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Na^+/HCO_3^- Cotransport in Normal and Cystic Fibrosis Intestine

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Summary

In a search for the HCO_3^- supply mechanisms to the enterocyte we cloned and sequenced an intestinal subtype of the $Na^+HCO_3^-$ cotransporter isoform I (dNBC1), which turned out to be identical to the pancreatic NBC1 subtype (pNBC1). Within the intestine, we found particularly high NBC1 expression levels in the duodenum and proximal colon. Experiments with stripped rabbit duodenum in Ussing-chambers revealed that $Na^+HCO_3^-$ cotransport (NBC) and CO_2 hydration/Na^+/H^+ exchange were equally important duodenal HCO_3^- supply pathways and were both upregulated during cAMP-mediated secretion. In the proximal colon, however, HCO_3^- secretion was low but NBC1 expression even higher than in the duodenum. Ussing-chamber experiments with an NBC-specific inhibitor revealed that NBC, coupled to basolateral Cl^-/HCO_3^- exchange, was an important alternative Cl^- supply pathway to $Na^+K^+2Cl^-$ cotransport (NKCC) during cAMP-stimulated colonic Cl^- secretion.

To investigate the functional integrity of anion uptake pathways in the absence of cystic fibrosis transmembrane conductance regulator (CFTR), we fluorometrically assessed NBC and NKCC transport rates and cell volume before and during forskolin-stimulation in isolated colonic crypts from normal and CFTR (-/-) mice. Although forskolin stimulation decreased cell volume only in normal, not in CFTR (-/-) crypts, it activated NBC and NKCC to a similar degree in both normal and CFTR (-/-) crypts.

We conclude that, depending on the intestinal segment, NBC1 plays an important role in basolateral HCO_3^- or Cl^- uptake. Expression and activation by cAMP is preserved in CFTR (-/-) intestine.

In cystic fibrosis (CF) patients and CFTR (-/-) mice, intestinal, pancreatic and biliary HCO_3^- secretion is impaired. In all segments of *in vitro* CFTR (-/-) mouse intestine, we found a reduction in basal HCO_3^- secretory rate and a striking absence of a HCO_3^- secretory response to all tested physiologic agonists [1].

Since small intestinal peptic damage, malabsorption, and obstruction of biliary and pancreatic ducts as well as the intestinal lumen in CF patients may, at least in part, be secondary to the HCO_3^- secretory defect, we further investigated intestinal HCO_3^- transport pathways in normal and CF epithelium. The curious observation was that despite the molecular or functional presence of non-CFTR anion channels in the intestine of CFTR (-/-) mice, and despite the fact that HCO_3^- secretion could be stimulated in CFTR (-/-) intestine using tools to alter enterocyte membrane potential, no physiologic stimulus was found that caused significant HCO_3^- secretion.

Recent investigations have revealed a plethora of secondary defects in cells not expressing the CFTR protein, including defective expression and/or regulation of ion transport proteins [2-8]. We therefore speculated that downregulation of expression or defective regulation of basolateral HCO_3^- uptake mechanisms may intensify the HCO_3^- secretory defect in CF intestine and possibly have secondary effects on the intracellular pH (pH_i) and volume regulatory or absorptive function of CF enterocytes. The HCO_3^- supply pathways during intestinal HCO_3^- secretion were ill defined, but some functional evidence existed that a stilbene-sensitive transport pathway, possibly a $Na^+HCO_3^-$ cotransporter, was involved in HCO_3^- secretion in the duodenum, the intestinal segment with the highest HCO_3^- secretory rate [9, 10, 11].

We therefore aimed to 1) functionally identify the major base uptake mechanisms in isolated duodenal basolateral membranes, 2) clone and sequence potential intestinal base uptake mechanisms and demonstrate their physiological function in the HCO_3^- secretory process and 3) study their expression levels and regulation in normal and CF intestine.

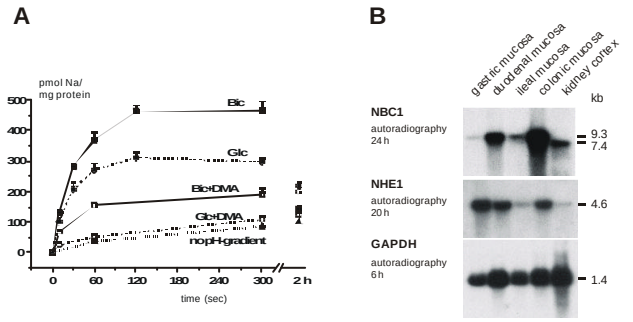

Figure 1. A) pH-gradient driven ^{22}Na-uptake into duodenal BLMvs in the presence and absence of HCO_3^-, with and without DMA. B) High-stringency Northern Blot analysis of about 10 µg once purified poly (A+) RNA from rabbit gastric mucosa, duodenal mucosa, ileal mucosa, colonic mucosa, and kidney cortex. A glycerol aldehyde trisphosphate dehydrogenase (GAPDH) probe confirms loading of intact RNA in all lanes.

$Na^+HCO_3^-$ Cotransport in the Duodenum

Functional identification of the major base uptake mechanisms in duodenal basolateral membrane vesicles. Previous experiments had demonstrated that duodenal HCO_3^- secretion [9, 11] and base uptake into isolated duodenal cells [12, 13, 14] is Na^+-dependent. Thus, the investigation of pH_i-gradient driven Na^+ uptake should assess *all* physiological base uptake mechanisms in the duodenocyte. Figure 1A shows $^{22}Na^+$ uptake into rabbit duodenal basolateral membrane vesicles (BLMvs) in the absence and presence of HCO_3^-, with and without dimethyl-amiloride (DMA), an inhibitor of Na^+/H^+ exchange, and in the absence and presence of a pH-gradient. It is obvious that pH_i-dependent $^{22}Na^+$ uptake consists of a HCO_3^--dependent, DMA insensitive one ($Na^+HCO_3^-$ cotransport) and a DMA-sensitive, HCO_3^- independent one (Na^+/H^+ exchange). Consistent with this concept was the finding that 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) inhibited the HCO_3^- dependent, DMA insensitive, pH-gradient driven $^{22}Na^+$ uptake, whereas more than 80% of DMA-sensitive, HCO_3^--independent $^{22}Na^+$ uptake was inhibited by 1 µM Hoe 642 (Hoechst compound 642) and therefore mediated by the NHE1 isoform of the Na^+/H^+ exchanger (NHE) gene family (data shown in [15]).

Molecular characterization and expression levels of duodenal basolateral HCO_3^- transporters. Having obtained functional evidence for the presence of a $Na^+HCO_3^-$ cotransporter in the basolateral membrane of rabbit duodenocytes, we wanted to establish its molecular identity. We therefore cloned and sequenced cDNA fragments for all recently cloned NBC isoforms, and established their expression levels by a semiquantitative PCR protocol (data shown in [15]). Although other isoforms like NBC2 [16] and NBCn1 [17] were expressed in the intestinal tract, the NBC1

isoform had the highest expression levels. Figure 1B shows a Northern Blot with poly (A+) RNA from different gastrointestinal segments and kidney cortex, hybridized with cDNA fragments for NBC1 and NHE1, the two major basolateral base uptake mechanisms in rabbit duodenocyte. Both NBC1 and NHE1 are strongly expressed in duodenal mucosa. Since we observed a difference in mRNA size between NBC1 expressed in the intestine and the kidney, we cloned the complete NBC1 sequence from rabbit duodenum and pancreas and the N-terminal sequence from rabbit kidney and found that the gastrointestinal and the kidney isoform differ in their N-termini. Primers were chosen for the selective amplification of the different N-termini from the different gastrointestinal segments and kidney cortex. Figure 2 demonstrates that both NBC1 subtypes are expressed in intestine and kidney, but with a strong predominance of the intestinal NBC1 (dNBC1) subtype in the intestine and the kidney NBC1 (kNBC1) subtype in the kidney.

Thus, it is clear that $Na^+HCO_3^-$ cotransport in the intestine is mediated by different molecular entities, among which the intestinal subtype of NBC1 has the highest expression levels.

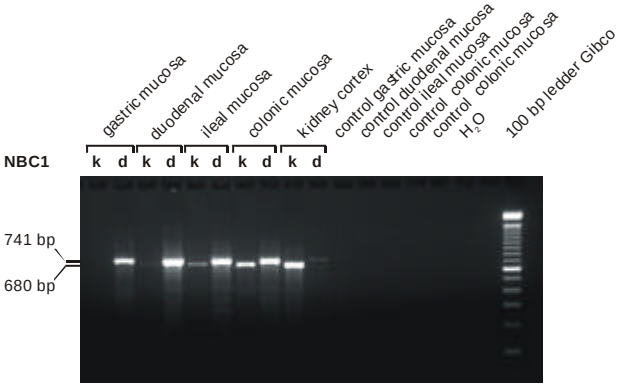

Figure 2. RT-PCR analysis of rabbit NBC1 mRNA variants, using specific forward primers for kNBC1 and dNBC1. EtBr-stained NBC1 amplification products (34 cycles) from rabbit gastric mucosa, duodenal mucosa, ileal mucosa, colonic mucosa, and kidney cortex RNA.

Physiological role of $Na^+HCO_3^-$ cotransport and NHE1 in HCO_3^- secretion. After having identified a $Na^+HCO_3^-$ cotransporter and NHE1 as the major basolateral base importers in rabbit duodenocyte basolateral membrane, we studied their importance for basal and stimulated duodenal HCO_3^- secretion. 1 mM DIDS, which in this concentration will inhibit all currently known $Na^+HCO_3^-$ cotransporters, caused a strong reduction of basal ouabain-sensitive HCO_3^- secretion to about 50%, demonstrating that a major part of the actively secreted HCO_3^- ions are supplied by uptake via $Na^+HCO_3^-$ cotransport. Unexpectedly, the 8-Br-cAMP-stimulated increase in HCO_3^- secretion was unchanged from the control conditions (Figure 3AB). This suggested that another system for HCO_3^- supply was activated during secretion. Since the removal of CO_2/HCO_3^- diminished basal and stimulated HCO_3^- secretion by approximately 85% (data shown in [15]), only 15% can be generated by CO_2 hydration from intracellular sources or by import and secretion of a base other than HCO_3^-. Thus, the likely alternative system was CO_2 uptake and hydration via carboanhydrase, and basolateral extrusion of the generated protons by Na^+/H^+ exchange. 1 mM acetazolamide caused ouabain-sensitive basal secretory rate to drop to approximately 50% of control value, but surprisingly, the 8-Br-cAMP-stimulated increase in HCO_3^- secretion was not diminished compared to the control, suggesting upregulation of $Na^+HCO_3^-$ cotransport (Figure 3C). Only the combination of DIDS and acetazolamide, or DIDS and 1 µM Hoe 642, which will inhibit NHE1 and thereby the basolateral extrusion of protons generated during CO_2 hydration, strongly reduced both the peak and the duration of the secretory response (Figure 3DE). These results demonstrate that $Na^+HCO_3^-$ cotransport and CO_2 hydration/Na^+/H^+ exchange *via* NHE1 are equally important pathways for duodenal HCO_3^- supply and are upregulated during cAMP-mediated stimulation

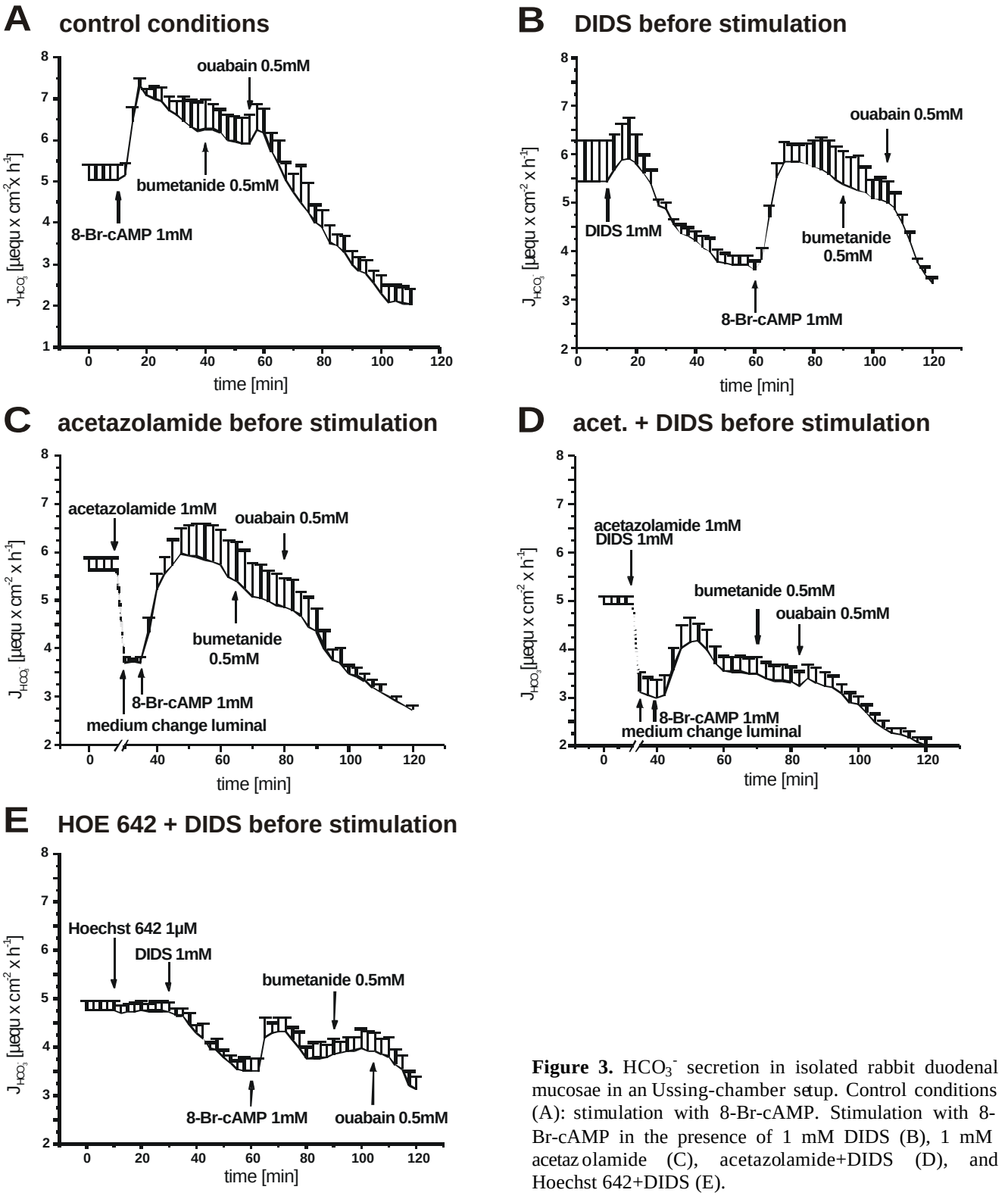

Figure 3. HCO_3^- secretion in isolated rabbit duodenal mucosae in an Ussing-chamber setup. Control conditions (A): stimulation with 8-Br-cAMP. Stimulation with 8-Br-cAMP in the presence of 1 mM DIDS (B), 1 mM acetazolamide (C), acetazolamide+DIDS (D), and Hoechst 642+DIDS (E).

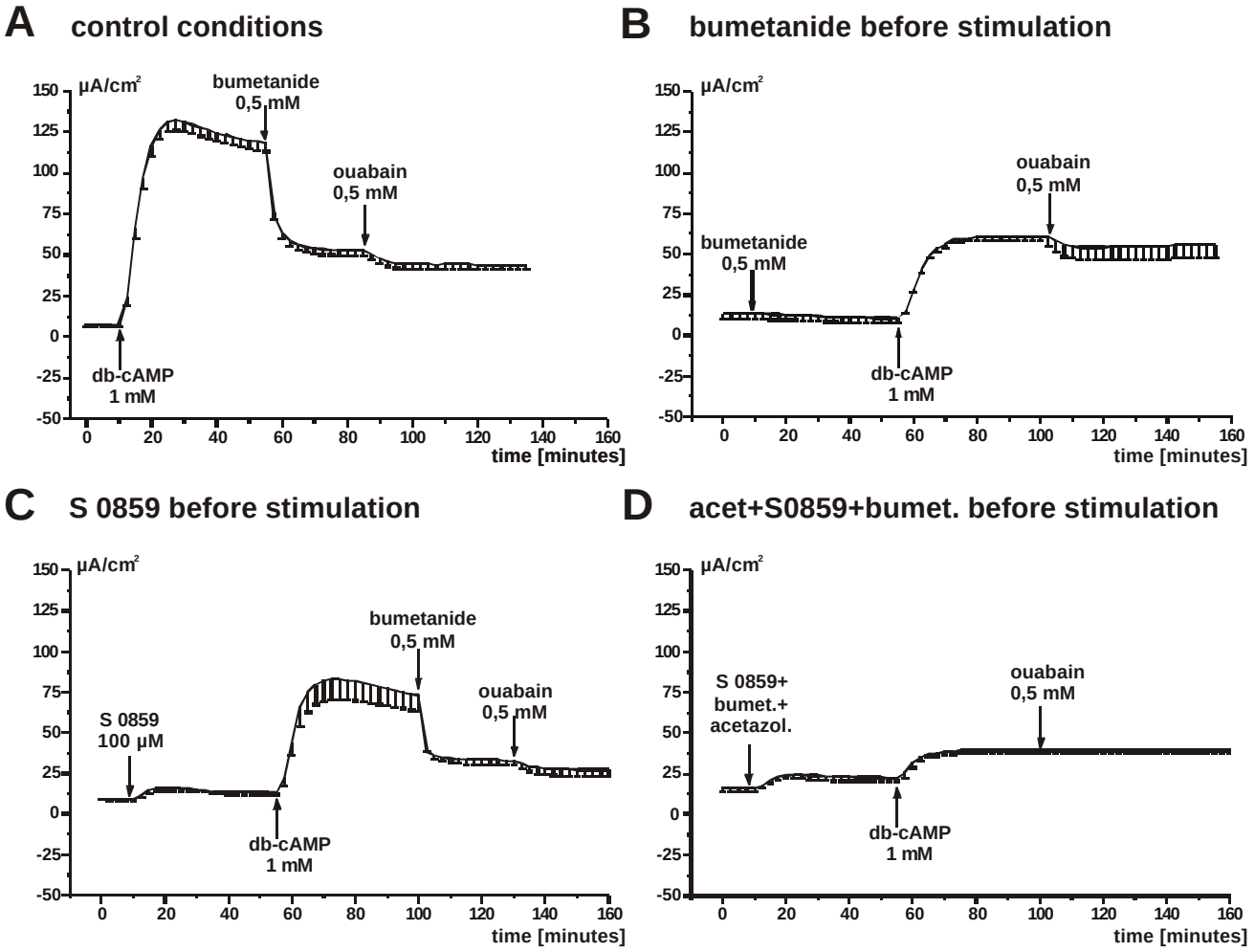

Figure 4. Short circuit current in isolated mouse colonic mucosae in an Ussing-chamber setup. Control conditions (A): stimulation with dB-cAMP. Stimulation with dB-cAMP in the presence of 0.5 mM bumetanide (B), 100 µM of the NBC-inhibitor S 0859 (C), and S 0859 + bumetanide + acetazolamide (D).

$Na^+HCO_3^-$ Cotransport in the Colon

The Northern Blot in Figure 1 shows a strong NBC1 expression in the proximal colon as well as the duodenum. However, HCO_3^- secretion is low in this part of the intestinal tract. We therefore wondered what might be the physiological significance of this transporter in the colon. HCO_3^- ions are exchanged for luminal Cl^- in the process of electroneutral salt absorption, and HCO_3^- ions for this process could be in part taken up by $Na^+HCO_3^-$ cotransport. However, this would imply the concomitant uptake of Na^+ ions from the interstitium, a process that would seem somewhat counterproductive for NaCl absorption. On the other hand, $Na^+HCO_3^-$ cotransport, coupled to an electroneutral Cl^-/HCO_3^- exchange mechanism in the basolateral membrane, could serve as an alternative Cl^- uptake mechanism to the $Na^+K^+2Cl^-$ cotransporter during Cl^- secretion. This concept would be consistent with earlier observations of residual anion secretion after pharmacological or gene technologic inhibition of $Na^+K^+2Cl^-$ cotransport [18, 19], as well as explain the high colonic expression levels for the basolateral anion exchanger AE2 [20]. To test this hypothesis, we measured 8-Br-cAMP-stimulated short-circuit current (I_{sc}) and HCO_3^- secretion in murine muscle-stripped proximal colon in the Ussing-chamber. Serosal

bumetanide (100 µM) or the NBC-specific inhibitor S0859 (100 µM) reduced db-cAMP-induced ΔI_{sc} by 60% and 45%, respectively, the combination of both plus bilateral acetazolamide inhibited ΔI_{sc} to the same extent as bumetanide in the absence of CO_2/HCO_3^- (Figure 4A-D). Acetazolamide augmented the inhibitory effect of S0859 but not of serosal DIDS (inhibits NBC and AE2), suggesting that the HCO_3^- ions used for basolateral Cl^-/HCO_3^- exchange (*via* AE2) are predominantly supplied by basolateral $Na^+HCO_3^-$ cotransport and to a minor extent by CO_2 hydration (data not shown). These results demonstrate that in the colon, basolateral $Na^+HCO_3^-$ cotransport is involved in Cl^- uptake during colonic anion secretion.

NBC1 and NKCC1 Expression and Regulation in Normal and CF Colonic Crypts

$Na^+HCO_3^-$ and $Na^+K^+2Cl^-$ cotransport is activated during cAMP-stimulated anion secretion, but whether this is a direct effect of the second messenger or secondary to cell volume reduction subsequent to Cl^- channel opening (which by itself can activate protein kinases [21, 22, 23]), or both, is under debate [24-30]. We wondered if reduced expression levels or defective regulation of these anion uptake pathways may be a component of the secretory defect in CF epithelia. We did indeed observe a reduction in NBC1 and NKCC1 expression in the intestine of adult CFTR (-/-) mice in relation to the 18s rRNA, but not when compared to villin, a cytosceletal protein of a brush border membrane, suggesting that structural changes may occur in the intestine of these mice (Figure 5). Therefore, we searched for a model in which the activity of $Na^+HCO_3^-$ and $Na^+K^+2Cl^-$ cotransport could be assessed at a cellular level. Colonic cypts were isolated from the proximal colon of CFTR (-/-) mice and their normal littermates and NBC and NKCC activity were assessed fluorometrically

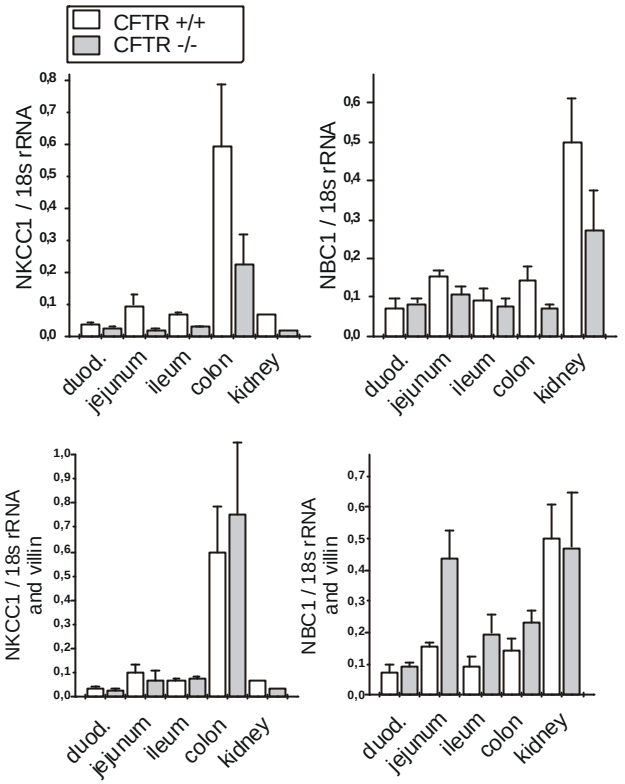

Figure 5. Expression of NBC1 and NKCC1 in relationship to 18s rRNA (upper panel) and normalized to villin (lower panel) in rabbit intestine and kidney in CFTR (+/+) and (-/-) mice as detected by semiquantitative RT-PCR.

after loading the crypts with the pH_i-sensitive dye 2'7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF). Crypt volume was measured by determining the cross sectional area of calcein-loaded crypts using confocal microscopy. We found that although basal NBC and NKCC activity were slightly reduced in CF crypts, forskolin activated both NBC and NKCC transport activity to the same degree in CFTR (+/+) and (-/-) crypts (data not shown). Forskolin stimulation caused a marked reduction in the crypt cross sectional area in CFTR (+/+) crypts, whereas no change in crypt volume was seen in CFTR (-/-) cells. This demonstrates that changes in cell volume is not the mechanism by which forskolin activates NKCC and NBC transport activity.

In summary, we found that the intestinal subtype of the NBC1 is strongly expressed in both the duodenum and colon. In the

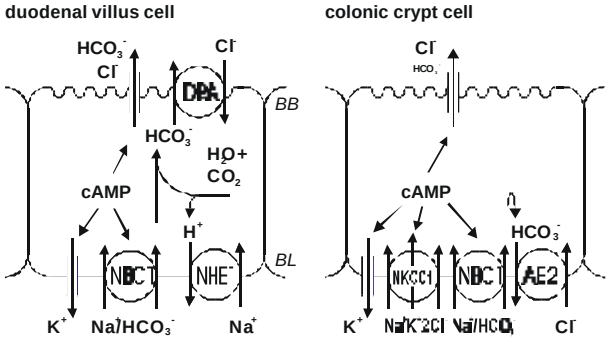

Figure 6. Cellular model for $Na^+HCO_3^-$ cotransport action in the intestine.4

duodenum, its expression is in the villus region and it is a major HCO_3^- supply pathway during basal and stimulated HCO_3^- secretion. In the colon, it is expressed predominantly in the crypts and one of its physiological functions is to serve as an alternative Cl^- uptake mechanism in conjunction with a basolateral anion exchanger. Our current concept of the anion transport mechanisms in duodenum and colon is depicted in Figure 6.

Methods

BLMV isolation. Basolateral membrane vesicles from rabbit duodenum were isolated by a combination of differential and sucrose density gradient centrifugation as previously described [15, 31].

$^{22}Na^+$ uptake experiments. To load BLMvs with the appropriate intravesiclar buffer, BLMvs were suspended, centrifuged and revesiculated in the appropriate buffer. To avoid the buildup of a diffusion potential, 20 µM valinomycin and high and equal K^+ concentrations were present in the intravesiclar and uptake buffer. Details of the method and the appropriate buffer compositions are described elsewhere [15].

Cloning and expression studies To clone rabbit NBC1, degenerate primers were chosen based on the published sequence from ambystoma and human kidney [32, 33]. The N-termini of the intestinal and kidney NBC1 subtype were cloned by rapid amplification of cDNA ends PCR (RACE PCR). Based on established cDNA sequences, appropriate primers for quantitative PCR analysis were searched for and tested, and a semiquantitative PCR protocol was established in which the amplification of the gene of interested was compared to that of a control gene in the exponential phase of the reaction [34].

Ussing-chamber experiments Muscle-stripped intestinal segments were placed in conventional Ussing-chambers, electrical parameters were recorded during continuous or intermittent voltage-clamp, and HCO_3^- secretion was measured by pH-stat titration.

Colonic surface cell and crypt isolation Colon crypts were isolated by vibration of everted proximal colon in EDTA-containing solutions, and sequential isolation of surface cells, then a few fractions that were discarded, and eventially whole crypts.

Fluorescence measurements To measure NBC transport rates, BCECF-loaded cypts were acid-loaded by an ammonium prepulse, and pH_i-recovery measured in the presence of 700 µM dimethyl-amiloride (DMA), which blocks all sodium-proton-exchanger isoforms in the colon. The corresponding flux rates were calculated from the pH_i recovery rate and the buffering capacity at the appropriate pH. To measure NKCC activity, we utilized the fact that NH_4^+ can enter the cell via the NKCC, using the K^+ transport site, and thereby causes a proton influx. After superfusion of the crypts with ammonium chloride, pH_i first increases sharply, because the cell permeable NH_3 causes an alkalinization. The pH_i recovery that follows is in part mediated by NH_4 uptake by NKCC. The base flux rate after ammonium pulse is mainly composed of an azosemide fraction, representing NKCC activity, and a DIDS-sensitive fraction which reflects the activation of a Cl^-/base exchanger.

Crypt volume measurements Confocal images of the crypt cross sectional area were used as a measure for the crypt volume as described by Heitzmann *et al.* [27].

Key words Anions; Bicarbonates; Colon; Cystic Fibrosis; Duodenum; Intestinal Mucosa; Intestinal Obstruction; Intestinal Secretions; Ion Transport

Abbreviations AE: anion exchanger; BCECF: 2'7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; BLMvs: basolateral membrane vesicles; $[Cl^-]_i$; intracellular Cl^- concentration; CF: cystic fibrosis; CFTR: cystic fibrosis transmembrane conductance regulator; DIDS: 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid; DMA: dimethyl-amiloride; dNBC1: $Na^+HCO_3^-$ cotransporter isoform I; GAPDH: glycerol aldehyde trisphosphate dehydrogenase; Hoe: Hoechst compound; I_{sc}: short-circuit current; kNBC1: kidney NBC1 subtype; NBC: $Na^+HCO_3^-$ cotransport; NHE: Na^+/H^+ exchanger; NKCC: $Na^+K^+2Cl^-$ cotransport; pH_i: intracellular pH; pNBC1: pancreatic NBC1 subtype; RACE PCR: rapid amplification of cDNA ends PCR

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References

1. Seidler U, Blumenstein I, Kretz A, Viellard-Baron D, Rossmann H, Colledge WH, et al. A functional CFTR protein is required for mouse intestinal cAMP-, cGMP- and Ca^{2+}-dependent HCO_3^- secretion. J Physiol 1997; 505:411-23. [98085162]

2. Gabriel SE, Clarke LL, Boucher RC, Stutts MJ. CFTR and outward rectifying chloride channels are distinct proteins with a regulatory relationship. Nature 1993; 363:263-8. [93254577]

3. Greger R, Mall M, Bleich M, Ecke D, Warth R, Riedemann N, Kunzelmann K. Regulation of epithelial ion channels by the cystic fibrosis transmembrane conductance regulator. J Mol Med 1996; 74:527-34. [97047138]

4. Ismailov II, Awayda MS, Jovov B, Berdiev BK, Fuller CM, Dedman JR, et al. Regulation of epithelial sodium channels by the cystic fibrosis transmembrane conductance regulator. J Biol Chem 1996; 271:4725-32. [96214891]

5. Jovov B, Ismailov II, Berdiev BK, Fuller CM, Sorscher EJ, Dedman JR, et al. Interaction between cystic fibrosis transmembrane conductance regulator and outwardly rectified chloride channels. J Biol Chem 1995; 270:29194-200. [96094308]

6. Schwiebert EM, Benos DJ, Egan ME, Stutts MJ, Guggino WB. CFTR is a conductance regulator as well as a chloride channel. Physiol Rev 1999; 79:S145-66. [99126616]

7. Schwiebert EM, Benos DJ, Fuller CM. Cystic fibrosis: a multiple exocrinopathy caused by dysfunctions in a multifunctional transport protein. Am J Med 1998; 104:576-90. [98337479]

8. Stutts MJ, Canessa CM, Olsen JC, Hamrick M, Cohn JA, Rossier BC, Boucher RC. CFTR as a cAMP-dependent regulator of sodium channels. Science 1995; 269: 847-850.

9. Goddard PJ, Takahashi S, Milbank AJ, Silen W, Soybel DI. HCO_3^- secretion by bullfrog duodenum: dependence on nutrient Na^+ during secretory stimulation. Can J Physiol Pharmacol 1998; 76:882-90. [99164021]

10. Guba M, Seidler U, Kuhn M, Forssmann WG, Classen M. HCO_3^- and Cl^- flux through guanylin-stimulated apical anion conductance depends on basolateral anion importers in rat duodenum. Gut 1995; 37:A231. (Abstract)

11. Simson JN, Merhav A, Silen W. Alkaline secretion by amphibian duodenum. I. General characteristics. Am J Physiol 1981; 240:G401-8. [81204708]

12. Ainsworth MA, Amelsberg M, Hogan DL, Isenberg JI. Acid-base transport in isolated rabbit duodenal villus and crypt cells. Scand J Gastroenterol 1996; 31:1069-77. [97093337]

13. Ainsworth MA, Hogan DL, Rapier RC, Amelsberg M, Dreilinger AD, Isenberg JI. Acid/base transporters in human duodenal enterocytes. Scand J Gastroenterol 1998; 33:1039-46. [99044822]

14. Isenberg JI, Ljungstrom M, Safsten B, Flemstrom G. Proximal duodenal enterocyte transport: evidence for Na^+-H^+ and Cl^--HCO_3^- exchange and $NaHCO_3$ cotransport. Am J Physiol 1993; 265:G677-85. [94056770]

15. Jacob P, Christiani S, Rossmann H, Lamprecht G, Vieillard-Baron D, Muller R, et al. Role of $Na^+HCO_3^-$ cotransporter NBC1, Na^+/H^+ exchanger NHE1 and carbonic anhydrase in rabbit duodenal bicarbonate secretion. Gastroenterology 2000; 119:406-19. [20389977]

16. Ishibashi K, Sasaki S, Marumo F. Molecular cloning of a new sodium bicarbonate cotransporter cDNA from human retina. Biochem Biophys Res Commun 1998; 246:535-8. [98273317]

17. Choi I, Aalkjaer C, Boulpaep EL, Boron WF. An electroneutral sodium/bicarbonate cotransporter NBCn1 and associated sodium channel. Nature 2000; 405:571-5. [20306889]

18. Flagella M, Clarke LL, Miller ML, Erway LC, Giannella RA, Andringa A, et al. Mice lacking the basolateral Na-K-2Cl cotransporter have impaired epithelial chloride secretion and are profoundly deaf. J Biol Chem 1999; 274:26946-55. [99410432]

19. Schultheiss G, Horger S, Diener M. The bumetanide-resistant part of forskolin-induced anion secretion in rat colon. Acta Phys Scand 1998; 164:219-28. [99021925]

20. Alper SL, Rossmann H, Wilhelm S, Stuart-Tilley AK, Shmukler BE, Seidler U. Expression of AE2 anion exchanger in mouse intestine. Am J Physiol 1999, 277:G321-32. [99375101]

21. Lang F, Waldegger S. Regulating cell volume. Am Scientist 1997: 85:456-63.

22. Lytle C. A volume-sensitive protein kinase regulates the Na-K-2Cl cotransporter in duck red blood cells. Am J Physiol 1998; 274:C1002-10. [98236710]

23. Waldegger S, Barth P, Forrest JN Jr, Greger R, Lang F. Cloning of sgk serine-threonine protein kinase from shark rectal gland - a gene induced by hypertonicity and secretagogues. Pflügers Arch 1998; 436:575-80. [PMID 9683731]

24. Bleich M, Heitzmann D, Hug MJ, Hoffmann EK, Greger R, Warth R. Regulation of the $Na^+2Cl^-K^+$ co-transporter - mechanisms in the rectal gland of Squalus acanthias with implications for the thick ascending limb of Henle. Nephrol Dial Transplant 2000; 15(Suppl.6):16-8. [21018267]

25. D'Andrea L, C. Lytle C, J.B. Matthews JB, P. Hofman P, B. Forbush B, and J.L. Madara JL. Na^+:$2Cl^-$:K^+ cotransporter (NKCC) of intestinal epithelial cells - Surface expression in response to cAMP. J Biol Chem 1996; 271:28969-76. [97067145]

26. Forbush B 3rd, Haas M, Lytle C. Na-K-Cl cotransport in the shark rectal gland. I. Regulation in the intact perfused gland. Am J Physiol 1992; 262:C1000-8. [92230751]

27. Heitzmann D, Warth R, Bleich M, Henger A, Nitschke R, Greger R. Regulation of the $Na^+2Cl^-K^+$ cotransporter in isolated rat colon crypts. Pflügers Arch 2000; 439:378-84. [20114413]

28. Lytle C, Forbush B 3rd. The Na-K-Cl cotransport protein of shark rectal gland. II. Regulation by direct phosphorylation. J Biol Chem 1992; 267:25438-43. [93094263]

29. Lytle C, Forbush B 3rd. Regulatory phosphorylation of the secretory Na-K-Cl cotransporter: modulation by cytoplasmic Cl. Am J Physiol 1996; 270:C437-48. [96373614]

30. Torchia J, Lytle C, Pon DJ, Forbush B 3rd, Sen AK. The Na-K-Cl cotransporter of avian salt gland. Phosphorylation in response to cAMP-dependent and

calcium-dependent secretogogues. J Biol Chem 1992; 267:25444-50. [93094264]

31. Nader M, Lamprecht G, Classen M, Seidler U. Different regulation by pHi and osmolarity of the rabbit ileum brush-border and parietal cell basolateral anion exchanger. J Physiol 1994; 481:605-15. [95222435]

32. Burnham CE, Amlal H, Wang Z, Shull GE, Soleimani M. Cloning and functional expression of a human kidney Na^+:HCO_3^- cotransporter. J Biol Chem 1997, 272:19111-4. [97382229]

33. Romero MF, Hediger MA, Boulpaep EL, Boron WF. Expression cloning and characterization of a renal electrogenic Na^+/HCO_3^- cotransporter. Nature 1997; 387:409-13. [97305959]

34. Rossmann H, Bachmann O, Vieillard-Baron D, Gregor M, Seidler U. Na^+ HCO_3^- cotransport and expression of NBC1 and NBC2 in cultured rabbit parietal and surface cells. Gastroenterology 1999; 116:1389-98. [99278252]