Heat-stable enterotoxin of *Escherichia coli* stimulates a non-CFTR-mediated duodenal bicarbonate secretory pathway


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Sellers, Zachary M., Debbie Childs, Jimmy Y. C. Chow, Anders J. Smith, Daniel L. Hogan, Jon I. Isenberg, Hui Dong, Kim E. Barrett, and Vijaya S. Pratha. Heat-stable enterotoxin of *Escherichia coli* stimulates a non-CFTR-mediated duodenal bicarbonate secretory pathway. *Am J Physiol Gastrointest Liver Physiol* 288: G654–G663, 2005. First published October 28, 2004; doi:10.1152/ajpgi.00386.2004.—The cystic fibrosis (CF) transmembrane conductance regulator (CFTR) is an important pathway for duodenal mucosal bicarbonate secretion. Duodenal biopsies from CF patients secrete bicarbonate in response to heat-stable enterotoxin from *Escherichia coli* (STa) but not cAMP. To explore the mechanism of STa-induced bicarbonate secretion in CF more fully, we examined the role of CFTR in STa-stimulated duodenal bicarbonate secretion in mice. In vivo, the duodenum of CFTR (−/−) or control mice was perfused with forskolin (10−4 M), STa (10−7 M), uroguanylin (10−7 M), 8-bromoguanosine 3′,5′-cGMP (8-Br-cGMP) (10−3 M), genistein (10−6 M) plus STa, or herbimycin A (10−6 M) plus STa. In vitro, duodenal mucosae were voltage-clamped in Ussing chambers, and bicarbonate secretion was measured by pH-stat. The effect of genistein, DIDS (10−4 M), and chloride removal was also studied in vitro. Control, but not CF, mice produced a significant increase in duodenal bicarbonate secretion after perfusion with forskolin, uroguanylin, or 8-Br-cGMP. However, both control and CF animals responded to STa with significant increases in bicarbonate output. Genistein and herbimycin A abolished this response in CF mice but not in controls. In vitro, STa-stimulated bicarbonate secretion in CF tissues was inhibited by genistein, DIDS, and chloride-free conditions, whereas bicarbonate secretion persisted in control mice. In the CF duodenum, STa can stimulate bicarbonate secretion via tyrosine kinase activity resulting in apical Cl−/HCO3− exchange. Further studies elucidating the intracellular mechanisms responsible for such non-CFTR mediated bicarbonate secretion may lead to important therapies for CF.

tyrosine kinase; cystic fibrosis; chloride/bicarbonate exchange

DUODENAL MUCOSAL BICARBONATE secretion is an important defensive factor in preventing acid-peptic injury (1). It has been well-established that duodenal mucosal bicarbonate secretion plays a vital role in neutralizing secretory products introduced from the stomach (16) and is attenuated in individuals with duodenal ulcers (27). The duodenal mucosa secretes bicarbonate in response to a variety of cAMP-, cGMP-, and Ca2+–mediated pathways. Both cAMP- and Ca2+-mediated duodenal bicarbonate secretion involve a DIDS-insensitive, apical membrane electrogenic conductance pathway most likely via the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) (10, 24).

CF is caused by defects in the CFTR, which result in abnormal electrolyte transport in secretory epithelia, including the airways and intestine (4). CFTR is a nonrectifying, low conductance chloride channel (5, 31) located in the apical membrane and is regulated by cAMP-dependent protein kinase A, hydrolysable nucleoside triphosphates, and protein phosphatases (3, 18, 39). CFTR is abundantly expressed in duodenal epithelial cells, with decreasing gradients of expression observed along the crypt-villus and proximal-distal axes (2) and is permeable to bicarbonate as well as chloride (35).

The heat-stable enterotoxin of *Escherichia coli* (STa) is an important cause of secretory diarrhea worldwide, responsible for about half of all *E. coli*-related diarrheal diseases, which include traveler’s diarrhea and epidemic diarrhea in newborns (21). STa binds to its receptor, membrane-bound guanylyl cyclase C (CG-C), and increases chloride and net fluid secretion, principally through a cGMP/cGMP-dependent protein kinase II/CFTR pathway (45). Work in jejunal and colonic tissue from CF patients and mice showed that STa-stimulated chloride and bicarbonate secretion is impaired (20, 42); however, there has been little work related to bicarbonate transport in the proximal small intestine. Because duodenal bicarbonate secretion is important for normal duodenal function, the effect of STa on duodenal bicarbonate secretion is an important area of study. In a previous study (36), we reported that human CF duodenal biopsies gave similar short-circuit current (Isc) and bicarbonate responses to STa as control tissues; however, we were unable to verify whether this was due to residual CFTR activity in CF patients or an alternate CFTR-independent pathway. Therefore, it was our goal to determine the importance of CFTR in STa-stimulated duodenal bicarbonate secretion both in vitro and in vivo in a CFTR knockout model and to characterize the underlying mechanism.

In the present study, we found that STa stimulated duodenal mucosal bicarbonate secretion equally in both control and CF mice, indicating a CFTR-independent bicarbonate secretory pathway. However, surprisingly, both uroguanylin- and 8-bromoguanosine 3′,5′-cGMP (8-Br-cGMP)-stimulated bicarbonate secretion were CFTR-dependent. Addition of genistein or herbimycin A, both tyrosine kinase inhibitors, abolished STa-stimulated bicarbonate secretion in CF mice with no effect in control mice. Likewise, we observed in vitro differences in the electrogenicity of bicarbonate secretion. Whereas STa-stimulated bicarbonate secretion in control mice was electrogenic, chloride-independent, and DIDS-insensitive, in CF mice STa stimulated electroneutral, DIDS-inhibitable, chloride-depen-

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dent bicarbonate secretion. In whole, we provide evidence for the existence of a cGMP-, CFTR-independent mechanism of StA-stimulated bicarbonate secretion through tyrosine kinase-mediated Cl⁻/HCO₃⁻ exchange.

MATERIALS AND METHODS

Chemicals. Forskolin, 8-Br-cGMP, STa, uroguanylin, genistein, herbiomycin A, and DIDS were purchased from Sigma-Aldrich (St. Louis, MO).

Animals. A murine CF colony, cfrtm¹¹⁰ UNC, was established by mating animals heterozygous for the CFTR gene disruption [CFTR (+/-); Jackson Laboratories, Bar Harbor, ME]. Wild-type [WT; CFTR (+/+) ] mice were produced by mating heterozygous [Het; CFTR (+/-/)] mice or homozygous [CFTR (+/+) ] mice. Because homozygous CF [CFTR (-/-) ] mice often die prematurely due to intestinal obstruction and perforation (43), an electrolyte solution containing polyethylene glycol 3350 (GoLYTELY; Braintree Laboratories, Braintree, MA) was administered in drinking water to all mice ad libitum. This solution has been shown to increase survival significantly in CF mice, without altering the histomorphological integrity of the intestine (9). This solution was also made available to littersmates to maintain experimental consistency.

Genotyping. PCR was performed to determine the genotype of mouse progeny. Approximately 1 cm of tail was clipped from each pup at weaning and digested with lysis buffer and proteinase K (DNasey kit; QIagen, Valencia, CA) at 55°C overnight. DNA was extracted by using DNasey Tissue kit according to the manufacturer’s instructions (Qiagen). PCR reaction was carried out according to a protocol obtained from Jackson Laboratories (http://jaxmice.jax.org/ pub-cgi/protocols/protocols.sh?objtype=protocol&protocol_id=423). In brief, 1 × PCR buffer (10× PCR Reaction Buffer, Invitrogen, Carlsbad, CA), 2.5 mM MgCl₂ (Invitrogen), oligonucleotide primers (In- vitrogen), 2-deoxynucleotide 5’-triphosphate (dNTPs; 1 mM each; Invitrogen), and Taq DNA polymerase (5 U/μl; Invitrogen), was executed in a DNA thermal cycler (Hybaid). DNA loading buffer was added to the samples before samples were run on a 1.5% agarose gel, for 45 min at 150 V. Resulting bands (526 and 357 bp) were visualized under ultraviolet light. Wild-type genotype was reflected by a single band at 526 bp, heterozygous by two bands at 526 and 357 bp, and knockout by a single band at 357 bp.

Basal and stimulated bicarbonate secretion in vivo. In vivo experiments were performed by using a well-validated technique as previously described (24). Briefly, after anesthesia, the proximal 7–10 mm of duodenum was isolated and perfused with forskolin (10⁻⁴ M), STa (10⁻⁷ M), uroguanylin (10⁻⁷ M), 8-Br-cGMP (10⁻³ M), genistein (10⁻⁶ M) plus STa, or herbiomycin A (10⁻⁶ M) plus STa. In all animals (WT, Het, and CF), six separate experimental series were performed. After an initial 20-min washout and recovery period, basal bicarbonate secretion was measured for 30 min. Subsequently, each agonist was perfused intragamentally for 30 min. To measure their inhibitory effects, genistein or herbiomycin A were perfused for 30 min after the basal period, after which, STa plus genistein or herbiomycin A was perfused for an additional 30 min. The segment was then gently flushed to remove residual stimulatory agents, and bicarbonate secretion was measured for an additional 45 min. After each experiment, length of the duodenal test segment was measured in situ to the nearest 0.5 mm. As shown previously, animals could be sustained for >3 h under these experimental conditions (24).

Sample volumes were measured by weight to the nearest 0.01 mg. The amount of bicarbonate in the effluents was quantitated by a validated micro back-titration method (Radiometer, Copenhagen, Denmark). Briefly, 100 μl of 50 mM HCl was added to the sample with 2 ml of distilled H₂O. Samples were then gassed with N₂, prewarmed in Ba(OH)₂ to remove all CO₂, and back-titrated with 2.5 mM NaOH to an end point of pH 7.0. As reported previously (24), a series of in vitro standards were performed by using the corresponding agonist perfuses, which showed excellent correlation between the amounts of bicarbonate added and those recovered.

Bicarbonate outputs were determined in 15-min periods and expressed as micromoles per centimeter per hour. Stimulated bicarbonate outputs are presented as bicarbonate output over time and as net bicarbonate output (peak output minus average basal). Percent inhibition was calculated by comparing net peak values.

Measurement of bicarbonate secretion in vitro. Stripped duodenal mucosae from WT, Het, and KO mice were mounted between two Lucite half chambers with an exposed area of 0.1 cm² and placed in Ussing chambers. Experiments were performed under continuous short-circuited conditions (voltage-current clamp model VCC 600; Physiologic Instruments, San Diego, CA), as previously described (44). Duodenal tissue from each animal was divided and examined in three chambers. Both mucosal and serosal solutions contained (in mM): 140 Na⁺, 5.4 K⁺, 1.2 Ca²⁺, 1.2 Mg²⁺, and 120 Cl⁻. The serosal bath contained (in mM) 25 HCO₃⁻, 2.4 HPO₄²⁻, and 10 glucose. The luminal solution contained 25 mM gluconate and 10 mM mannitol. To measure the effects of chloride removal, gluconate was isosmotically substituted for chloride in both mucosal and serosal bathing tissue. The osmolalities of all solutions were ~284 osmol/kg.

After a 30-min measurement of basal parameters, STa (10⁻⁷ M) was added to the mucosal side of tissue in Ussing chambers for 75 min. To measure the inhibitory effects of genistein or DIDS, after basal measurements, genistein (10⁻⁶ M) or DIDS (10⁻⁴ M) was added to the mucosal side of tissue for 30 min before STa addition. Measurements were recorded at 5-min intervals, and mean basal and peak values for consecutive 10-min periods were averaged. The rate of luminal bicarbonate secretion is expressed as micromoles per square centimeter per hour. The Le was measured in microammeter and converted into micromolar equivalents per square centimeter per hour.

Statistics. Results are expressed as means ± SE for a series of n experiments. Statistical analysis was performed by using the Student’s t-test for unpaired data or by ANOVA with the Student-Newman-Keuls post hoc test, as appropriate. P values of <0.05 were considered significant.

RESULTS

WT and Het animals displayed similar basal and stimulated bicarbonate secretory responses to all agonists tested in the present study, as in previous studies (24, 25), and thus were combined as controls for comparison to CF mice. As far as possible, control and CF animals were matched for physiological parameters. There was no significant difference among sex, weight, or length of duodenal segment between control and CF mice for each group (data not shown).

Similar to previous studies (25, 35), in vivo basal bicarbonate secretion in CF mice (n = 31) was significantly diminished (P < 0.001) compared with normal animals (n = 35), 2.23 ± 0.41 vs. 6.70 ± 0.66 μmol·cm⁻¹·h⁻¹, respectively.

Forskolin-stimulated bicarbonate secretion. In control animals (n = 7), intraduodenal perfusion of forskolin (10⁻⁴ M) produced an immediate and significant increase in duodenal mucosal bicarbonate secretion (P < 0.05). However, in CF animals (n = 6), forskolin did not elicit any increase in bicarbonate secretory response, resulting in a significant difference in responses between control and CF mice (Fig. 1, A and B).

Lack of an increase in bicarbonate output in response to forskolin in CF animals was caused solely by a decrease in bicarbonate electrolyte secretion and not due to an increase in fluid secretion that might dilute secreted bicarbonate. In control animals, net effluent bicarbonate concentration after luminal addition of forskolin was significantly greater than in CF...
animals (0.87 ± 0.13 vs. 0.33 ± 0.16 mM, respectively; \( P < 0.05 \)), but there was no significant change in net fluid secretion in CF mice compared with control animals (213 ± 148 vs. 260 ± 128 \( \mu l \cdot cm^{-1} \cdot h^{-1} \), respectively; \( P > 0.05 \)).

**STa-stimulated bicarbonate secretion.** To examine the role of CFTR in STa-stimulated bicarbonate secretion, STa (10^{-7} M) was luminally perfused through the proximal duodenal segment. Control animals (\( n = 8 \)) responded to STa with a significant increase in bicarbonate output (\( P < 0.05 \)). Similarly, in CF mice (\( n = 7 \)), STa elicited a significant increase in bicarbonate output (\( P < 0.05 \)) comparable with that in control animals when the lower baseline in the CF group was taken into account (Fig. 2A). As a result, there was no difference in net peak bicarbonate secretion in CF animals, compared with control animals (Fig. 2B).

Perfusion with STa increased effluent bicarbonate concentration in control animals (0.39 ± 0.16 vs. 0.85 ± 0.26 mM; \( P < 0.05 \)) and to a comparable extent in CF animals (0.15 ± 0.05 vs. 0.55 ± 0.15 mM; \( P < 0.05 \)). Thus increased bicarbonate output was apparently similar in both groups and unrelated to differences in fluid secretion.

**Uroguanylin-stimulated bicarbonate secretion.** To provide additional insight into the mechanism(s) whereby STa can evoke bicarbonate secretion in CF mice, the effect of uroguanylin (10^{-7} M), an endogenous ligand for the GC-C receptor, on duodenal bicarbonate secretion was studied. Uroguanylin significantly stimulated bicarbonate secretion in control animals (\( P < 0.05 \)), but failed to alter bicarbonate output in CF mice, resulting in significant differences between the two groups (Fig. 3A). Thus in contrast to responses to STa, there...
was a significant attenuation in net peak bicarbonate output evoked by uroguanylin between control and CF animals (Fig. 3B). Similar to forskolin, the failure of CF animals to secrete bicarbonate in response to uroguanylin was the result of an attenuation in bicarbonate concentration, not net fluid secretion. In CF animals, net peak effluent bicarbonate concentration was significantly diminished from that in control animals (0.11 ± 0.04 vs. 0.36 ± 0.07 mM, respectively; *P < 0.05). Net fluid secretion induced by uroguanylin in CF mice, 1,416 ± 875 μl·cm⁻¹·h⁻¹ was not significantly different from that in control animals, 1,281 ± 618 μl·cm⁻¹·h⁻¹ (*P > 0.05).

8-Br-cGMP-stimulated bicarbonate secretion. Given the disparity between STa- and uroguanylin-stimulated duodenal bicarbonate secretion in CF mice, we sought to determine whether the STa-stimulated bicarbonate secretory response in CF mice was likely due to an increase in cGMP levels. Thus the effect of 8-Br-cGMP (10⁻³ M), a cell-permeable analog of cGMP, on bicarbonate secretion was studied to bypass second messenger generation. Luminal addition of 8-Br-cGMP resulted in a significant and sustained increase of duodenal bicarbonate secretion in control mice (n = 9). In contrast, basal bicarbonate was largely unaffected by the addition of 8-Br-cGMP in CF mice (n = 6). *Significant increases over baseline values (*P < 0.05). Bicarbonate output differed significantly between both groups at all points (*P < 0.05; ANOVA). The net peak response to 8-Br-cGMP in control animals was significantly different from that in CF mice (**P < 0.01; Student’s t-test). All results are means ± SE.
not net fluid secretion (260 ± 128 vs. 499 ± 199 μL⋅cm⁻¹⋅h⁻¹, respectively; P > 0.05). In control animals, net effluent bicarbonate concentration was significantly increased over that seen in CF mice (0.87 ± 0.13 vs. 0.18 ± 0.12 mM, respectively; P < 0.01). Thus the effect of STα on bicarbonate secretion in CF mice is likely not attributable to an increase in cGMP, because it was not reproduced by 8-Br-cGMP.

Effect of tyrosine kinase inhibition on STα-stimulated bicarbonate secretion. Because the effect of STα on bicarbonate secretion in CF mice was apparently not mediated by cGMP, we sought an alternate mechanism of action. Pathways mediated by cAMP or calcium were not candidates, because bicarbonate secretion in response to forskolin (24) and carbachol (24) is known to be absent in CF mice. We therefore studied the effects of genistein (10⁻⁶ M) and herbimycin A (10⁻⁶ M), both broad-spectrum tyrosine kinase inhibitors, on STα-stimulated duodenal bicarbonate secretion. Genistein alone slightly increased bicarbonate secretion in control but not CF mice. Perfusion of genistein before STα addition abolished the bicarbonate secretory response to STα in CF animals (n = 4) but only partially inhibited STα-stimulated bicarbonate secretion in control animals (n = 4) (Fig. 5A). In the presence of genistein, STα produced a significant increase in peak bicarbonate output over baseline in control mice (3.97 ± 0.37 vs. 9.82 ± 0.55 μmol⋅cm⁻¹⋅h⁻¹, P < 0.001), which was comparable with that evoked by STα alone (4.29 ± 0.82 vs. 11.43 ± 1.62 μmol⋅cm⁻¹⋅h⁻¹; 22% inhibition). In contrast, genistein abolished the effect of STα in CF mice with no significant increase in peak bicarbonate output over baseline (2.03 ± 0.49 vs. 2.48 ± 0.62 μmol⋅cm⁻¹⋅h⁻¹, P > 0.05), corresponding to 85% inhibition compared with the effects of STα alone (2.06 ± 0.66 vs. 7.74 ± 2.77 μmol⋅cm⁻¹⋅h⁻¹). Similarly, genistein alone caused a significant decrease in basal bicarbonate output by CF mice and overall abolished STα-stimulated bicarbonate secretion in CF mice (Fig. 5B).

Similar to genistein, incubation with herbimycin A, before STα stimulation, resulted in attenuation of STα-stimulated duodenal bicarbonate secretion in CF mice (n = 4) with no significant inhibitory effect in control mice (n = 4) (Fig. 6A). In the presence of herbimycin A, STα produced a significant increase in peak bicarbonate output over baseline in control mice (3.79 ± 0.31 vs. 10.90 ± 0.99 μmol⋅cm⁻¹⋅h⁻¹, P < 0.001), which was comparable with that produced by STα alone (4.29 ± 0.82 vs. 11.43 ± 1.62 μmol⋅cm⁻¹⋅h⁻¹; 105% increase). In CF mice, herbimycin A significantly inhibited the effect of STα, preventing a significant increase in peak bicarbonate output over baseline (1.27 ± 0.35 vs. 2.16 ± 0.92 μmol⋅cm⁻¹⋅h⁻¹, P > 0.05), which corresponded to 85% inhibition compared with the effects of STα alone (2.06 ± 0.66 vs. 7.74 ± 2.77 μmol⋅cm⁻¹⋅h⁻¹).

Lack of bicarbonate output in CF animals was unrelated to net fluid secretion. In control animals treated with genistein or herbimycin A, STα caused a significantly greater increase in net peak bicarbonate concentration than in CF animals (genistein: 0.54 ± 0.05 vs. 0.10 ± 0.05 mM, respectively; P < 0.001; herbimycin A: 0.82 ± 0.09 vs. 0.15 ± 0.08 mM, respectively, P < 0.005), with no difference in net fluid secretion (genistein and control: 828 ± 230 vs. CF: 788 ± 119 μL⋅cm⁻¹⋅h⁻¹; P > 0.05; herbimycin A and control: 283 ± 99 vs. CF: 172 ± 72 μL⋅cm⁻¹⋅h⁻¹; P > 0.05).

STα-stimulated bicarbonate secretion in vitro. To determine the electrogenicity of STα-stimulated bicarbonate secretion, duodenal mucosae from control (n = 8) and CF (n = 8) mice were stripped and mounted in Ussing chambers. Similar to our findings in vivo, mucosal addition of STα (10⁻⁷ M) resulted in a significant increase in bicarbonate secretion in both control and CF mice (Fig. 7A). STα also produced a significant increase in Iₑ in control mice, but no change in Iₑ occurred in CF mice, implicating electroneutral Cl⁻/HCO₃⁻ exchange (Fig. 7B). Thus to determine whether STα-stimulated bicarbonate
Fig. 6. STa-stimulated bicarbonate secretion is inhibited by herbimycin A in CF mice. A: luminal perfusion of herbimycin A (10^{-6} M) abolished STa-stimulated bicarbonate secretion in CF mice (n = 4), while not affecting control mice (n = 4). **Significant increases over baseline values, before and after herbimycin A addition (P < 0.05; ANOVA). Bicarbonate secretion in CF mice was significantly attenuated at all time points compared with that in control mice (P < 0.05). B: Net peak bicarbonate response to herbimycin A alone (black bars) or herbimycin A plus STa (gray bars). ***Net peak response to STa in the presence of herbimycin A was significantly different in CF mice compared with control mice (P < 0.001). Statistically significant differences were assessed by Student’s t-test. All results are means ± SE.

secretion in control (n = 8) and CF (n = 6) mice was indeed dependent on chloride, duodenal tissue was stripped and mounted in Ussing chambers containing chloride-free solutions. STa-stimulated bicarbonate secretion in control mice was unaffected by chloride removal (net peak: 1.50 ± 0.20 vs. 1.38 ± 0.19 μmol·cm^{-1}·h^{-1}, P > 0.05). On the other hand, chloride removal significantly decreased STa-stimulated bicarbonate secretion in CF mice (net peak: 1.17 ± 0.06 vs. 0.45 ± 0.14 μmol·cm^{-1}·h^{-1}, P < 0.0005), supporting a chloride-dependent mechanism for STa-stimulated bicarbonate secretion in these animals (Fig. 8A). Whereas chloride removal resulted in attenuated I_{sc} compared with experiments done in the presence of chloride in both groups (control net peak: 0.63 ± 0.12 vs. 2.16 ± 0.17 μeq·cm^{-2}·h^{-1}, P < 0.0001; CF net peak: -0.09 ± 0.03 vs. 0.30 ± 0.14 μeq·cm^{-2}·h^{-1}, P < 0.05), STa continued to elicit a small but significant increase in I_{sc} over baseline in control mice, but not in CF mice (Fig. 8B). To confirm the role of an apical Cl⁻/HCO₃⁻ exchanger in STa-stimulated bicarbonate secretion in CF mice, DIDS (10^{-4} M) was added to the mucosal bath 30 min before STa addition. In control tissues, DIDS failed to prevent STa-stimulated increases in I_{sc} (basal: 1.69 ± 0.28 vs. peak: 3.89 ± 0.32 μeq·cm^{-2}·h^{-1}, P < 0.001, n = 5) or bicarbonate secretion (basal: 2.27 ± 0.43 vs. peak: 4.19 ± 0.34 μmol·cm^{-2}·h^{-1}, P < 0.01). However, DIDS inhibited significant increases in STa-stimulated bicarbonate secretion in CF tissues (basal: 2.31 ± 0.97 vs. peak: 2.98 ± 0.96 μmol·cm^{-2}·h^{-1}, P > 0.05, n = 5). Likewise, there was no significant change in I_{sc} upon

Fig. 7. STa-stimulated bicarbonate secretion in vitro is CFTR-independent. Duodenal mucosae from control (n = 8) and CF (n = 8) mice were stripped of seromuscular layers and mounted in Ussing chambers. A: STa stimulated a significant and comparable increase in peak bicarbonate secretion (gray bars) over baseline (black bars) in both control and CF mice (***P < 0.05). B: STa stimulated a significant increase in peak short-circuit current (I_{sc}) (gray bars) over baseline (black bars) in control mice (***P < 0.001). However, in CF mice, basal I_{sc} values were reduced and no change in I_{sc} occurred after STa stimulation. Basal and stimulated values were significantly different in control mice compared with those in CF mice, as denoted (basal, #P < 0.05; peak, ++ + + P < 0.001). All results are means ± SE, and significant differences were assessed by Student’s t-test.
STa addition (basal: 0.64 ± 0.14 vs. peak: 1.16 ± 0.31 μEq·cm⁻²·h⁻¹, P > 0.05, n = 5) in CF tissues pretreated with DIDS.

Effect of tyrosine kinase inhibition on STa-stimulated bicarbonate secretion in vitro. To confirm that genistein inhibition of STa-stimulated duodenal bicarbonate secretion in vivo was likely due to tyrosine kinase inhibition rather than systemic effects of the drug, duodenal mucosae of control (n = 5) and CF (n = 5) mice were mounted in Ussing chambers. Similar to findings obtained in vivo, genistein (10⁻⁶ M) alone slightly increased basal bicarbonate secretion, but had no inhibitory effect on STa-stimulated bicarbonate secretion in control tissues. In contrast, genistein alone had no basal effect on bicarbonate secretion in CF tissues, but inhibited STa-stimulated bicarbonate secretion, with a significant decrease in the net peak response of CF tissues to STa compared with control tissues (0.96 ± 0.08 vs. 2.33 ± 0.49 μmol·cm⁻²·h⁻¹, respectively, P < 0.005) (Fig. 9A). Similar results were observed for Iₛ. In control animals, there was no inhibitory effect on Iₛ with addition of genistein, resulting in a significant increase in Iₛ.

Fig. 8. STa-stimulated bicarbonate secretion is chloride-dependent in mice. Similar to previous experiments, duodenal mucosae from control mice (n = 8) and CF mice (n = 6) were stripped and mounted in Ussing chambers. However, to study the effect of chloride on STa-stimulated bicarbonate secretion, chloride was removed from both mucosal and serosal solutions as described in MATERIALS AND METHODS. A: chloride removal had no inhibitory effect on STa-stimulated duodenal bicarbonate secretion in control mouse tissues, resulting in a significant increase in peak bicarbonate secretion (gray bars) over baseline (black bars) (**P < 0.01), with no difference from chloride-containing solutions. However, in CF mice, peak STa-stimulated bicarbonate secretion was essentially abolished by chloride removal, resulting in no change in peak bicarbonate secretion from baseline bicarbonate secretion. Response to STa in CF mice was significantly less than that seen in controls (+P < 0.01). B: STa stimulated a significant increase in peak Iₛ (gray bars) over baseline (black bars) in control mice (*P < 0.05), despite decreased values compared with chloride-containing solutions. STa elicited no increase in peak Iₛ over baseline Iₛ in CF mice. ###Basal and + + + peak Iₛ in CF mice was significantly less than that seen in controls (P < 0.001). All results are means ± SE, and statistically significant differences were assessed by Student’s t-test.

Fig. 9. STa-stimulated bicarbonate secretion is inhibited by genistein in vitro. Duodenal mucosae from control (n = 5) and CF (n = 5) mice were stripped of seromuscular layers and mounted in Ussing chambers. A: addition of genistein resulted in an increase over baseline in bicarbonate secretion (gray bars) over chloride-containing solutions. However, in CF mice, peak STa-stimulated bicarbonate secretion was essentially abolished by chloride removal, resulting in no change in peak bicarbonate secretion from baseline bicarbonate secretion. Response to STa in CF mice was significantly less than that seen in controls (+P < 0.01). B: STa stimulated a significant increase in peak Iₛ (gray bars) over baseline (black bars) in control mice (*P < 0.01), despite decreased values compared with chloride-containing solutions. STa elicited no increase in peak Iₛ over baseline Iₛ in CF mice. ###Basal and + + + peak Iₛ in CF mice was significantly less than that seen in controls (P < 0.001). All results are means ± SE, and statistically significant differences were assessed by Student’s t-test.
upon STa addition. Similar to previous experiments, there was no change in $I_{sc}$ upon addition of genistein or STa in CF mice (Fig. 9B).

**DISCUSSION**

CF is the most common fatal autosomal recessive disorder in Caucasians and is characterized clinically by chronic obstructive pulmonary disease, insufficient pancreatic exocrine function, intestinal malabsorption, and elevated sweat electrolyte levels (6). Although lung disease is currently the primary cause of mortality in CF patients, gastrointestinal complications account for a significant proportion of the morbidity of the disease, including gastroesophageal reflux disease, intestinal obstruction, and peptic ulcers (14), which continue postlung transplant (19). Decreased bicarbonate secretion by the pancreas and the duodenal mucosa, due to the lack of CFTR function, leads to an acidic environment in the duodenum, with fasting and postprandial intraluminal duodenal pH 1–2 pH units lower than normal (46). This alteration in duodenal pH can lead to duodenal mucosal damage, insufficient enzyme function, and fat malabsorption in CF patients, even in those that are otherwise pancreatic sufficient (41). Despite the presence of other bicarbonate secretory pathways (i.e., Cl⁻/HCO₃⁻ exchangers), the functional loss of CFTR is proposed to be the main defect responsible for the pathophysiologic failure to alkalinize the duodenum. In particular, the ability of CFTR to transport both chloride and bicarbonate is integral to duodenal mucosal bicarbonate secretion (15).

Guanylin and uroguanylin, endogenous ligands of the GC-C receptor, stimulate duodenal bicarbonate secretion in rats, likely through CFTR (22, 30). Based on data from the jejunum and colon, it is thought that STa, along with guanylin and uroguanylin, binds to GC-C and stimulates a cGMP/cGMP-dependent protein kinase II/CFTR pathway to stimulate chloride and/or bicarbonate transport. Recent evidence suggests that GC-C, Na⁺/H⁺ exchanger 3 (NHE3), and CFTR are interconnected via PSD95-Dlg-zona occludens-1 domains, which allows for NHE3 inhibition and CFTR stimulation after GC-C activation (45). To date, the majority of studies with STa have focused on the distal small intestine and colon (20, 42); however, we have previously shown that duodenal biopsies from CF patients secrete bicarbonate in response to STa, which we hypothesized might be due to residual CFTR in CF patients (36). However, in our current and more comprehensive study in which we have used a murine CFTR knockout model, we have been able to show that STa stimulates bicarbonate secretion, both in vitro and in vivo, in the absence of CFTR. We found that STa-stimulated bicarbonate secretion in CF mice is mechanistically distinct from the response in normal mice. Whereas under normal conditions STa elicits an electrogenic bicarbonate secretory pathway likely through CFTR, murine CF duodenal mucosa is able to achieve normal levels of STa-stimulated bicarbonate secretion through a pathway that apparently involves tyrosine kinase activity and electroneutral Cl⁻/HCO₃⁻ exchange.

When viewed in light of the current hypothesis that both STa and uroguanylin induce ion secretion through CFTR, our results are unexpected. However, there is evidence to suggest the presence of at least two distinct STa-binding sites in the intestine, one that binds STa with low affinity but is highly expressed and another that exhibits high STa binding at concentrations three- to fourfold less but that is expressed at lower levels (11, 23, 26, 28). Whereas some studies have likewise shown that GC-C exhibits high and low affinity binding states (12, 13), others have attributed these additional sites to non-GC-C STa-binding receptors. Whereas non-GC-C STa-binding receptors have not been cloned, there is evidence to suggest their existence. Despite lacking GC-C receptors, GC-C deficient mice continue to exhibit a small (10%) but reproducible degree of specific binding of STa to colonic mucosal cell membranes (8, 34). Additionally, in a comparison between Caco-2 and IEC-6 cells, Mann et al. (33) showed that Caco-2 cells, which can be considered a model for villous enterocytes, expressed a single STa-binding receptor with GC-C activity. However, in IEC-6 cells, which resemble intestinal crypt cells, a STa-binding receptor was found that did not display GC-C activity. Similar to Mann et al. (33), Hakki et al. (23) have likewise observed an STa-binding protein in rat intestinal membranes that is not linked to GC-C activity. Recently, work investigating GC-C-independent natriuretic responses to STa/uroguanylin/guanylin in kidney epithelium has shown mRNA expression for a GC-C-like protein that is distinct from GC-C (7). It is as yet unknown whether the renal mRNA transcripts found in this study are also present in the intestines. To date, the ability of uroguanylin to bind to alternative binding sites has not been reported. Despite use of a submaximal dose, uroguanylin stimulated a robust increase in duodenal mucosal bicarbonate secretion in control mice but not CF mice. These results suggest that STa may be able to stimulate duodenal bicarbonate secretion in a manner distinct from uroguanylin. Although we did not address the specific role of GC-C in our studies, the ability of STa to stimulate significant CFTR-independent duodenal bicarbonate secretion in CF mice should prompt a detailed examination of the receptor(s) involved in mediating this phenomenon.

In the present study, we found that genistein and herbimycin A differentially affected STa-stimulated duodenal bicarbonate secretion in CF mice compared with control mice. Despite the fact that GC-C contains a kinase homology domain similar to that of receptor tyrosine kinases, it is thought to be devoid of protein kinase activity, because critical residues required for phosphotransfer activity in many kinases are absent from GC-C. Biochemically, it has been shown that tyrphostin 82, a potent tyrosine kinase inhibitor, can inhibit GC-C activity, although the functional relevance of this has yet to be examined (29). In other studies, tyrosine kinases have been implicated in the regulation of bicarbonate secretion. In vivo studies by us and others have shown that genistein (but not genistin) and the MEK inhibitor PD-98059 inhibit acid-stimulated duodenal bicarbonate secretion (17, 38). Likewise, studies have shown that Src, a cytoplasmic nonreceptor tyrosine kinase, can affect Na⁺-HCO₃⁻ cotransporter and Cl⁻/HCO₃⁻ exchanger activity (37, 40). On the basis of preliminary Western blot analysis (unpublished data), we found that differential tyrosine phosphorylation occurs in CF mice compared with control mice, both at baseline and in response to STa. On the basis of these results, p⁶⁰⁰ Src and p³⁸ are possible candidates for downstream regulators of tyrosine kinase-mediated bicarbonate secretion in CF mice. In particular, our finding that herbimycin A, which preferentially inhibits Src, coupled with previous findings that Src mediates Na⁺-HCO₃⁻ cotransporter and
Cl⁻/HCO₃⁻ exchanger activity (37, 40), make Src a likely candidate in mediating this response. However, further detailed studies are necessary to identify the specific role of tyrosine kinases in mediating STa-stimulated duodenal bicarbonate secretion and whether this activity is a result of GC-C activity at the kinase homology domain or due to alternative signaling intermediates.

In these studies, we also found that STa-stimulated bicarbonate secretion was chloride independent and DIDS insensitive in control mice, whereas STa-stimulated bicarbonate secretion was chloride dependent and DIDS sensitive in CF mice. This is in accordance with our previous work (36), where base extrusion from CF duodenoocytes was chloride dependent, whereas that in cells from normal volunteers was chloride independent. In contrast, whereas both CF human and murine tissues secrete normal levels of bicarbonate in response to STa, the electrical nature of the responses differed. In CF human tissues, STa-stimulated bicarbonate secretion was accompanied by an increase in $I_{sc}$, whereas in CF murine tissues, STa increased bicarbonate secretion without altering $I_{sc}$ significantly. It is unknown whether this is explained simply by species differences, differences between mutations and whole gene knockout, or whether it implies more than one type of CFTR-independent bicarbonate secretory mechanism. Although it is beyond the scope of this study, further work determining the identity of the Cl⁻/HCO₃⁻ exchanger(s) involved in mediating STa-stimulated duodenal bicarbonate secretion in CF tissue is an important area of research. Whereas downregulated adenoma and putative anion transporter 1 have been implicated as the major anion exchangers in the duodenum, recent work has shown that these transporters are electrogenic (32) compared with electroneutral anion exchangers such as anion exchangers 3 and 4. From our studies, we were not able to identify the specific anion exchangers involved; however, we hypothesize that the electroneutral bicarbonate secretory response elicited by STa is either due to classic electroneutral anion exchangers or electroneutral coupling of electrogenic exchangers of the SLC26 family. An understanding of the transporter(s) involved in STa-stimulated duodenal bicarbonate secretion will provide important therapeutic opportunities for increasing bicarbonate secretion in CF epithelia.

The mechanism of basal and agonist-stimulated duodenal bicarbonate secretion is important not only for the maintenance of normal physiological function within the duodenal micro-environment but for understanding pathological changes that result in the loss of bicarbonate secretion. In particular, the knowledge of this mechanism may allow the development of methods to rectify defects in bicarbonate secretion that occur in CF. Our studies show that STa can induce bicarbonate secretion in vivo and in vitro in mice that do not express CFTR. Additionally, the magnitude of this secretory response is similar to that seen in normal mice. Despite similar levels of secretion, our findings suggest that in the absence of CFTR, STa can stimulate bicarbonate secretion in CF mice via a cGMP-, CFTR-independent mechanism. It remains to be understood whether this pathway also occurs in normal animals or whether it represents an adaptive response due to the loss of CFTR. On the basis of our data, we hypothesize that STa stimulates a distinct bicarbonate secretory mechanism in the absence of CFTR that differs from that occurring in the presence of this channel. In the presence of CFTR, STa likely stimulates electrogenic bicarbonate secretion through cGMP generation leading to PKG phosphorylation and CFTR opening. However, in the absence of CFTR, STa stimulates a tyrosine kinase-mediated electroneutral bicarbonate secretory pathway via Cl⁻/HCO₃⁻ exchange. Further characterization of the receptors involved and the exact mechanism by which they evoke bicarbonate secretion could potentially be important in treating symptoms of CF that arise from diminished bicarbonate secretion, such as malabsorption and mucosal injury.

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REFERENCES

NON-CFTR-MEDIATED DUODENAL HCO₃⁻ SECRETORY PATHWAY