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Intestinal Bicarbonate Secretion in Cystic Fibrosis Mice

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Summary

Gene-targeted disruption of the cystic fibrosis transmembrane conductance regulator (CFTR) in mice results in an intestinal disease phenotype that is remarkably similar to bowel disease in cystic fibrosis patients. In the intestinal segment downstream from the stomach (i.e., the duodenum), CFTR plays an important role in bicarbonate secretion that protects the epithelium from acidic gastric effluent. In this report, we examine the role of CFTR in cAMP-stimulated bicarbonate secretion in the murine duodenum and the mechanisms of acid-base transport that are revealed in CFTR knockout (CF) mice. Ion substitution, channel blocker and pH stat studies comparing duodena from wild-type and CF mice indicate that CFTR mediates a HCO_3^- conductance across the apical membrane of the epithelium. In the presence of a favorable cell-to-lumen HCO_3^- gradient, the CFTR-mediated HCO_3^- current accounts for about 80% of stimulated HCO_3^- secretion. Exposure of the duodenal mucosa to acidic pH reveals another role of CFTR in facilitating HCO_3^- secretion via an electroneutral, 4,4'-diisothiocyanato-stilbene-2,2' disulfonic acid (DIDS) sensitive-Cl^-/HCO_3^- exchange process. In CF duodenum, other apical membrane acid-base transporters retain function, thereby affording limited control of transepithelial pH. Activity of a Cl^--dependent anion exchanger provides near-constant HCO_3^- secretion in CF intestine, but under basal conditions the magnitude of secretion is lessened by simultaneous activity of a Na^+/H^+ exchanger (NHE). During cAMP stimulation of CF duodenum, a small increase in net base secretion is measured but the change results from cAMP inhibition of NHE activity rather than increased HCO_3^- secretion. Interestingly, a small inward current that is sensitive to the anion channel blocker, 5-nitro-2(3-phenylpropyl amino)-benzoate (NPPB), is also activated during cAMP stimulation of the CFTR-null intestine but the identity of the current is yet to be resolved. Studies to identify the proteins involved in non-CFTR mediated HCO_3^- secretion are on-going and potentially will provide targets to correct deficient HCO_3^- secretion in the CF intestine.

In cystic fibrosis patients and CFTR knockout mice, duodenal bicarbonate transport is greatly diminished, resulting in abnormal pH regulation at the mucosal surface. Two transport pathways at the apical cell membrane are involved in bicarbonate secretion - an anion conductance(s) and Cl^-/HCO_3^- (OH^-) exchanger(s). Stimulation of intracellular cAMP yields electrogenic bicarbonate secretion that requires the activity of CFTR to either provide a bicarbonate conductance and/or a chloride conductance that recycles Cl^- entering the cell *via* a Cl^-/HCO_3^- exchanger. Although patch clamp and apical membrane preparation studies have shown that CFTR is moderately permeable to HCO_3^-

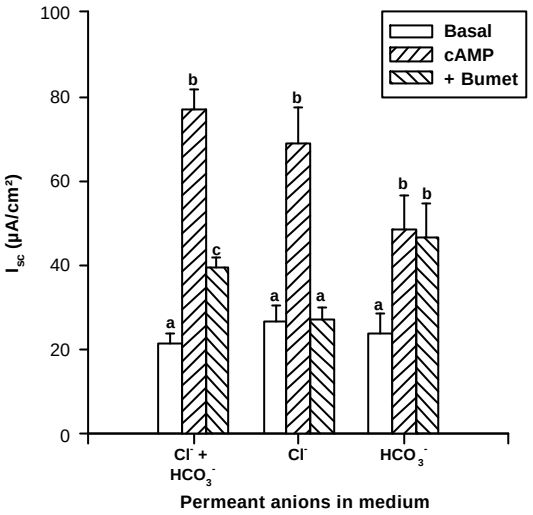

Figure 1. Effect of anion substitution in the bathing medium on basal and cAMP-stimulated short-circuit current across wild-type murine duodenum. Transepithelial short-circuit current (I_{sc}, an index of anion secretion) was measured in Ringers media containing both chloride and bicarbonate (Cl^-+ HCO3-), only chloride (Cl^-) or only bicarbonate (HCO_3^-) as CFTR-permeant anions. Measurements were made during sequential periods: Basal, during cAMP stimulation by bilateral addition of 10 µM forskolin + 100 µM isobutyl methylxanthine (cAMP), and following addition of 100 µM bumetanide (an inhibitor of Cl^- secretion) to the serosal bath (Bumet). The data provide evidence that CFTR carries bicarbonate current during cAMP stimulation. First, a significant bumetanide-insensitive I_{sc} is present only when HCO_3^- is present in the bathing medium. Second, a significant cAMP current is stimulated when HCO_3^- is the only permeant anion available for transport by CFTR. In contrast, these responses are minimal in CFTR knockout duodenum (data not shown). Letters indicate differences between means within each group, $p < 0.05$.

(P_{Cl}:P_{HCO_3} = about 0.25) [1, 2, 3] it has been difficult to determine whether CFTR mediates a bicarbonate conductance under physiological conditions in native duodenal epithelium. Two lines of evidence have emerged. First, *in vivo* measurements and pH stat studies have shown that CFTR is required for electrogenic bicarbonate secretion under conditions that inhibit apical membrane Cl^-/HCO_3^- exchange activity [4, 5, 6, 7, 8]. Second, anion substitution studies of intact duodenal mucosa, such as the study of murine duodenum shown in Figure 1, indicate that cAMP-stimulated CFTR can carry a bumetanide-insensitive HCO_3^- current when other anions that have significant permeability in CFTR are removed from the bathing medium.

Interestingly, careful examination of CFTR knockout duodenum reveals a finite increase in bicarbonate (base) secretion in response to cAMP stimulation. As shown in Figure 2a, bicarbonate secretion ($J_{sm}^{HCO_3}$) is minimal under basal conditions. Following treatment with forskolin (cAMP), $J_{sm}^{HCO_3}$ increases by 1 µEq/cm²·h and this change is accompanied by a similar increase in short-circuit current (I_{sc}). Note, however, that the cAMP-induced changes

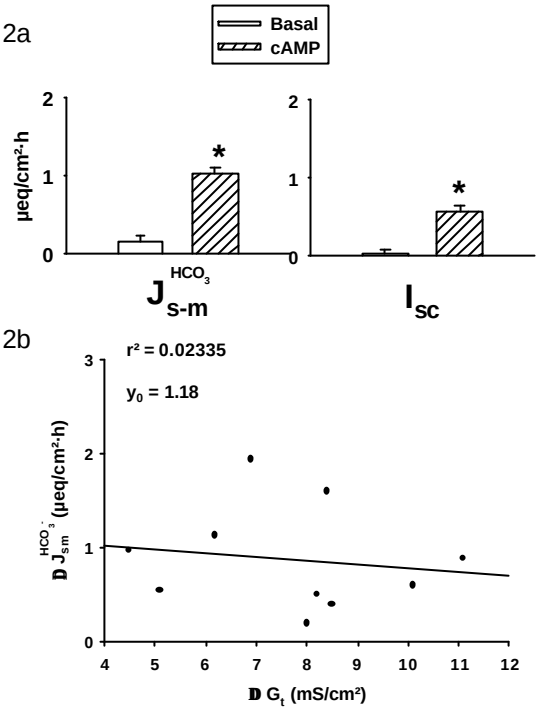

Figure 2. Effect of intracellular cAMP stimulation on bicarbonate secretion, I_{sc} and transepithelial conductance in CF muri ne duodenum. In pH stat experiments (2a) performed under voltage-clamped conditions, stimulation of intracellular cAMP with a forskolin/IBMX cocktail induces simultaneous increases in the serosal-to-mucosal flux of bicarbonate ($J_{sm}^{HCO_3}$) and the I_{sc}. However, the transepithelial conductance, G_t, was significantly increased by stimulation of cAMP (Basal G_t = 45.2 ±0.7; cAMP G_t = 553 ± 0.7, $p < 0.05$, n = 10). * Significantly different from Basal, $p < 0.05$). To estimate the effect of transepithelial conductance (2b) on the $J_{sm}^{HCO_3}$, the ΔG_t from the CF mice experiments were regressed against the $\Delta J_{sm}^{HCO_3}$. However, a significant correlation was not apparent.

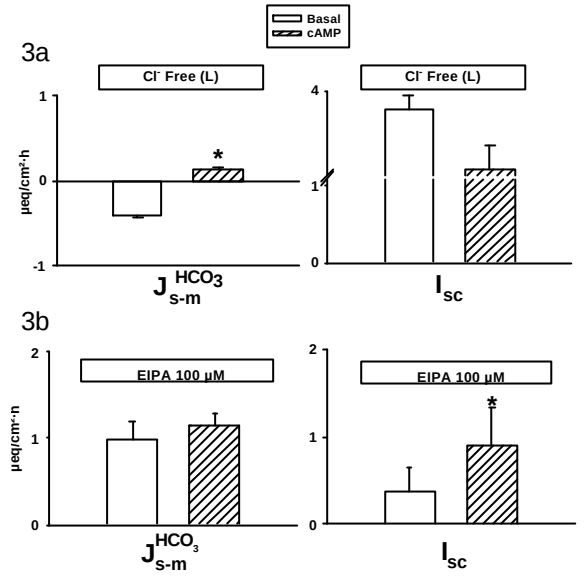

Figure 3. Effects of luminal Cl^- removal and EIPA on bicarbonate secretion and I_{sc} across CF murine duodenum. Replacing luminal Cl^- content (3a) with the poorly permeable anion, isethionate, resulted in net acid secretion by CF duodenum that was abolished by subsequent cAMP stimulation using a forskolin/IBMX cocktail. The imposed cell-to-lumen and mucosal-to-serosal chloride gradient resulted in high basal I_{sc} in the CF duodenum. However, cAMP stimulation did not increase Isc under this condition (n = 13). To determine whether a change in proton secretion via a luminal membrane Na^+/H^+ exchanger was responsible for the change in $J_{sm}^{HCO_3}$, the CF duodenum was treated with EIPA (3b), an inhibitor of intestinal Na^+/H^+ exchangers (NHE2 and NHE3). EIPA treatment resulted in a stable increase in the basal bicarbonate secretion and prevented cAMP stimulation of $J_{sm}^{HCO_3}$. However, a significant increase in I_{sc} was still apparent following forskolin/IBMX in the presence of EIPA (n = 8).
* Significantly different from Basal.

in the bioelectric properties include a significant increase in total tissue conductance (G_t), a measure of the paracellular pathway in the intestine [9]. To evaluate the possibility that the cAMP-induced $\Delta J_{sm}^{HCO_3}$ is a consequence of both the imposed transepithelial bicarbonate gradient (see Methods) and an increase in paracellular permeability, the cAMP-induced $\Delta J_{sm}^{HCO_3}$ was correlated with the ΔG_t. As shown in Figure 2b, no relationship existed between the $\Delta J_{sm}^{HCO_3}$ and ΔG_t under either condition. This finding confirms earlier studies of murine duodenum showing that $J_{sm}^{HCO_3}$ does not correlate with G_t during cAMP stimulation [10].

The above findings indicated an active bicarbonate secretory process, therefore, the involvement of luminal Cl^-/HCO_3^- exchange activity during cAMP stimulation of the CF duodenum was investigated by replacing Cl^- in the luminal bath with the poorly permeable solute, isethionate. Interestingly, this maneuver resulted in net acid secretion during the basal period that was then abolished by cAMP treatment (Figure 3a). The basal I_{sc} of the duodenum was greatly accentuated under these conditions but did not increase with cAMP treatment. Acid secretion during inhibition of luminal Cl^-/HCO_3^- exchange is consistent with activity of luminal Na^+/H^+ exchange activity as recently suggested by pH measurements of the luminal content in wild-type and NHE3 knockout mice [11]. We tested this hypothesis by exposing the luminal membrane to the NHE exchange inhibitor, 5-(N-Ethyl-N-isopropyl) amiloride (EIPA), at a concentration (100 µM) that inhibits the major intestinal isoforms, NHE2 and NHE3, in media containing physiological concentrations of Na^+ [12]. As shown in Figure 3b, EIPA treatment increased the basal $J_{sm}^{HCO_3}$ to about 1 µEq/cm²·h and prevented the increase in $J_{sm}^{HCO_3}$ during cAMP stimulation. Thus, the increase in $J_{sm}^{HCO_3}$ measured during cAMP stimulation of the CF duodenum is likely due to cAMP inhibition of NHE activity, which reveals activity of a Cl^--dependent anion exchanger(s). This latter conclusion was confirmed by the lack of an EIPA effect on the CF duodenum during Cl^- substitution in the luminal bath (data not shown).

Although the cAMP change in $J_{sm}^{HCO_3}$ was abolished by the amiloride analog EIPA, forskolin stimulated a small but significant increase in the I_{sc} of the CF duodenum. Thus, the cAMP-induced ΔI_{sc} in the CFTR knockout duodenum was dissociated from the $\Delta J_{sm}^{HCO_3}$ in both the luminal Cl^- substitution and EIPA experiments, indicating that the current is not

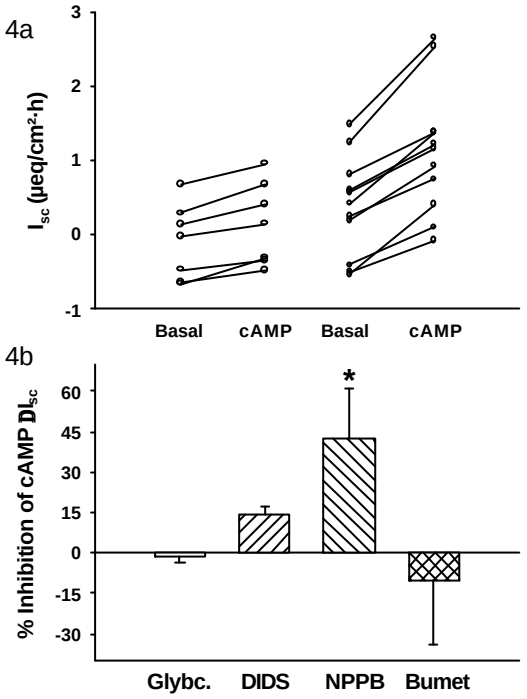

Figure 4. Inhibitor studies of the cAMP -induced I_{sc} in CF murine duodenum. Analysis of the cAMP-induced I_{sc} in the duodena from individual CF mice (4a) indicated a subpopulation of mice with robust responses to forskolin/IBMX (right). The effects of anion transport inhibitors on the cAMP-induced I_{sc} response (4b) indicated that NPPB significantly reduced the I_{sc} by 39% (n = 4 - 6).
*Significantly different from I_{sc} before treatment.

carried by HCO_3^-. Evaluation of the individual cAMP-induced ΔI_{sc} indicates the presence of a subpopulation of CF mice that have robust responses (see Figure 4a), suggesting that a cohort of CF mice surviving to adulthood may be selected for the expression of an alternate conductance. Inhibitor studies of the cAMP stimulated I_{sc} in CF duodenum indicate partial blockade by the anion conductance inhibitors, DIDS and NPPB (Figure 4b). Although the identity of the conductive pathway has not been resolved, these findings indicate the presence of an alternate cAMP-sensitive anion channel that may modify the physiological consequences of gene-targeted deletion of CFTR in murine intestine.

Methods

Animals. Wild-type (WT) and CFTR knockout (CF) mice 2-4 months of age were used. The mice were fasted overnight before experimentation (water was provided *ad-libitum*).

Ussing chamber studies. Freshly-excised duodenum was stripped of the underlying muscle layers and mounted on standard Ussing chambers with 0.25 cm² exposed surface area. All sections were treated with 1 µM indomethacin and 0.1 µM tetrodotoxin (serosal) prior to experimentation. The duodenal sections were voltage-clamped using an automatic voltage clamp (Physiologic Instruments, San Diego, CA, USA).

pH stat. The duodenal studies consisted of two sequential 30 min flux periods: a basal period and a treatment period using either 10 µM forskolin (cAMP) or 100 µM EIPA (EIPA).
All drugs were obtained from Sigma Chemicals (St. Louis, USA). The luminal surface of duodenum was bathed with 4 mL of an unbuffered NaCl solution containing (in mM): Na^+, 144.0; Cl^-, 154.0; K^+, 5.2; Ca^{2+}, 1.2; Mg^{2+}, 1.2. The mucosal bath pH was clamped at 7.4 by neutralizing the appearance of base with 5 mM HCl or acid with 5 mM NaOH using an automatic titrator (Radiometer, Radiometer Analytical, Lyon, France). The mucosal solution was gassed with 100% O_2. The serosal surface was bathed with Krebs bicarbonate Ringers solution (KBR) containing (in mM): Na^+, 140.0; Cl^-, 120.0; HCO_3^-, 25.0; $H_2PO_4^-$, 0.4; HPO_4^{2-}, 2.4; K^+, 5.2; Ca^{2+}, 1.2; Mg^{2+}, 1.2; glucose, 10; pH 7.4 (gassed with 95% O_2: 5% CO_2). Both solutions were warmed to 37 °C by water-jacketed reservoirs. For Cl^- free lumen experiments, chloride was replaced with 91 mM gluconate and either 25 mM SO_4^{2-} plus 25 mM mannitol or 50 mM isethionate.

Statistics. Paired *t*-test was used to compare two sequential treatment periods and an unpaired *t*-

test was used to compare two treatment groups. Repeated measures ANOVA was used to compare three sequential treatment periods and a one-way ANOVA was used to compare multiple treatments groups. A *p* value less than 0.05 was considered statistically significant. All data are given as means±SEM.

Key words Antiporters; Chloride Channels; Cyclic AMP; Cystic Fibrosis Transmembrane Conductance Regulator; Duodenum; Sodium-Hydrogen Antiporter

Abbreviations CF: CFTR knockout mice; CFTR: cystic fibrosis transmembrane conductance regulator; DIDS: 4,4'-diisothiocyanato-stilbene-2,2' disulfonic acid; EIPA: 5-(N-Ethyl-N-isopropyl) amiloride; G_t: total tissue conductance; I_{sc}: short-circuit current; $J_{sm}^{HCO}{}_3$: serosal-to-mucosal bicarbonate flux; KBR: Krebs bicarbonate Ringers solution; NHE: Na^+/H^+ exchanger: NPPB: 5-nitro-2(3-phenylpropyl amino)-benzoate; WT: wild-type

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