Independence of apical Cl⁻/HCO₃⁻ exchange and anion conductance in duodenal HCO₃⁻ secretion

S. Spiegel,¹ M. Phillipper,¹ H. Rossmann,¹ B. Riederer,¹,² M. Gregor,¹ and U. Seidler¹,²
¹First Department of Medicine, Eberhard-Karls-Universität, 72076 Tübingen; and ²Zentrum Innere Medizin, Abteilung VI, Medizinische Hochschule Hannover, 30625 Hannover, Germany

Submitted 19 February 2003; accepted in final form 26 June 2003

Spiegel, S., M. Phillipper, H. Rossmann, B. Riederer, M. Gregor, and U. Seidler. Independence of apical Cl⁻/HCO₃⁻ exchange and anion conductance in duodenal HCO₃⁻ secretion. Am J Physiol Gastrointest Liver Physiol 285: G887–G897, 2003. First published July 3, 2003; 10.1152/ajpgi.00083.2003.—Reduced gastrointestinal HCO₃⁻ secretion contributes to malabsorption and obstructive syndromes in cystic fibrosis. The apical HCO₃⁻ transport pathways in these organs have not been defined. We therefore assessed the involvement of apical Cl⁻/HCO₃⁻ exchangers and anion conductances in basal and cAMP-stimulated duodenal HCO₃⁻ secretion. Muscle-stripped rat and rabbit proximal duodena were mounted in Ussing chambers, and electrical parameters, HCO₃⁻ secretion rates, and ³⁶Cl⁻, ²²Na⁺, and H⁺mannitol fluxes were measured. mRNA expression levels were measured by a quantitative PCR technique. Removal of Cl⁻ from or addition of 1 mM DIDS to the luminal perfusate markedly decreased basal HCO₃⁻ secretion but did not influence the HCO₃⁻ secretory response to 8-bromo-cAMP, which was inhibited by luminal 5-nitro-2-(3-phenylpropylamino)-benzoate. Bidirectional ²²Na⁺ and ³⁶Cl⁻ flux measurements demonstrated an inhibition rather than a stimulation of apical anion exchange during cAMP-stimulated HCO₃⁻ secretion. The ratio of Cl⁻ to HCO₃⁻ in the anion secretory response was compatible with both Cl⁻ and HCO₃⁻ being secreted via the CFTR anion channel. CFTR expression was very high in the duodenal mucosa of both species. We conclude that in rat and rabbit duodena, an apical Cl⁻/HCO₃⁻ exchanger mediates a significant part of basal HCO₃⁻ secretion but is not involved in the HCO₃⁻ secretory response to cAMP analogs. The inhibitor profile, the strong predominance of Cl⁻ over HCO₃⁻ in the anion secretory response, and the high duodenal CFTR expression levels suggest that a major portion of cAMP-stimulated duodenal HCO₃⁻ secretion is directly mediated by CFTR.

cystic fibrosis transmembrane conductance regulator; 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; 5-nitro-2-(3-phenylpropylamino)-benzoate; bicarbonate; cystic fibrosis; intestin...
Brunner’s glands. We also tested whether the CFTR anion channel itself is the likely transport pathway for agonist-stimulated HCO₃⁻ secretion.

**MATERIALS AND METHODS**

**Chemicals and Solutions**

H¹³⁶Cl and ²²NaCl were obtained from Amersham (Braunschweig, Germany); scintillation cocktail was from Packard (Frankfurt am Main, Germany); Rompun was from Bayer, and Ketanest was from Parke-Davis. Unless otherwise specified, all other reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany) including agarose type III high biology grade, or the highest grade available. (Darmstadt, Germany) at tissue culture grade, molecular hofen, Germany), Roth (Karlsruhe, Germany), or Merck (Darmstadt, Germany) at tissue culture grade, molecular biology grade, or the highest grade available.

The luminal solution contained 154 mmol/l NaCl, gassed with 100% O₂ and the nutrient solution contained (in mmol/l) 2.5 mmol/l glucose was replaced by mannitol in the luminal bath. The other reagents were purchased from Sigma-Aldrich and Ketanest was from Parke-Davis. Unless otherwise specified, all other reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany); scintillation cocktail was from Packard (Frankfurt am Main, Germany); Rompun was from Bayer, and Ketanest was from Parke-Davis. Unless otherwise specified, all other reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany) including agarose type III high biology grade, or the highest grade available. (Darmstadt, Germany) at tissue culture grade, molecular hofen, Germany), Roth (Karlsruhe, Germany), or Merck (Darmstadt, Germany) at tissue culture grade, molecular biology grade, or the highest grade available.

The luminal solution contained 154 mmol/l NaCl, gassed with 100% O₂ and the nutrient solution contained (in mmol/l) 140.5 Na⁺, 4.5 K⁺, 2 Ca²⁺, 1.3 Mg²⁺, 126 Cl⁻, 1.3 SO₄²⁻, 20 HCO₃⁻, 1.5 HPO₄⁻, 11.9 dextrose, and 10 sodium pyruvate plus 3×10⁻⁶ mol/l indomethacin and 10⁻⁶ mol/l TTX, gassed with 95% O₂-5% CO₂, pH 7.4 ± 0.03, both at 37°C. In the case of the Cl⁻-free studies, Cl⁻ was replaced by gluconate. When voltage clamp was applied, the luminal and serosal solutions were identical and were both gassed with 5% CO₂ except that glucose was replaced by mannitol in the luminal bath. The electrodes contained 3 M KCl agarose or, in the case of substitution of Cl⁻, 3 M potassium nitrate agarose.

**Experimental Methods**

**Animals.** Male New Zealand White rabbits weighing 2,500–3,000 g and female Wistar rats weighing 280–350 g were maintained under standard temperature (21–22°C) and light conditions (12:12-h light-dark cycle). Animals had access to tap water and pelleted food ad libitum.

**Duodenal isolation.** For rat duodenal isolation, the procedure has been described (13). Rabbits were preanesthetized by an intramuscular injection of 100 mg/kg ketamine, 10 mg/kg dihydroxylidin, and 0.2 mg/kg atropine. The abdomen was opened, a 3-cm-long segment of the duodenum was excised, and the animals were killed with an overdose of dihydroxylidin, and 0.2 mg/kg atropine. The abdomen was opened, a 3-cm-long segment of the duodenum was excised, and the animals were killed with an overdose of dihydroxylidin, and 0.2 mg/kg atropine. The abdomen was opened, a 3-cm-long segment of the duodenum was excised, and the animals were killed with an overdose of dihydroxylidin, and 0.2 mg/kg atropine. The abdomen was opened, a 3-cm-long segment of the duodenum was excised, and the animals were killed with an overdose of dihydroxylidin, and 0.2 mg/kg atropine. The abdomen was opened, a 3-cm-long segment of the duodenum was excised, and the animals were killed with an overdose of dihydroxylidin, and 0.2 mg/kg atropine. The abdomen was opened, a 3-cm-long segment of the duodenum was excised, and the animals were killed with an overdose of dihydroxylidin, and 0.2 mg/kg atropine. The abdomen was opened, a 3-cm-long segment of the duodenum was excised, and the animals were killed with an overdose of dihydroxylidin, and 0.2 mg/kg atropine. The abdomen was opened, a 3-cm-long segment of the duodenum was excised, and the animals were killed with an overdose of dihydroxylidin, and 0.2 mg/kg atropine. The abdomen was opened, a 3-cm-long segment of the duodenum was excised, and the animals were killed with an overdose of dihydroxylidin, and 0.2 mg/kg atropine.

**Experimental Methods**

**Animals.** Male New Zealand White rabbits weighing 2,500–3,000 g and female Wistar rats weighing 280–350 g were maintained under standard temperature (21–22°C) and light conditions (12:12-h light-dark cycle). Animals had access to tap water and pelleted food ad libitum.

**Duodenal isolation.** For rat duodenal isolation, the procedure has been described (13). Rabbits were preanesthetized by an intramuscular injection of 100 mg/kg ketamine, 10 mg/kg dihydroxylidin, and 0.2 mg/kg atropine. The abdomen was opened, a 3-cm-long segment of the duodenum was excised, and the animals were killed with an overdose of phenobarbital. The proximal part of the duodenum was stripped of external serosal and muscle layers (effectively removing the Brunner’s glands as well) with fine forceps under the stereomicroscope, mounted between two Lucite half-chambers of 0.636-cm² exposed area, and placed in an Ussing chamber. TTX (10⁻⁶ mol/l, to abolish the influence of endogenous prostaglandin production) were administered to the basolateral solution. Drugs were administered at the indicated times. Ouabain was applied to the basolateral solution. Drugs were administered at the indicated times. Ouabain was applied to the basolateral solution. Drugs were administered at the indicated times. Ouabain was applied to the basolateral solution.

**Electrophysiology.** The open-circuit transepithelial electrical potential difference (PD) was recorded (DVC-1000 dual voltage clamp; World Precision Instruments, Sarasota, FL) via agar 3 mol/l KCl bridges. Before the tissue was placed into the chamber, the series resistances of the solutions etc. were assessed, and a fluid resistance compensation was performed before each experiment. The direct-current electrical resistance was determined from the change in PD after sending a current of ~40 μA/cm² through the mucosa in either direction in a 200-ms interval. The open-circuit condition was chosen because to measure HCO₃⁻ secretion, no CO₂ or HCO₃⁻ may be in the luminal solution. This results in the presence of a concentration gradient, which should not be present under voltage-clamp conditions. In addition, the open-circuit condition is closer to the physiological situation. Under open-circuit conditions, short-circuit current (Isc) is calculated from PD and R, and is, of course, not a true short-circuit current, because the transepithelial PD is a driving force for passive ion movement. To assess whether changes in “calculated Isc” can be used as an approximate measurement for changes in true Isc, we applied voltage-clamp conditions to a complete set of experiments (including flux rates) for the control conditions. In all experiments, a positive PD or a positive Isc reflects a net anion movement from the serosal to the mucosal side. Because we often compare HCO₃⁻ secretion with changes in Isc, anion movement into the luminal bath is defined as positive in both cases.

**In vitro determination of duodenal HCO₃⁻ secretion.** Luminal pH was maintained at 7.4 by a continuous pH stat titration method (Radiometer, Copenhagen, Denmark), and the rate of alkalinization was calculated from the consumption volume of the HCl or H₂SO₄-containing titrant solution and is given as micromoles per hour per square centimeter.

**Isotope flux studies.** H¹³⁶Cl⁻, ²²Na⁺, and ³H⁻ mannitol flux studies were performed in the open-circuit mode and during voltage clamp to 0 PD. 74 kBq/ml H¹³⁶Cl for ²²Na⁺ and 62 kBq/ml ³H⁻mannitol (2 mmol/l), respectively, were added either to the serosal or the mucosal solution after it had reached stable HCO₃⁻ flux (JHCO₃⁻) and electrical parameters. For each isotope, neighboring pieces of rabbit duodenum and identical sections of age-, weight-, and sex-matched inbred rats were used. After a 30-min period of equilibration, aliquots were taken in 20-min intervals for rat and 15-min intervals for rabbit, radioactivity was determined in a liquid scintillation counter, and the bidirectional flux rates for the respective substance was calculated. The values for Isc and JHCO₃⁻ represent the average values of the 20- and 15-min periods, respectively.

**RNA isolation and semiquantitative RT-PCR.** The RNA isolation method, the semiquantitative RT-PCR procedure, and the sequence information of histone 3.3a and 18s rRNA isoforms were described in detail previously (1, 41). Homologous primers for rat (forward: 5'-ACAAATTCATCACAACCATCACC-TCC-3' ; reverse: 5'-CATTGCCTCATTCTGTGGTTC-3') and rat (forward: 5'-CTGAACTCAAAGTCTAGTGC-3'; reverse: 5'-CCACCTCAAAGAAAAAC-3') CFTR were deduced from published sequence information (GenBank accession nos. AF189720 and M89906). The obtained PCR fragments displayed the expected size (696 bp for rabbit and 717 bp for rat CFTR). The identity of the amplimers was confirmed by restriction analysis.

**Statistics**

All results are expressed as means ± SE; n is the number of separate experiments. Error bars are not shown when included within the symbol. All examinations were performed at least in triplicate. If applicable, P values were determined by using the Student’s t-test.

**RESULTS**

HCO₃⁻ Secretion, Isc, and Bilateral Cl⁻, Na⁺, and ³H-Mannitol Fluxes Under Basal and Camp-Stimulated Conditions

After stable tissue parameters were reached, basal HCO₃⁻ secretion was 1.02 ± 0.2 and mean Isc was 1.65 ±
and mucosal-to-serosal 

tion under open-circuit conditions, serosal-to-mucosal 

The data demonstrate the very different basal and stimulated short- 

circuit current (Isc) before and after stimulation with 8-bromo-cAMP (8-Br-cAMP). 

Moreover, the relationship of secreted HCO3− to Cl− strongly predominated in the anion secretory response. Moreover, the relationship of secreted HCO3− to Cl− found in these experiments is well within the values for the CFTR anion conductance in patch-clamp experiments (11, 35).

If an apical Cl−/HCO3− exchanger was the transporter for cAMP-induced HCO3− secretion, it should be stimulated during cAMP-stimulated anion secretion. We therefore measured bilateral flux rates for Cl− and Na+ under open-circuit conditions (Fig. 2B) and searched for an increase in mucosal-to-serosal Cl− flux. However, we found a marked decrease in the mucosal-to-serosal Cl− flux rate after stimulation with a cAMP analog in the rat duodenum and a very small decrease in rabbit duodenum (Fig. 2B). In both species, serosal-to-mucosal Cl− fluxes strongly increased. The Cl− movements were paralleled by Na+ movements, albeit not in a one-to-one fashion. This is most likely due to the open-circuit conditions, with a lumen-negative PD and the unequal ion concentrations in the luminal and serosal perfusate as additional driving forces for ion movement. The decrease in mucosal-to-serosal Cl− and Na+ flux is most likely due to the known inhibitory effect of cAMP on electroneutral Na+/Cl− absorption mediated by Na+/H+ exchanger NHE3 and an anion exchanger whose molecular identity in the duodenum is under debate. These data demonstrate that under our experimental conditions, apical anion exchange is inhibited (in rat) or unchanged (in rabbit) rather than stimulated during cAMP-mediated electrogenic anion secretion in rat and rabbit duodenum.

3H− mannitol flux experiments were performed to assess changes in paracellular flux under conditions of the experiments. A net mucosal-to-serosal flux was found for mannitol, and 8-Br-cAMP elicited no significant changes (data not shown). Thus cAMP-induced changes in paracellular flux cannot explain the observed changes in anion secretory rate.

We then performed the same experiments under short-circuit conditions and bilateral identical CO2/ 

HCO3−-containing solutions in rabbit duodenum (n = 4). Under these circumstances, HCO3− secretion cannot be measured. The mean Isc was 0.15 ± 0.18 under resting-state conditions (compared with the calculated Isc of 1.08 ± 0.16 during open-circuit conditions), and the ΔIsc after 8-Br-cAMP was 2.55 ± 0.16 (compared with 2.12 ± 0.18), which decreased to 0.25 ± 0.15 (compared with −0.9 ± 0.22) 1 h after ouabain application. For Na+ and Cl−, the mucosal-to-serosal flux rates were slightly lower than during open-circuit conditions (possibly due to lack of concentration gradient) and did not significantly change after 8-Br-cAMP application (data not shown), the serosal-to-mucosal Cl− flux was identical under open and short-circuit conditions, and the Cl− secretory response was similar (9.3 ± 3.5 vs. 7.1 ± 2.8). The serosal-to-mucosal Na+ flux was lower (16.8 ± 1.3 vs. 21.5 ± 0.5), and 8-Br-cAMP caused a markedly lower rise in serosal-to-mucosal Na+ flux (18.1 ± 3.7). The data demonstrate that both the measured ΔIsc under short-circuit conditions and the calculated ΔIsc under open-circuit conditions are relatively close, and both underrepresent the CAMP-induced anion secretion. They further demon-
strate that the likely driving force for the strong serosal-to-mucosal Na\(^+\) flux under open-circuit conditions is the strong increase in PD. Third, they show that the Cl\(^-\) secretory response in the presence of 5% CO\(_2\)/24 mM HCO\(_3\) in the luminal bath is identical to if not enhanced from that in its complete absence. These data suggest that HCO\(_3\) concentrations that are likely to occur physiologically within the intestinal lumen do not compromise the Cl\(^-\) secretory response [contrasting the recent report of a strong inhibition of the CFTR Cl\(^-\) conductance by external HCO\(_3\) in a cultured cell line (32)].
Ion Substitution Experiments

If cAMP-stimulated duodenal HCO$_3^-$ secretion is primarily mediated by an apical Cl$^-$/HCO$_3^-$ exchange process, removal of Cl$^-$ should strongly inhibit this process. Therefore, we first studied the effect of complete Cl$^-$ removal from the system. In bilateral Cl$^-$-free conditions, basal HCO$_3^-$ secretion decreased in both species to the same values as found in the absence of luminal Cl$^-$, and 8-Br-cAMP elicited a HCO$_3^-$ secretory response that was markedly higher than in the presence of Cl$^-$ (Fig. 3). This rules out an essential role of Cl$^-$/HCO$_3^-$ exchange in cAMP-stimulated duodenal HCO$_3^-$ secretion.

It could be argued that the CFTR channel is permeable for HCO$_3^-$ in the absence but not in the presence of intracellular Cl$^-$. Therefore, we next tested the effect of selective removal of luminal Cl$^-$. The luminal substitution of Cl$^-$ by the gluconate ion increased $I_{sc}$ in both epithelia but reduced basal ouabain-sensitive (as determined in the experiments of Fig. 1) HCO$_3^-$ secretory rate by $\sim$40% in rat (Fig. 4A) and rabbit duodenum (Fig. 4B), suggesting that a substantial part of basal HCO$_3^-$ secretion was mediated by luminal Cl$^-$/HCO$_3^-$ exchange. However, 8-Br-cAMP led to an increase of HCO$_3^-$ secretion that was even somewhat higher ($\sim$20% in both epithelia) than after stimulation in the presence of luminal Cl$^-$. In the absence of luminal Cl$^-$, ouabain does not inhibit $I_{sc}$. This could be either due to the fact that in the absence of luminal Cl$^-$, a negative membrane potential (which would be generated by basolateral K$^+$ channel and Na$^+$-K$^+$-ATPase activity) is not required for Cl$^-$ flux via apical Cl$^-$ channels.

Because Cl$^-$ is transported across the epithelium during luminal Cl$^-$/free conditions, and this Cl$^-$ could potentially be exchanged for intracellular HCO$_3^-$ via apical Cl$^-$/HCO$_3^-$ exchange, we measured the actual amount of Cl$^-$ that is transported or leaks across the mucosa into the luminal compartment. The rate of serosal-to-mucosal Cl$^-$/movement in the absence of luminal Cl$^-$ was 10.3 $\mu$mol·cm$^{-2}$·h$^{-1}$; thus the Cl$^-$ concentration in the luminal bulk solution was $\sim$2 $\mu$M at the time of 8-Br-cAMP addition. This value is far lower than any $K_d$ value for external Cl$^-$ reported for any apical anion exchange process within the intestine or for any cloned and expressed anion exchanger protein studies so far. This makes it unlikely that an apical Cl$^-$/HCO$_3^-$ is not inhibited by luminal Cl$^-$ substitution. 

Effect of Bumetanide in the Presence and Absence of Luminal Cl$^-$

Bumetanide inhibits the basolateral Na$^+$-K$^+$-2Cl$^-$-cotransporter, which could lead to a decrease in intracellular Cl$^-$ concentration ([Cl$^-$]$_i$) and an increase in Cl$^-$ import via the apical Cl$^-$/HCO$_3^-$ exchanger, in
exchange for intracellular HCO$_3^-$.

Indeed, bumetanide in the serosal bath resulted in a decrease in $I_{sc}$, an increase in HCO$_3^-$ secretion, and an increase in Cl$^-$ (and Na$^+$) absorption in rat duodenum (Fig. 5A), and the most likely explanation is that bumetanide results in a decrease in [Cl$^-$], which in turn stimulates apical Cl$^-$/HCO$_3^-$ exchange. Interestingly, in the rabbit duodenum, bumetanide also resulted in an increase in Na$^+$ and Cl$^-$ reabsorption (data not shown) but not in a fall in $I_{sc}$ or an increase in HCO$_3^-$ secretion.

Thus the use of bumetanide allowed us to test whether HCO$_3^-$ secretion mediated by the apical Cl$^-$/HCO$_3^-$ exchanger is indeed inhibited by removal of Cl$^-$ from the luminal bath. Of course, this hypothesis could only be tested in the rat duodenum. The increase in basal HCO$_3^-$ secretion by bumetanide was completely inhibited by removal of luminal Cl$^-$, whereas the subsequent HCO$_3^-$ secretory response to 8-Br-cAMP was slightly increased, as seen during substitution of luminal Cl$^-$ in the absence of bumetanide (Fig. 5B). The most likely explanation for this experiment is that the increased activity of apical anion exchange, induced by inhibition of basolateral Cl$^-$ uptake, is inhibited by luminal Cl$^-$ substitution. This demonstrates that luminal Cl$^-$ substitution is indeed effectively inhibiting apical Cl$^-$/HCO$_3^-$ exchange.

**DIDS and 5-nitro-2-(3-phenylpropylamino)-benzoate Experiments**

The brush-border membrane Cl$^-$/HCO$_3^-$ exchanger in rat and rabbit duodenum is inhibited by the stilbene derivative DIDS. The luminal application of DIDS in a concentration of 1 mmol/l approximately halved basal ouabain-sensitive HCO$_3^-$ secretion in rat and rabbit; but again, the 8-Br-cAMP-induced HCO$_3^-$ secretory response was slightly higher than in the absence of luminal DIDS (Fig. 6A for rabbit duodenum only, but

![Fig. 4. $J_{HCO_3}$ (right axis) and $I_{sc}$ (left axis) in rat (A) and rabbit (B) isolated duodenum before and after removal of Cl$^-$ from the luminal perfusate and subsequent stimulation with 8-Br-cAMP. Luminal Cl$^-$ removal resulted in a significant reduction of the basal HCO$_3^-$ secretory rate ($P < 0.05; n = 5$), suggesting that a substantial part of basal HCO$_3^-$ secretion is mediated by apical Cl$^-$/HCO$_3^-$ exchange. The secretory response to 8-Br-cAMP was even somewhat larger than in the control (not significant for $n = 5$).](http://ajpgi.org/)

![Fig. 5. $J_{HCO_3}$ (right axis) and $I_{sc}$ (left axis) in rat isolated duodenum before (A) and after (B) removal of Cl$^-$ from the luminal perfusate and subsequent application of bumetanide ($10^{-4}$ M) followed by stimulation with 8-Br-cAMP. A: as expected of an agent that blocks Na$^+$-K$^+$-2Cl$^-$ cotransport, bumetanide application resulted in a decrease in basal $I_{sc}$ and a reduced $I_{sc}$ response. HCO$_3^-$ secretion, however, increased after bumetanide application, both in the basal state and when applied during the plateau phase after 8-Br-cAMP addition (data not shown). This suggested that an inhibition of basolateral Cl$^-$ influx stimulates apical Cl$^-$ uptake via Cl$^-$/HCO$_3^-$ exchange. B: in the absence of Cl$^-$ in the luminal bath, no increase in HCO$_3^-$ secretion was observed after bumetanide application, suggesting that luminal Cl$^-$ removal is effective in reducing Cl$^-$ concentration near the apical membrane of the enterocytes to values low enough to inhibit the apical Cl$^-$/HCO$_3^-$ exchange. 8-Br-cAMP results in a significantly reduced $I_{sc}$ ($P < 0.01$) but normal HCO$_3^-$ secretory response (not significant) in both conditions, compared with the stimulation in the absence of bumetanide.](http://ajpgi.org/)
rat yielded qualitatively similar results). To test whether DIDS exerts its effect on basal HCO\textsubscript{3}\textsuperscript{-} secretion primarily via inhibition of apical anion exchange or via inhibition of a DIDS-sensitive anion conductance or the basolateral Na\textsuperscript{+}-HCO\textsubscript{3}\textsuperscript{-} cotransporter, we then tested the effect of luminal DIDS on basal and stimulated HCO\textsubscript{3}\textsuperscript{-} secretion in the complete absence of Cl\textsuperscript{-} in the system (Fig. 6B). In the absence of Cl\textsuperscript{-}, luminal DIDS had virtually no effect on basal or stimulated HCO\textsubscript{3}\textsuperscript{-} secretion, demonstrating that the majority of luminal DIDS action is likely due to inhibition of the same pathway (apical Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchange) as that which is inhibited by removal of luminal Cl\textsuperscript{-}.

When 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), another Cl\textsuperscript{-} channel blocker with a known inhibitory effect on CFTR anion channels, was applied luminally, a reduction in basal $I_{sc}$ and a very strong inhibition of both the magnitude and the duration of 8-Br-cAMP-induced $I_{sc}$ and HCO\textsubscript{3}\textsuperscript{-} secretory response was observed ($\Delta I_{sc} = 0.9$ for rat and 0.34 for rabbit and $\Delta J_{HCO3} = 0.18$ for rat and 0.52 for rabbit). These results suggest that an NPPB-sensitive, DIDS-insensitive anion conductance is responsible for a major part of cAMP-stimulated HCO\textsubscript{3}\textsuperscript{-} secretion. The similar NPPB sensitivity of both $I_{sc}$ and HCO\textsubscript{3}\textsuperscript{-} secretory response is compatible with, but certainly does not prove, secretion via CFTR.

**CFTR mRNA Expression in Rat and Rabbit Duodenum**

It is known that the conductivity of the CFTR channel is not very high (3, 33), and therefore, it has been suggested that CFTR-dependent anion secretion may occur via CFTR-regulated channels or transporters rather than via the CFTR channel itself. We studied CFTR mRNA expression in rat and rabbit duodenum and compared it to the expression of other organs with a known, and high, CFTR expression, such as the pancreas or the colon. Both rat and rabbit duodenum had similarly high or higher CFTR expression levels in comparison to the other tested organs (Fig. 7). Moreover, comparing the CFTR expression levels with those for ion transport proteins such as the electroneutral Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchanger downregulated in adenoma (DRA) in the brush-border membrane of duodenal enterocytes (19), the Na\textsuperscript{+}-HCO\textsubscript{3}\textsuperscript{-} cotransporter NBC1 (18), or the anion exchanger AE2 in the basolateral membrane (19), we found expression levels in the same order of magnitude. Even though protein abundance and/or function may not always correlate with mRNA levels (for example, mutated CFTR protein gets degraded quickly, whereas mRNA expression levels remain unchanged), we believe that the extremely high CFTR expression levels (for a channel protein) will easily balance the relatively low conductance of each individual CFTR channel protein.

**DISCUSSION**

This study investigates the involvement of an apical Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchange process in basal and cAMP-stimulated duodenal HCO\textsubscript{3}\textsuperscript{-} secretion. It was found that although a Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchanger mediates an important part of the basal HCO\textsubscript{3}\textsuperscript{-} secretory rate, its activity is neither necessary for, nor stimulated during, cAMP-induced HCO\textsubscript{3}\textsuperscript{-} secretion. This finding was observed in rats, a species with rather low pancreatic and duodenal HCO\textsubscript{3}\textsuperscript{-} secretory rates, and rabbits, one with high rates comparable with humans. In both species, a DIDS-insensitive, NPPB-sensitive, cAMP-activated anion conductance mediates a secretory response with a strong predominance of Cl\textsuperscript{-} over HCO\textsubscript{3}\textsuperscript{-} in the secreted anions.

Gastrointestinal and hepatobiliary HCO\textsubscript{3}\textsuperscript{-} secretion has been recognized as a crucial transport process for the maintenance of mucosal integrity, enzymatic digestion, and Cl\textsuperscript{-} absorption (8, 9, 29, 31). All HCO\textsubscript{3}\textsuperscript{-}-secreting epithelia in CF patients show abnormal function, likely related to pH changes of their secretions.
Recent evidence suggests that CFTR mutations with a strong $\text{HCO}_3^-$ secretory defect are associated with severe pancreatic and intestinal disease (6). Thus the question of whether the transport of $\text{HCO}_3^-$ occurs via the CFTR protein itself or via a CFTR-regulated anion transporter may have considerable clinical implications. In the first instance, no option exists to restore $\text{HCO}_3^-$ secretory function other than a repair of the defect in the CFTR protein itself. If another protein is responsible for $\text{HCO}_3^-$ transport, then the potential exists to activate this transport process independently of CFTR activation.

A number of theoretical considerations has suggested that the high agonist-induced $\text{HCO}_3^-$ secretory rates found in duodenal or pancreatic epithelium cannot be transported by the CFTR protein. Apical anion channels in epithelial cell lines with properties of the CFTR anion conductance, as well as CFTR-like channels in CFTR expression systems, had $\text{HCO}_3^-$-to-$\text{Cl}^-$ permeability ratios between 1:3 and 1:8 (11, 16, 35). By extrapolating intracellular anion concentrations and membrane potentials that had been measured in primitive epithelial cell lines to the conditions in native epithelia, it was calculated that under "physiological" driving forces these apical anion conductances would secrete $\text{Cl}^-$ rather than $\text{HCO}_3^-$ (12, 25). The actual intestinal and pancreatic $\text{HCO}_3^-$ transport pathways were therefore thought to be CFTR-dependent anion channels with a higher conductivity and $\text{HCO}_3^-$ permeability than CFTR channels or $\text{Cl}^-$/$\text{HCO}_3^-$ exchangers coupled to CFTR channel activity via the intra- or extracellular $\text{Cl}^-$ concentration. But these hypotheses were neither substantiated nor ruled out.

The most attractive hypothesis is a coupling between an apical anion exchanger and the CFTR anion channel. Both DRA, a recently identified intestinal anion transport protein whose mutational defects are the molecular basis for the Finnish familial chloride diarrhea, and putative anion transporter (PAT)1, a recently identified anion transport protein from the same gene family, are apically expressed in gastrointestinal and pancreatic epithelia (19, 46), and recent studies suggest both structural (27, 28) and functional interaction of CFTR and DRA/PAT1 (23).

Effective removal of $\text{Cl}^-$ from the lumen inhibits any apical $\text{Cl}^-$/$\text{HCO}_3^-$ exchange process, and the stilbene DIDS has been shown to inhibit DRA and PAT1. Both $\text{Cl}^-$ removal from and the addition of DIDS to the luminal perfusate inhibited a substantial part of the ouabain-sensitive basal $\text{HCO}_3^-$ secretion in the duodenum of rat and rabbit and makes it unlikely that the observed changes in basal $\text{HCO}_3^-$ secretion were secondary to changes in paracellular $\text{HCO}_3^-$ movement. The application of luminal DIDS has a minimal effect on the residual $\text{HCO}_3^-$ secretion after complete removal of $\text{Cl}^-$, demonstrating that indeed the same $\text{HCO}_3^-$ transport pathway is inhibited by these maneuvers, namely, an apical anion exchange process. It rules out

Fig. 7. Semiquantitative RT-PCR analysis of CFTR mRNA expression levels in different segments of the gastrointestinal tract in rat (B) and rabbit (C) intestine. Exemplary for all PCR experiments, Fig. 7A shows similar amplification efficiency of the gene of interest (CFTR) and the control gene (histone 3.3a) from rabbit duodenal mucosa. The bars depict relative expression levels of rat CFTR vs. 18S RNA (B) and rabbit CFTR vs. histone 3.3a (C) ($n = 3$). In rabbit, the expression levels are also given (as ODI ratios) in stomach and kidney, organs that are also known to express CFTR. By comparison, duodenal CFTR expression is high.
a substantial contribution of the highly DIDS-sensitive outwardly rectifying Cl$^-$ channel (ORCC) (10), a strong inhibition of the basolateral Na$^+-$HCO$_3^-$ cotransporter by luminal DIDS application, or a major effect of external DIDS on the HCO$_3^-$ conductivity of the CFTR channel.

In contrast, the cAMP-induced HCO$_3^-$ secretory response was unaffected by Cl$^-$ removal or luminal DIDS. The strong HCO$_3^-$ secretory response in the complete absence of Cl$^-$ was also seen in the duodenum of CFTR $+/-$ but not of $-/-$ mice (I. Blumenstein, unpublished data). The absence of HCO$_3^-$ in the luminal solution also makes an electrogenic 1 HCO$_3^-$-to-2 Cl$^-$ exchange unlikely (23). Together, the data argue against the concept that Cl$^-$/HCO$_3^-$ exchange is involved in cAMP-dependent duodenal HCO$_3^-$ secretion.

A lack of effect of luminal Cl$^-$ removal on intraluminal alkalization has been observed in isolated pancreatic and epididymal ducts (5, 17). However, it has been argued that movement of serosal Cl$^-$ through the epithelium may cause Cl$^-$ concentrations near the brush-border membrane sufficiently high to allow uninhibited operation of an apical anion exchanger. We therefore sought for a way to stimulate the apical anion exchanger. In rat duodenum, bumetanide inhibits basolateral Na$^+-$K$^+$-2Cl$^-$ cotransport and reduces $I_{sc}$ but stimulates HCO$_3^-$ secretion. This stimulation is completely dependent on the presence of Cl$^-$ in the luminal bath, whereas subsequent stimulation by cAMP analogs is unaffected. The likely explanation is that the inhibition of basolateral Na$^+-$K$^+$-2Cl$^-$ cotransport results in a decrease in [Cl$^-$_i], which in turn stimulates apical Cl$^-$ uptake by Cl$^-$/HCO$_3^-$ exchange. Thus the basic principle of apical Cl$^-$/HCO$_3^-$ exchange activation by low [Cl$^-$_i] is applicable. Cl$^-$ removal from the luminal bath appears to effectively inhibit apical Cl$^-$/HCO$_3^-$ exchange.

What then is the likely mechanism for agonist-stimulated duodenal HCO$_3^-$ secretion? Its complete absence in CFTR $-/-$ mice is indicative of a CFTR-dependent mechanism (42). The involvement of ORCCs seems improbable because of their DIDS sensitivity (10, 21, 22). Purinergic, receptor-activated, Ca$^{2+}$-dependent channels of the CaCC family are inhibited by CFTR activation (26, 48). Cl$^-$ channel CIC-2 expression has also been found to be apically located in the intestine of normal and CFTR $-/-$ mice (20), but these are acti-

### Table 1.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control Conditions</th>
<th>Addition of Stimulants</th>
<th>Mean PD [mV] ± SE</th>
<th>Mean R [Ohm × cm$^2$] ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Basal conditions, addition of 8-Br-cAMP</td>
<td>0.5 ± 0.1</td>
<td>25.8 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>Peak effect of 8-Br-cAMP</td>
<td>1.9 ± 0.1</td>
<td>27.4 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>Addition of bumetanide</td>
<td>1.8 ± 0.1</td>
<td>30.2 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>Addition of ouabain</td>
<td>1.6 ± 0.1</td>
<td>28.9 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>DIDS application</td>
<td>0.4 ± 0.1</td>
<td>22.6 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>85</td>
<td>Luminal chloride-substitution after 30 minutes of equilibration</td>
<td>-0.5 ± 0.1</td>
<td>23.3 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>B: Bilateral chloride-substitution before the start of the experiment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time (min)</td>
<td>Addition of Stimulants</td>
<td>Mean PD [mV] ± SE</td>
<td>Mean R [Ohm × cm$^2$] ± SE</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Equilibration after solution change, addition of 8-Br-cAMP</td>
<td>2.3 ± 0.2</td>
<td>43.9 ± 10.0</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>Peak effect of 8-Br-cAMP</td>
<td>4.2 ± 0.4</td>
<td>50.9 ± 7.2</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>Addition of bumetanide</td>
<td>3.7 ± 0.6</td>
<td>52.2 ± 7.3</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>Addition of ouabain</td>
<td>2.9 ± 0.9</td>
<td>48.3 ± 4.9</td>
<td></td>
</tr>
<tr>
<td>85</td>
<td>DIDS application</td>
<td>2.5 ± 1.1</td>
<td>45.8 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>C: Luminal chloride-substitution after 30 minutes of equilibration</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time (min)</td>
<td>Ion substitution/addition of stimulants</td>
<td>Mean PD [mV] ± SE</td>
<td>Mean R [Ohm × cm$^2$] ± SE</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>Solution change, addition of 8-Br-cAMP</td>
<td>0.7 ± 0.2</td>
<td>29.1 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>Addition of 8-Br-cAMP</td>
<td>0.2 ± 0.1</td>
<td>52.0 ± 3.7</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>15 minutes after addition of 8-Br-cAMP</td>
<td>2.8 ± 2.5</td>
<td>53.5 ± 3.4</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>Addition of 8-Br-cAMP</td>
<td>4.6 ± 2.8</td>
<td>50.9 ± 4.3</td>
<td></td>
</tr>
<tr>
<td>85</td>
<td>Addition of ouabain</td>
<td>11.1 ± 2.4</td>
<td>90.9 ± 15.8</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>DIDS application</td>
<td>11.0 ± 2.7</td>
<td>87.7 ± 15.6</td>
<td></td>
</tr>
<tr>
<td>115</td>
<td>Addition of ouabain</td>
<td>10.1 ± 2.9</td>
<td>66.6 ± 10.6</td>
<td></td>
</tr>
<tr>
<td>130</td>
<td>Addition of DIDS</td>
<td>10.3 ± 2.9</td>
<td>56.0 ± 8.0</td>
<td></td>
</tr>
<tr>
<td>145</td>
<td>Addition of 8-Br-cAMP</td>
<td>11.1 ± 2.8</td>
<td>53.5 ± 7.2</td>
<td></td>
</tr>
<tr>
<td>D: Addition of DIDS to the luminal perfusate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time (min)</td>
<td>Addition of DIDS</td>
<td>Mean PD [mV] ± SE</td>
<td>Mean R [Ohm × cm$^2$] ± SE</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>DIDS application</td>
<td>0.5 ± 0.1</td>
<td>27.7 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>Addition of bumetanide</td>
<td>0.8 ± 0.1</td>
<td>27.0 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>Peak effect of 8-Br-cAMP</td>
<td>1.9 ± 0.2</td>
<td>21.6 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>DIDS application</td>
<td>1.5 ± 0.2</td>
<td>25.1 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>85</td>
<td>Addition of ouabain</td>
<td>1.2 ± 0.2</td>
<td>23.2 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>Addition of 8-Br-cAMP</td>
<td>0.5 ± 0.1</td>
<td>19.7 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>115</td>
<td>Addition of ouabain</td>
<td>-0.1 ± 0.1</td>
<td>22.3 ± 1.4</td>
<td></td>
</tr>
</tbody>
</table>

---

AJP-Gastrointest Liver Physiol • VOL 285 • NOVEMBER 2003 • www.ajpgi.org
vated by osmotic swelling and hyperpolarization (36, 40, 49), the opposite of which occurs during cAMP-dependent stimulation of enterocytes. Moreover, recently a tight-junctional localization of CIC-2 has been described in small intestine (14). Very recently, a CIC-4 channel has been colocalized with CFTR in mouse and human intestine (30), but again, its properties make it unlikely that this channel mediates the cAMP-stimulated HCO$_3^-$ current.

Thus none of the anion transport mechanisms discussed above appears to be a more likely candidate for agonist-stimulated duodenal HCO$_3^-$ secretion than the CFTR protein itself. The percentage of HCO$_3^-$ to Cl$^-$ in the rat and rabbit (as well as mouse; data not shown) duodenal secretory response is fully compatible with any HCO$_3^-$/Cl$^-$ permeability ratio measured for CFTR expression systems. NPPB sensitivity is certainly not specific for the CFTR channel, but a lack of NPPB inhibition on $I_{sc}$ and HCO$_3^-$ secretion would have been noteworthy; however, this was not found. Duodenal CFTR mRNA expression levels were found to be in the range of those for electroneutral ion exchangers like NHE3 (19), which, given the high transport capacity of a channel compared with an exchanger, forms the basis for substantial intestinal anion flux via CFTR.

For the measurement of HCO$_3^-$ secretion, we studied the tissues in the open-circuit mode. In a leaky tissue like the duodenum, changes in PD could elicit considerable ion fluxes via the tight junctions. However, the marked increase in tissue resistance after the removal of Cl$^-$ did not decrease the HCO$_3^-$ secretory response to cAMP analogs. Because an increase in PD negativity would inhibit paracellular HCO$_3^-$ secretion, the observed increase is likely transcellular.

To better understand the observed increase in cAMP-induced Na$^+$ flux under open-circuit conditions, we measured all parameters except HCO$_3^-$ secretion under voltage-clamp conditions in rabbit duodenum (in this instance with CO$_2$/HCO$_3$ on both sides of the epithelium). We found a markedly lower cAMP-induced serosal-to-mucosal Na$^+$ efflux, demonstrating that the strong cAMP-induced serosal-to-mucosal Na$^+$ flux under open-circuit conditions is indeed due to the stimulation-associated increase in PD negativity and that short circuiting can actually be achieved quite successfully in rabbit duodenum. The Cl$^-$ secretory response was not significantly affected. The surprising finding was that the accompanying $I_{sc}$ increase still was markedly lower than the Cl$^-$ secretory response. We do not understand this difference. Possible explanations are a relatively large conductive pathway for K$^+$ in the brush-border membrane or basolateral anion uptake via extracellular pathways.

We conclude that an apical Cl$^-$/HCO$_3^-$ exchange process mediates a substantial part of basal duodenal HCO$_3^-$ secretion. Wherever present in the apical membrane, this exchanger will allow the generation of high luminal HCO$_3^-$ (or CO$_2$ concentrations, if coupled with a Na$^+$/H$^+$ exchanger) and low luminal Cl$^-$ concentrations. The Cl$^-$/HCO$_3^-$ exchanger, however, is not the transport pathway for agonist-stimulated HCO$_3^-$ secretion. While stimulated by low [Cl$^-$], it is not stimulated by an activation of electrogenic anion secretion, possibly because basolateral Cl$^-$ uptake mechanisms effectively prevent a secretion-associated decrease in [Cl$^-$].

We gratefully acknowledge the expert technical assistance of Christina Neff, the technical support of Drs. Markus Guba and Irina Blumenstein, and the constructive criticism of Prof. Dr. Michael Sessler and Dr. G. Lamprecht.

This article includes experimental work performed by Stefanie Spiegel and Michael Phillipper in fulfillment of the requirements for their doctoral theses.

**DISCLOSURES**

This work was supported by Deutsche Forschungsgemeinschaft grant Se 460/13-1 and Se 460/13-2 and by a grant from the Interdiszplinäres Zentrum für Klinische Forschung Tübingen (Project IIIC1).

**REFERENCES**


15. Illek B, Fischer H, and Machen TE. Genetic disorders of membrane transport. II. Regulation of CFTR by small molecules.


