLM and LM (18). Expression of CFTR in the LM of SMG ducts (16) indicates that CFTR should stimulate AE activity present in the LM but not in the BLM of SMG ducts. Hence, AE activity in the BLM, although lower than that in the LM (18), increases the background against which the stimulation of AE activity by CFTR is evaluated. This reasoning is reinforced by the finding

that CFTR stimulates the luminal but not the basolateral AE activity of the pancreatic duct. Notably, in the SMG duct, when the AE activity of both membranes contributed to the measurement, stimulation of CFTR increased AE activity by about 43% (Fig. 7). Under the same depolarized conditions CFTR increased luminal AE activity of the pancreatic duct by about 96% (Fig. 8).

The exclusive expression of CFTR in the luminal membrane of the SMG duct and other CFTR-expressing cells indicates that also in these tissues CFTR regulates the luminal AE. To date the protein responsible for the luminal AE activity of CFTR-expressing cells has not been identified. Good immunocytochemical evidence indicates that the housekeeping AE2 is expressed exclusively in the basolateral membrane of SMG duct and acinar cells (19). This excludes AE2 as the isoform regulated by CFTR. It has been suggested that a variant of AE1 is expressed alternatively in the basolateral or luminal membrane of intercalated cells of collecting duct based on the metabolic state of the animal (41). However, preliminary reverse transcriptase-polymerase chain reaction analysis of the AE isoforms expressed in the cell lines used in the present paper and in Ref. 28 indicates that these cells express only AE2 and AE3 (not shown). Hence, it is possible that CFTR regulates AE3 and that AE3 is the isoform expressed in the luminal membrane of the SMG duct. Another alternative is that a protein other than the known AE isoforms mediates the luminal AE activity. Using expression systems we are attempting to test the effect of CFTR on the activity of each AE isoform.

Our findings provide new insight into the mechanisms of fluid and electrolyte secretion by CFTR-expressing cells and into the pathophysiology of cystic fibrosis. An important function of CFTR-expressing cells is the secretion of $HCO_3^-$ (1–3, 7, 9, 12, 25–27). $HCO_3^-$ is a chaotropic anion that is commonly used to dissolve, and thus, strip membranes of peripheral proteins. Moreover, $HCO_3^-$ regulates the pH of biological fluids. Fluids secreted by CFTR-expressing cells are rich in proteins, in particular mucins (1–3, 7, 25–27). Mucin solubility may be increased in high $HCO_3^-$, high pH fluids (42). In the special case of the exocrine pancreas, the high pH is also needed to prevent premature activation of harmful digestive enzymes (1). Impaired $HCO_3^-$ secretion may cause precipitation of mucins and thus play a major role in obstruction of almost all ductal systems in cystic fibrosis (43). Every model of luminal $HCO_3^-$ secretion in CFTR-expressing cells suggests that AE activity mediates the electroneutral portion of $HCO_3^-$ secretion (1–3, 7). By demonstrating regulation of AE activity by an activated CFTR, our findings indicate that CFTR directly regulates the entire process of $HCO_3^-$ secretion. Furthermore, the suggested regulation of luminal $Na^+$ channel by CFTR (44) allows the regulation of $Na^+$ absorption by CFTR. Several CFTR-expressing cells, such as the SMG duct, while absorbing $Na^+$ secrete isotonic $K^+$ (2). The luminal pathway responsible for $K^+$ efflux is believed to be an inward rectifier $K^+$ channel such as ROMK II in the kidney (3). CFTR has also been implicated as a regulator of ROMK II (45). Hence, CFTR must be viewed as a global regulator of epithelial fluid and electrolyte transport through its ability to function as a $Cl^-$ channel (46) and regulate $Cl^-$ absorption, regulate the epithelial $Na^+$ channel to regulate $Na^+$ absorption, regulate the luminal $K^+$ channel to regulate $K^+$ secretion, and regulate AE activity to regulate $HCO_3^-$ secretion.

The global role of CFTR indicates that its action is not likely to be replaced solely by activation of luminal $Cl^-$ channels through stimulation of selective $P_2$ purinoceptors. Again, the finding that cholinergic stimulation of intestinal $HCO_3^-$ secretion is abolished in the intestines of CFTR −/− mice (24) supports the global role of CFTR in epithelial fluid and electrolyte secretion. Cholinergic stimulation is expected to activate the luminal $Ca^{2+}$-activated $Cl^-$ channel found in many epithelial cells (47). Activation of this $Cl^-$ channel was not sufficient to cause normal $HCO_3^-$ secretion in cholinergically stimulated intestinal epithelium (24). Hence, although stimulation of luminal purinoceptors may induce $Cl^-$ secretion, it is not likely to be sufficient in itself in alleviating the symptoms of cystic fibrosis. Thus, improved expression of CFTR in the luminal membrane seems to be the best option in this endeavor.

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# Cystic Fibrosis Transmembrane Conductance Regulator Regulates Luminal $Cl^-/HCO_3^-$ Exchange in Mouse Submandibular and Pancreatic Ducts

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