Expression and regulation of the Na+–K+–2Cl− cotransporter NKCC1 in the normal and CFTR-deficient murine colon

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Defective regulation and/or reduced expression of the Na+–K+–2Cl− cotransporter NKCC1 may contribute to the severe secretory defect that is observed in cystic fibrosis, but data concerning the expression and function of NKCC1 in cystic fibrosis transmembrane conductance regulator (CFTR)-deficient cells are equivocal. We therefore investigated NKCC1 mRNA expression, Na+–K+–2Cl− cotransport activity and regulation by cAMP in crypts isolated from the proximal colon of CFTR-containing (CFTR (+/+)) and CFTR-deficient (CFTR (−/−)) mice. mRNA expression levels were determined by semiquantitative PCR, transport rates were measured fluorometrically in 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein acetomethylester (BCECF)-loaded crypts, cytoplasmic volume changes were assessed by confocal microscopy, and [Cl−]$_i$ changes were examined by *N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) quenching. NKCC1 mRNA expression levels were not significantly reduced in CFTR (−/−) crypts compared to controls. Azosemide-sensitive NH$_4^+$ influx (used as a measure of Na+–K+–2Cl− cotransport) was 2.23 ± 0.72 *vs.* 1.56 ± 0.16 mM min^{-1}, and increased by 63.6 % in (+/+) and 87.3 % in (−/−) crypts upon stimulation for 5 min with forskolin. After 20 min of stimulation with forskolin, the Na+–K+–2Cl− cotransport rates in (−/−) and (+/+) crypts were identical. Crypt cross-sectional area and [Cl−]$_i$ decreased only in (+/+) crypts upon stimulation. In conclusion, normal NKCC1 expression levels, somewhat reduced Na+–K+–2Cl− cotransport rates, but preserved activation by cAMP were found in colonic crypts from CFTR (−/−) mice, ruling out a severe dysfunction of the Na+–K+–2Cl− cotransporter in the CF intestine. Furthermore, these studies establish the existence of a direct, cell-volume- and [Cl−]$_i$-independent activation of colonic NKCC1 by an increase in intracellular cAMP.

When Cl− secretion is activated in response to cAMP in intestinal epithelia, basolateral uptake must also increase to maintain cellular electrolyte composition. The importance of basolateral Cl− uptake as the rate-limiting step in the onset of apical Cl− secretion has been demonstrated in shark rectal glands (Lytle & Forbush, 1996). Colonic basolateral anion uptake is mediated predominantly by the bumetanide-sensitive Na+–K+–2Cl− cotransporter NKCC1 and by the alternative pathway of Na+–HCO$_3^−$ cotransport coupled to basolateral anion exchange (Jacob *et al.* 2001).

In cystic fibrosis (CF), epithelial anion secretion is severely hampered, resulting in mucus accumulation in the pancreatic, biliary and epididymal ducts, and in the smaller airways and intestinal tract. Functional, molecular and immunohistochemical evidence exists for the presence of non-CF transmembrane conductance regulator (CFTR) anion conductances or transporters in

the apical membrane of either intact intestine or intestinal cell lines (Hagos *et al.* 1999; Joo *et al.* 1999; Gaspar *et al.* 2000). Numerous attempts have been made by several laboratories to activate these alternative anion efflux pathways pharmacologically, but this has remained largely unsuccessful in the CFTR-deficient intestine (Seidler *et al.* 1997; Joo *et al.* 1998; Grubb & Boucher, 1999).

Based on experiments in CFTR-deficient and CFTR-transfected pancreatic tumour cells, Shumaker & Soleimani (1999) suggested that CFTR transcriptionally regulates NKCC1. They hypothesized that it is the CF-associated dysfunction of basolateral anion uptake, rather than reduced apical CFTR anion conductance, that may underlie the severe anion secretory defect observed in the pancreatic ducts of many CF patients (Soleimani & Ulrich, 2000). If this is true, even successful opening of alternative apical anion channels would not result in a substantial anion secretory response. In contrast, Haas *et al.* (1993)

found that [³H]bumetanide binding increased upon addition of cAMP analogues in nasal epithelial cells from CF patients, although these cells did not display an increase in Cl^- efflux, suggesting a direct activation of $Na^+–K^+–2Cl^-$ cotransport by cAMP.

Most CF patients and cells from these patients have some residual CFTR expression, which could potentially explain the discrepant results reported by Shumaker & Soleimani (1999) and Haas *et al.* (1993). The only native epithelia with zero CFTR expression are those of the CFTR null mutant mice (Clarke *et al.* 1992; Ratcliff *et al.* 1993). These CFTR $(-/-)$ mice display ATP-activated anion conductances in their bronchial, biliary and pancreatic systems, but not in the intestine (Clarke *et al.* 1992). To measure the expression, transport rates and cAMP-dependent regulation of the $Na^+–K^+–2Cl^-$ cotransporter in the same cellular system, we isolated colonic crypts from the proximal colon of CFTR $(-/-)$ mice and their normal (CFTR $(+/+)$) littermates, determined NKCC1 mRNA expression levels and assessed $Na^+–K^+–2Cl^-$ activity fluorometrically in $2',7'$-biscarboxyethyl-5(6)-carboxyfluorescein acetomethylester (BCECF AM)-loaded crypts in the absence and presence of forskolin. To assess potential cAMP-evoked changes in cell volume and $[Cl^-]_i$ unrelated to CFTR channel opening, we determined volume changes in CFTR $(+/+)$ and $(-/-)$ crypts using confocal microscopy, and cAMP-induced changes of $[Cl^-]_i$ using the anion-sensitive dye *N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE).

METHODS

Materials
Azosemide was supplied by Sanofi-Synthelabo (Berlin, Germany), bovine serum albumin (BSA; cell culture grade) from Paesel and Lorei (Frankfurt, Germany) and BCECF AM, calcein AM and MQAE were from Molecular Probes (Leiden, The Netherlands). All other chemicals were either obtained from Sigma (Deisenhofen, Germany) or from Merck (Darmstadt, Germany) at tissue culture grade or the highest grade available.

Animal breeding and genotyping
The CF null mice (Cftr[tm1Cam]) were established in the laboratories of R. Ratcliff, W. H. Colledge and M. Evans and had been characterized previously (Ratcliff *et al.* 1993); the NKCC1-knockout mice were established in the laboratories of G. Shull (Flagella *et al.* 1999). Both strains were bred in the animal facility of the Department of Biochemistry in Tübingen. The CF null mice were genotyped as described previously (Seidler *et al.* 1997). For genotype analysis of the NKCC1-knockout mice, genomic DNA was prepared from tail samples of 1- to 2-week-old mice using the QIAamp tissue kit (Qiagen, Hilden, Germany) and added to 25 μl of a PCR mixture: 2 U Qiagen *Taq* DNA polymerase; 2.5 μl Qiagen PCR buffer B; 2.5 mM Qiagen $MgCl^-$; 20 mM of each dNTP; 20 μM of forward and reverse primers. For detection of the wild-type allele, the oligonucleotides (5′-GGAACATTCCATA-CTTATGATAGATG-3′) and (5′-CTCACCTTTGCTTCCCAC-TCCATTCC-3′) were used as primers and amplified a 105 base pair (bp) product. The primers (5′-GGAACATTCCATACTT-

ATGATAGATG-3′) and (5′-GACAATAGCAGGCATGCTGG-3′) amplified a 156 bp product of the mutant allele. The PCR was performed using the following temperature parameters: 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s Heterozygous breeding pairs were maintained on a NMRI (Naval Medical Research Institute) background by periodically backcrossing to avoid impaired viability due to inbreeding. Animal experiments followed approved protocols at the University of Tübingen and the local authorities for the regulation of animal welfare (Regierungspräsidium).

Preparation of colonic crypts and surface cells
Crypts were prepared from the proximal colon because our previous Ussing-chamber data on the relevance of NKCC1 and other anion transporters for colonic anion secretion were obtained from this segment of the colon. Mice were anaesthetized with a rising concentration of CO_2 and killed by cervical dislocation. A 3 cm segment of proximal colon was excised, washed with ice-cold buffer A gassed with 100 % O_2 (mM: 120 NaCl, 14 2-Hepes, 7 Tris, 3 KH_2PO_4, 2 K_2HPO_4, 1.2 $MgSO_4$, 1.2 calcium gluconate, 20 glucose; pH 7.4), everted and filled with Ca^{2+}-free buffer B: (mM: 127 NaCl, 5 KCl, 1 $MgCl_2$, 5 sodium pyruvate, 10 Hepes, 5 EDTA, with 1 % BSA; pH 7.4). The segment was placed into a tube filled with solution B and vibrated at 10 Hz at room temperature. At defined intervals the solution was replaced and epithelial fragments harvested by low-speed centrifugation. Early fractions contained surface cells, later fractions crypts, both easily identified under a microscope and their viability tested by trypan blue exclusion.

Semiquantitative RT-PCR
Isolation of total and purified poly (A⁺) RNA was carried out according to the method of Chomczyinski & Sacchi (1987), as described in Rossmann *et al.* (1999). Homologous primers for murine NKCC1 were designed from published sequence information (NKCC1: forward primer 5′-GGGCTAATGAAC-AACTTTCAGG-3′, reverse primer 5′-GGAGAACCTCTCATT-ACAAGGG-3′). An 18 s rRNA fragment was amplified as an internal control with the use of primers supplied by Ambion (Austin, TX, USA). Semiquantitative RT-PCR was performed as described previously (Rossmann *et al.* 1999, 2000), but using different annealing temperatures (56 °C for NKCC1, and 58 °C for 18 s rRNA). The relationship between the studied gene and 18 s rRNA was calculated and regarded as the relative expression level.

Ussing-chamber experiments
Mice were anaesthetized with ether and killed by cervical dislocation. Proximal mouse colon was muscle-stripped with the aid of a microscope and the mucosa was mounted between two specially designed oval lucite half-chambers with an exposed area of 0.62 cm² and placed in an Ussing apparatus. Both half-chambers were perfused with a buffer containing (mM): 140.5 Na^+, 4.5 K^+, 2 Ca^{2+}, 1.3 Mg^{2+}, 116 Cl^-, 1.3 SO_4^{2-}, 20 HCO_3^-, 1.5 HPO_4^{2-}. The serosal perfusate also contained 11.9 mM dextrose, 10 mM pyruvate and 0.01 mM indomethacin, and the luminal perfusate contained mannitol instead of dextrose. The osmolarity of both solutions was 308 mosmol l⁻¹. Before placing the tissue into the chamber, the series resistance of the solutions and the chamber were assessed and a fluid resistance compensation performed before each experiment. Subsequently, the epithelium was continuously short-circuited using the EVC4000 voltage-clamp apparatus (World Precision Instruments) and the short-circuit current (I_{SC}) was recorded.

Assessment of NKCC1 activity in murine colonic crypts

NKCC1 activity was measured fluorometrically in the presence of Ba^{2+} (except for the experiments to investigate the difference between flux rates in the absence and presence of Ba^{2+}) and in the absence of HCO_3^-, as described by Heitzmann *et al.* (2000). This technique is based on the fact that NKCC1 can transport NH_4^+ instead of K^+, with a resultant change in intracellular pH (pH_i). Crypt cell pH_i was measured as described previously (Bachmann *et al.* 1998; Rossmann *et al.* 1999) after loading crypts for 30 min at room temperature with 5 μM BCECF AM in buffer A. Crypts were fixed between a glass coverslip and a 0.3 μm polycarbonate membrane (Osmonics, Minnetonka, MN, USA) on the stage of an inverted microscope (Nikon Diaphot TMD, Nikon, Düsseldorf, Germany) and superfused following the appropriate protocol with buffer A and buffer C (40 mM NaCl was replaced by 40 mM NH_4Cl). Crypts were alternately excited at 440 ± 10 nm and 490 ± 10 nm at a rate of 100 s^{-1}. Data acquisition and processing was performed using the software provided by the manufacturer (Photon Technology International, Lawrenceville, NJ, USA). Emitted light from an individual crypt was collected through a 510 nm dichroic mirror, a 530 nm long-pass filter and an adjustable diaphragm, and was recorded by a photomultiplier. At the end of each experiment, the 490 : 440 nm ratio was calibrated to pH_i using the high K^+–nigericin method (mM: 100 potassium gluconate, 40 KCl, 7 Hepes, 1.2 calcium gluconate, 1.2 $MgSO_4$, 20 glucose, 0.01 nigericin; two buffers with pH values of 6.6 and 7.4 were prepared for two-point calibration). Background fluorescence was found to be negligible. CO_2/HCO_3^--free buffers were used to minimize pH_i recovery by HCO_3^--dependent transport mechanisms, and 1 mM Ba^{2+} was used to minimize NH_4^+ influx via K^+ channels (Heitzmann *et al.* 2000).

Determination of the intrinsic intracellular buffering power

Determination of the intracellular buffering power (β_i) was performed as described by Boyarsky *et al.* (1988) using increasing or decreasing concentrations of NH_4Cl (TMA-Cl instead of NaCl, and 0.5–80 mM TMA-Cl was replaced by NH_4Cl in buffer A) and measuring the resulting pH_i changes. β_i was calculated over a wide pH_i range (6.6–7.8), resulting in buffering curves that were not different between CFTR (+/+) and CFTR (−/−) crypts (Fig. 1).

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.* (2000). Single crypts were selected with the aid of a binocular microscope, transferred into a perfusion chamber on the stage of a Zeiss LSM 410 confocal microscope, fixed with two holding pipettes, loaded with the fluorescent dye calcein AM (10 μM for 15 min) and then perfused with buffer A containing agonists and inhibitors where appropriate. Internal validation was performed by an osmotic challenge at the end of the experiment: omitting 100 mM NaCl or adding 100 mM mannitol. Cross-sectional images were obtained by *z*-scanning with an excitation wavelength of 488 nm and a pinhole of 33 nm every 5 s. Cross-sectional areas were determined using the Metafluor software (Universal Imaging, Downingtown, PA, USA), with the initial value normalized to 100 %.

[Cl⁻]ᵢ measurements

Changes in $[Cl^-]_i$ were measured using the fluorescent dye MQAE as a qualitative tool for measuring concentration changes, not absolute Cl^- concentrations. The same experimental set-up as for the intracellular pH measurements was used (see above). Crypts were loaded with 10 mM MQAE for 1 h and then excited with 370 ± 10 nm at a rate of 100 s. The fluorescence signal was measured at an emission wavelength of 460 nm, with the initial value normalized to 100 %.

Statistics and calculation of proton fluxes

Results are given as means ± S.E.M. Proton fluxes were calculated by multiplying the initial steep pH_i slope determined by regression analysis after addition of Na^+ or removal of NH_4Cl, with the total buffering capacity (β_t) at the initial pH_i (Bachmann *et al.* 1998). Statistical significance was determined using Student's *t* test in its paired or unpaired version as appropriate.

RESULTS

NKCC1 expression levels in colonic crypts of CFTR (+/+) and (−/−) mice

The expression levels of NKCC1 were measured in colonic crypts from CFTR (+/+) and CFTR (−/−) mice using a semiquantitative PCR technique. Primers were chosen that displayed a similar amplification rate as the primers for 18 s rRNA, which was used as an internal control (Fig. 2*A*). As anticipated from the *in situ* expression data (Grubb *et al.* 2000), NKCC1 mRNA abundance was 12- and 18-fold higher in crypts than in surface cells in CFTR (+/+) and CFTR (−/−) mice, respectively (Fig. 2*B*). We found a slight but not statistically significant reduction in NKCC1 expression levels in CFTR (−/−) crypts (Fig. 2*B*). Thus, the absence of functional CFTR does not cause a severe reduction in the NKCC1 expression level in mouse colon.

Specificity of azosemide for Na⁺–K⁺–2Cl⁻ cotransport

Azosemide was used to inhibit NKCC1 (Heitzmann *et al.* 2000) because unlike bumetanide it does not exhibit
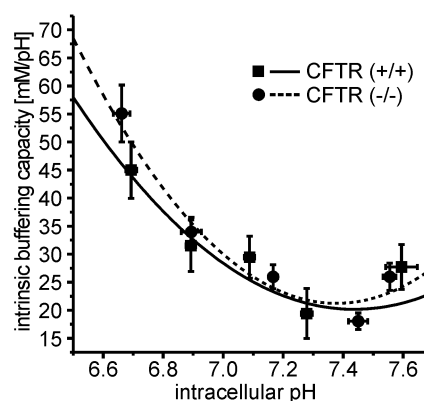
Figure 1. Intrinsic buffering capacity of colonic crypts from CFTR (+/+) and CFTR (−/−) mice

To calculate proton–base flux rates, β_i was determined in murine colonic crypts from CFTR (+/+) (■—) and CFTR (−/−) mice (●---) between pH_i 6.6 and 7.8, as described in Methods. The buffering curves with a β_i of 45.1 mM (pH unit)$^{-1}$ and 49.5 mM (pH unit)$^{-1}$ at pH_i 6.7, 24.7 mM (pH unit)$^{-1}$ and 26.0 mM (pH unit)$^{-1}$ at pH_i 7.1 and 26.7 mM (pH unit)$^{-1}$ and 24.3 mM (pH unit)$^{-1}$ at pH_i 7.6 in CFTR (+/+) and CFTR (−/−) mice, respectively, resemble that found in other cell types. Data points were fitted with a second-order polynomial function. ($n = 13$ crypts from 9 mice for normal mice, $n = 11$ crypts from 6 mice for knockout animals).
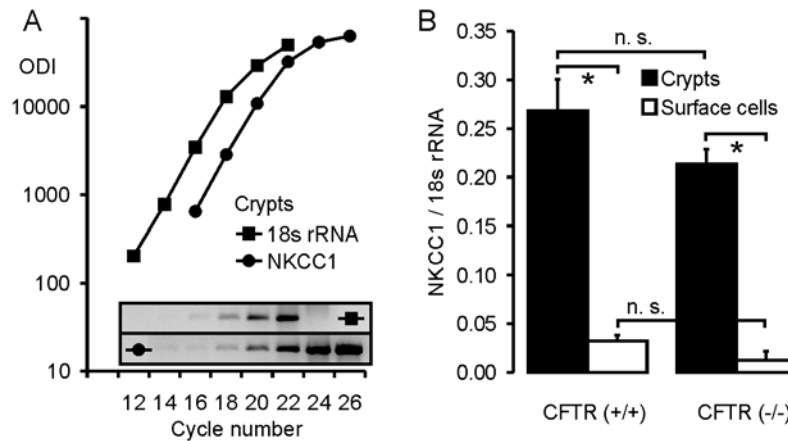
Figure 2. Method of the semiquantitative RT-PCR (*A*) and expression analysis of NKCC1 in colonic crypts and surface cells from CFTR (+/+) and CFTR (−/−) mice (*B*)

A, determination of NKCC1 expression in colonic crypts from CFTR (+/+) mice, using 18 s rRNA as an internal control. Amplification curves were created for both the NKCC1 and 18 s rRNA primer pairs, and the virtual relationship optical density integrated (ODI) of the studied gene *vs.* the ODI of 18 s rRNA was used as a measure for the expression level of the gene of interest after correction of the values for the different PCR products according to their length. *B*, relative expression levels of NKCC1 in colonic crypts (■) and surface cells (□) from CFTR (+/+) (left panel) and CFTR (−/−) mice (right panel). NKCC1 was significantly more abundant in crypts than in surface cells ($P < 0.01$, Student's unpaired *t* test); the expression levels in CFTR (−/−) tissues were slightly, but not significantly reduced ($n = 4$–6 mice).

autofluorescence. In previous experiments in isolated rat colonic mucosa, its inhibitory potency on I_{SC} stimulated by cAMP activation was similar to that of bumetanide (Schultheiss *et al.* 1998). The inhibitory potency and specificity of azosemide for NKCC1 inhibition in murine proximal colon was tested in isolated colonic mucosa of normal mice (C57/B6) as well as in NKCC1-knockout mice and their normal littermates, in Ussing-chamber experiments. The addition of 100 μM azosemide to the

serosal side of normal colonic tissue prior to stimulation of electrogenic anion secretion by dibutyryl-cAMP (db-cAMP) or forskolin resulted in the same reduction in I_{SC} response compared to control than the application of 100 or 500 μM bumetanide (Fig. 3). When either 100 μM azosemide was added after 500 μM bumetanide, followed by forskolin, or when 500 μM bumetanide was added after 100 μM azosemide, followed by forskolin, no decrease in I_{SC} was seen, indicating that both substances fully inhibited
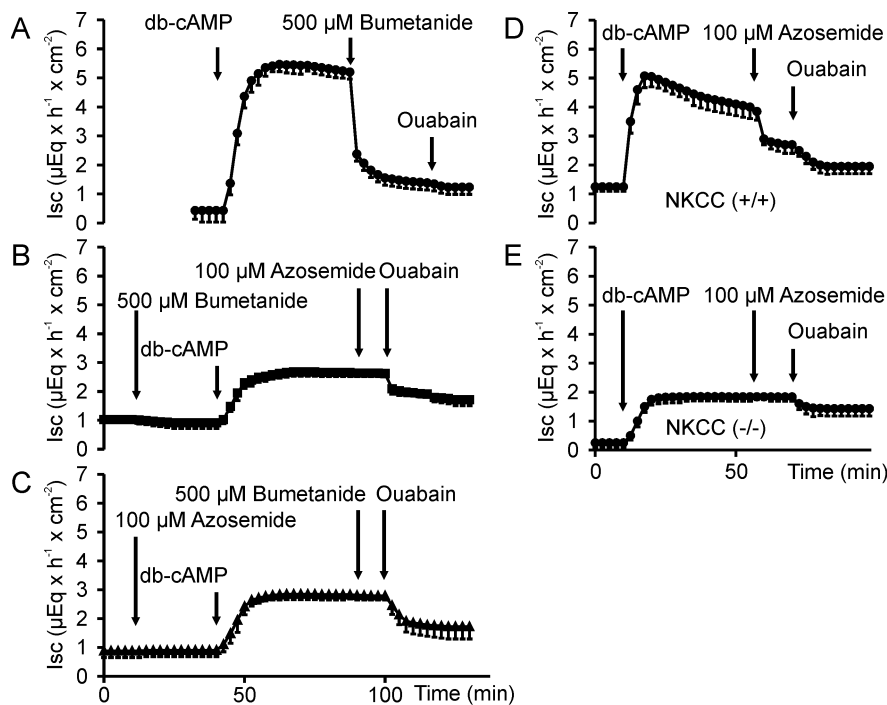
**Figure 3. I_{SC} measurements in Ussing-chamber experiments with isolated colonic mucosa**

I_{SC} measurements in Ussing-chamber experiments with isolated colonic mucosa from normal mice (Fig. 3*A*–*C*), from NKCC1 (−/−) mice and from their NKCC1 (+/+) littermates (Fig. 3*D* and *E*). cAMP-dependent stimulation of I_{SC} (*A*) was inhibited to the same extent by 100 μM (not shown) or 500 μM bumetanide (*B*) as by 100 μM azosemide (*C*), and neither compound resulted in further I_{SC} inhibition after the other one was added (*B* and *C*). Furthermore, 100 μM azosemide had no effect on I_{SC} (*D* and *E*) or HCO_3^- flux (not shown) in NKCC1 (−/−) colon ($n = 3$–5).

NKCC1 (Fig. 3*B* and *C*). Neither azosemide nor bumetanide had any effect on NKCC1 in the (−/−) colon (Fig. 3*D* and *E*), as reported by others for bumetanide (Flagella *et al.* 1999). The lack of effect of azosemide on I_{SC} and the HCO_3^- secretory response in bumetanide-pretreated normal colonic mucosa, or in the NKCC1 (−/−) mice suggests that azosemide does not interfere with the alternative Cl^- uptake pathways or with the cAMP-

activated K^+ channels in the colon, and can be used to assess NKCC1 activity with the same specificity as bumetanide.

cAMP-dependent stimulation of NKCC1 activity

Application of 40 mM NH_3/NH_4^+ caused rapid alkalization to pH 7.61 ± 0.2 and a subsequent slower recovery, which was azosemide sensitive, and therefore in part attributable to NH_4^+ uptake by the $Na^+–K^+–2Cl^-$ cotransporter (Fig. 4*A*
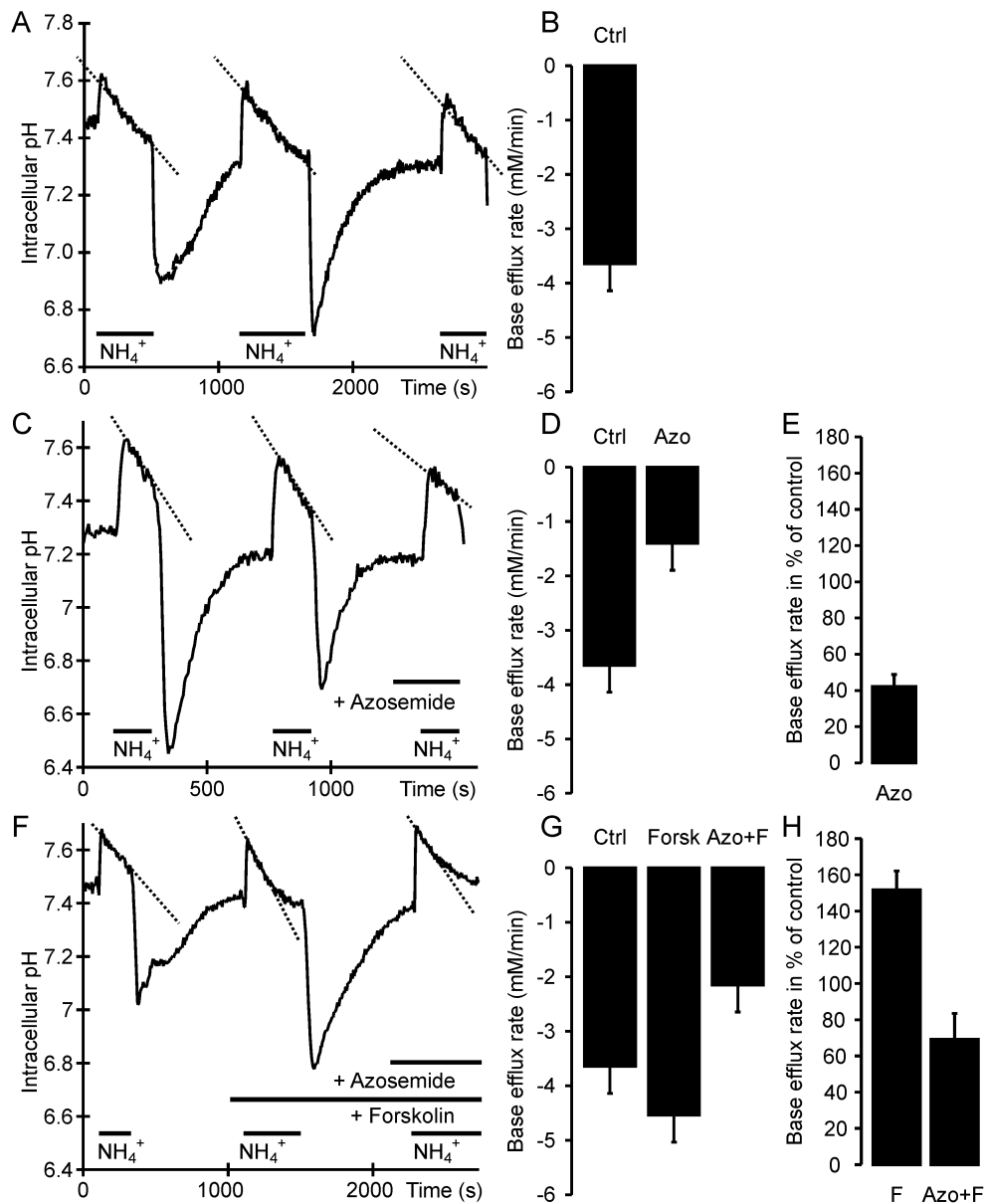
Figure 4. NKCC1 activity in colonic crypts, measured as the azosemide-sensitive fraction of pH_i recovery from a NH_3/NH_4^+ load

A, *C* and *F*, time course of changes in pH_i; *B*, *D* and *G*, corresponding base efflux rates; *E* and *H*, relative NKCC1 activity. After demonstrating equal base efflux rates during multiple NH_4^+ pulses (*A*, *B*; -3.65 ± 0.5 mM min^{-1}), NKCC1 activity was measured using azosemide, which decreased the $\Delta pH_i/\Delta t$ (*C*) and the flux rate (*D*; -1.4 ± 0.3 mM min^{-1}). Cotransport activity is expressed as a percentage of the initial flux(es) in each experiment as an internal control, to better standardize the results (*E*; $42 \pm 7\%$). When stimulated, the total and azosemide-sensitive $\Delta pH_i/\Delta t$ (*F*) as well as the base efflux rates (*G*; -4.54 ± 0.67 and -2.15 ± 0.45 mM min^{-1}) and the relative NKCC1 activity (*H*; $70 \pm 14\%$) was higher (Ctrl = control, Azo = azosemide, F/Forsk = forskolin; $n = 8–10$ crypts from 5–7 mice).

Table 1. Azosemide- and DIDS-sensitive base efflux rates in colonic crypts in the presence of Ba^{2+} upon the addition of NH_4Cl

|	Control flux rate for each group ($mM\ min^{-1}$)	Flux rate after inhibition ($mM\ min^{-1}$)	% Inhibition by the substance†
Azosemide	-3.35 ± 0.50	$-1.34 \pm 0.17^\star$	58 ± 7
DIDS	-2.56 ± 0.44	$-1.63 \pm 0.22^\star$	35 ± 5
Azosemide+DIDS	-2.67 ± 0.35	$-0.36 \pm 0.08^\star$	84 ± 5

$n = 8–10$ crypts from 5–7 mice, $^\star P < 0.01$ compared with the respective control group (Student's paired t test). † Calculated individually for each experiment in a group.

and *B*). In order to better standardize the results and compare the forskolin-stimulated NKCC1 transport rates with unstimulated NKCC1 rates, obtained in different experiments, we calculated the base efflux rates after application of forskolin and azosemide in relation to the total base efflux after NH_3/NH_4^+ application. The procedure is explained in Fig. 4. The azosemide-sensitive part of the base efflux expressed as a percentage of the control value reflects NKCC1 transport during stimulated and unstimulated conditions.

DIDS also inhibited a fraction of NH_4^+-induced base efflux (Table 1), suggesting that in addition to NKCC1, a DIDS-sensitive base-extruding process is also present, most likely a Cl^-–OH^- exchanger, and both contribute to pH_i recovery after NH_4^+ prepulse. The combination of both virtually abolished base efflux, demonstrating that in the presence of Ba^{2+}, NH_4^+ entry via other routes plays a minor role in this model.

Na^+–K^+–$2Cl^-$ cotransporter activity was stimulated by a rise in intracellular cAMP

Administration of 10 μM forskolin 5 min prior to alkalization increased the relative azosemide-sensitive NH_4^+ influx rates by 73 % in the absence of 1 $mM\ Ba^{2+}$, and by 63 % in its presence (Fig. 5*C*). The rationale for the use of 1 $mM\ Ba^{2+}$ was taken from the literature (Heitzmann *et al.* 2000), and was done to minimize NH_4^+ influx via K^+ channels. However, 1 $mM\ Ba^{2+}$ will not inhibit all K^+ channels, and K^+ channel inhibition may induce cell swelling, which in turn could inhibit NKCC1 activity. To assess the magnitude of these potentially conflicting parameters, we also performed experiments in the absence of Ba^{2+} (Fig. 5). A higher total base efflux rate as well as the azosemide-sensitive fraction of NH_4^+ influx was observed in the absence of 1 $mM\ Ba^{2+}$, indicating that Ba^{2+} inhibits NH_4^+ influx both by blocking K^+ channels and by slightly inhibiting the NKCC1 cotransporter, probably by
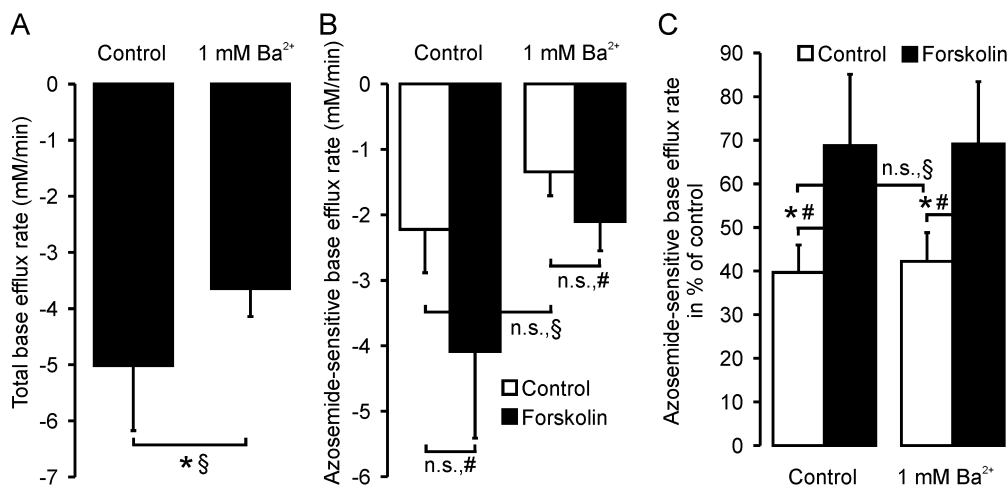
Figure 5. Base efflux rates in murine colonic crypts in the absence and presence of Ba^{2+}

Total (*A*) and azosemide-sensitive (*B* and *C*) base efflux rates in murine colonic crypts. In *C*, the azosemide-sensitive flux rates are expressed in relation to the first NH_4 pulse as an internal control (see Fig. 4*A*). Ba^{2+} slightly decreased total flux rates (*A*, $P < 0.05$) as well as azosemide-sensitive base efflux (n.s.; *B* and *C*), indicating an inhibitory effect of 1 $mM\ Ba^{2+}$ on both non-NKCC1-related base efflux and on basal NKCC1 activity. However, forskolin stimulation increased the azosemide-sensitive base efflux rates to a similar extent both in the presence and absence of Ba^{2+} (*C*; 42 ± 7 % *vs.* 69 ± 14 % and 40 ± 6 % *vs.* 69 ± 16 %, $n = 6–8$ crypts from 4–5 mice, $^\star P < 0.05$: § Student's unpaired t test, # Student's paired t test). When values are given as the flux rate as a percentage of the control measured within the same experiment (*C*), the variability is somewhat smaller, and therefore forskolin stimulation was significant at a relatively low value of n.

increasing $[K^+]_i$ and/or cell volume. Nevertheless, the azosemide-sensitive stimulatory effect of forskolin on the relative azosemide-sensitive NH_4^+ influx rate was virtually identical in both conditions.

NKCC activity in CFTR (+/+) and CFTR (−/−) mice

Na^+–K^+–$2Cl^-$ cotransporter activity was slightly reduced in CFTR (−/−) crypts compared to (+/+) crypts, although this difference was not statistically significant ($42 \pm 7\%$ in (+/+) *vs.* $24 \pm 3\%$ in (−/−) crypts, Fig. 6). Five minutes after the addition of 10 μM forskolin, NKCC1 activity was stimulated by 63.6 % in (+/+) and 87.3 % in (−/−) crypts. Interestingly, a 20 min period of forskolin stimulation, the effect of which was no different to that of a 5 min period in (+/+) crypts, was able to increase the absolute NKCC1 activity in (−/−) crypts to the level found in forskolin-stimulated (+/+) crypts. This indicates that the reduced basal activity of Na^+–K^+–$2Cl^-$ cotransporter in (−/−) crypts is not due to an absolute lack of NKCC1 protein, but to some downregulation of function. This is in agreement with our finding of almost normal expression levels for NKCC1 in CFTR (−/−) crypts.

Changes in intracellular volume and $[Cl^-]_i$ in colonic crypts of CFTR (+/+) and CFTR (−/−) mice upon cAMP-dependent stimulation

It has been suggested that Na^+–K^+–$2Cl^-$ cotransporter activity, including stimulation by cAMP, is mediated by changes in intracellular volume and $[Cl^-]_i$ caused by apical Cl^- secretion (Matthews *et al.* 1994; Lytle & Forbush, 1996). Although several investigators have demonstrated that cAMP does not stimulate Cl^- secretion in the murine CFTR (−/−) intestine (Clarke *et al.* 1992; Cuthbert *et al.* 1994; Seidler *et al.* 1997), changes in cellular volume or $[Cl^-]_i$ that are mediated by intracellular cAMP may still occur in CFTR (−/−) crypts. We therefore measured volume changes in colonic crypts of CFTR (+/+) and (−/−) mice, using the protocol of Heitzmann *et al.*(2000). Figure 7*A* shows the time course of changes in the cross-sectional area of colonic crypts from CFTR (+/+) and CFTR (−/−) mice. Azosemide, added to prevent rapid

volume recovery, did not change the cross-sectional area, but forskolin caused a decrease by $9.4 \pm 1.2\%$ in (+/+) crypts and no change in (−/−) crypts (Fig. 7*A* and *B*). This shows that a rise in intracellular cAMP decreases crypt cell volume only when CFTR expression is intact. This finding is in agreement with previous data from Valverde *et al.* (1993) for murine small intestinal crypts.

Measurements with the fluorescent dye MQAE were performed to exclude a possible decrease of $[Cl^-]_i$ by mechanisms other than CFTR-mediated Cl^- secretion. Forskolin caused no significant change in MQAE quenching in colonic crypts in the absence of azosemide, but in its presence, subsequent forskolin stimulation increased the emitted fluorescence intensity by $14.8 \pm 0.35\%$ in CFTR (+/+) crypts, compared to unstimulated crypts. There was no fluorescence change in forskolin-treated *vs.* untreated crypts from CFTR (−/−) crypts (Fig. 7*C*). Although the change in MQAE fluorescence may be due, at least in part, to cellular volume changes and does not give a quantitative measurement for the magnitude of the change in $[Cl^-]_i$, the lack of change in CFTR (−/−) crypts rules out that a change in $[Cl^-]_i$ is the reason for the forskolin-induced increase in NKCC1 activity in CFTR (−/−) crypts.

Role of basolateral K^+ channels

K^+ channel activation can potentially activate Na^+–K^+–$2Cl^-$ cotransporter via cellular shrinkage and/or a decrease in $[K^+]_i$. This could be the mechanism by which cAMP activates Na^+–K^+–$2Cl^-$ cotransporter. Since the Ba^{2+} sensitivity of the colonic K^+ channels activated by cAMP has not been established, we used the chromanol Hoe 293b, which has been shown to inhibit the basolateral cAMP-dependent K^+ channels involved in intestinal Cl^- secretion (Barrett & Keely, 2000; Greger, 2000; Kunzelmann *et al.* 2001*b*). In the presence of 100 μM Hoe 293b, which is fully inhibitory for the KCNQ1 K^+ channel (Kunzelmann *et al.* 2001*a*), Na^+–K^+–$2Cl^-$ cotransport rates were significantly reduced (Fig. 8*A*). This could either be due to a higher $[K^+]_i$ and/or $[Cl^-]_i$ in the crypt, or

Figure 6. NKCC activity in colonic crypts from CFTR (+/+) and (−/−) mice, in the presence and absence of forskolin stimulation

cAMP-dependent stimulation with forskolin markedly enhanced the relative azosemide-sensitive flux rate in CFTR (+/+) crypts ($42 \pm 4\%$ to $69 \pm 14\%$, $P < 0.05$: Student's paired *t* test; $n = 8$ crypts from 5 mice) as well as in CFTR (−/−) crypts ($24 \pm 2\%$ to $45 \pm 9\%$, $P < 0.05$: Student's paired *t* test, $n = 8$ crypts from 6 mice). Interestingly, a longer duration of stimulation with forskolin yielded a much more pronounced stimulation on top of the brief forskolin stimulation in CF mice ($72 \pm 12\%$, $n = 10$ crypts from 8 mice) than in normal mice ($76 \pm 11\%$, $n = 9$ crypts from 6 mice; * $P < 0.05$: § Student's unpaired *t* test, # Student's paired *t* test).

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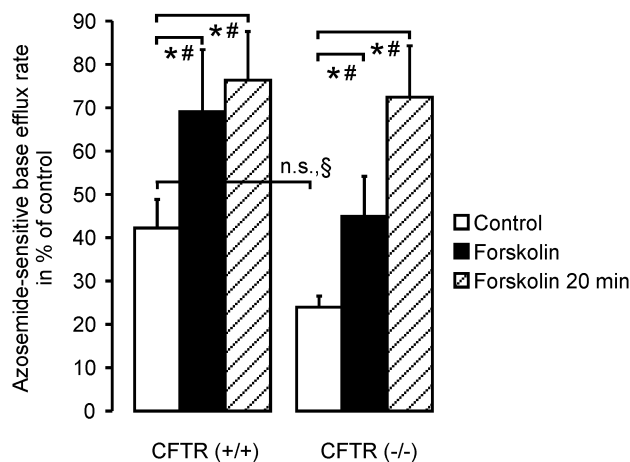
to cell swelling. We therefore measured crypt volume in response to addition of Hoe 293b. The compound caused a minor increase in cross-sectional area of $2.01 \pm 0.31\%$ (Fig. 8*B*), suggesting that these K^+ channels are only minimally active in the basal state. Forskolin still increased $Na^+-K^+-2Cl^-$ cotransporter activity 1.6-fold in the presence of Hoe 293b (Fig. 8*A*), demonstrating that the stimulatory effect of cAMP on $Na^+-K^+-2Cl^-$ cotransporter activity is fully preserved after Hoe 293b-sensitive K^+ channel blockade.

DISCUSSION

$Na^+-K^+-2Cl^-$ cotransport, mediated by the NKCC1 isoform in enterocytes, is considered to be the

predominant Cl^- uptake mechanism underlying intestinal anion secretion (Haas, 1994). Recent studies have found reduced expression levels and/or defective activation of NKCC1 in a CFTR-deficient pancreatic cell line (Shumaker & Soleimani, 1999). This finding was of great interest since several attempts to stimulate anion secretion in CF epithelia failed, despite functional, molecular or immunohistochemical evidence for the presence of non-CFTR apical anion conductances (Hagos *et al.* 1999; Joo *et al.* 1999; Gaspar *et al.* 2000). Failure to activate apical anion secretion may be caused by the absence of the rate-limiting basolateral anion uptake proteins. It was therefore important to clarify whether downregulation of NKCC1 also occurs in native intestinal epithelia in the absence of functional CFTR. As early as 1993, Haas *et al.* showed
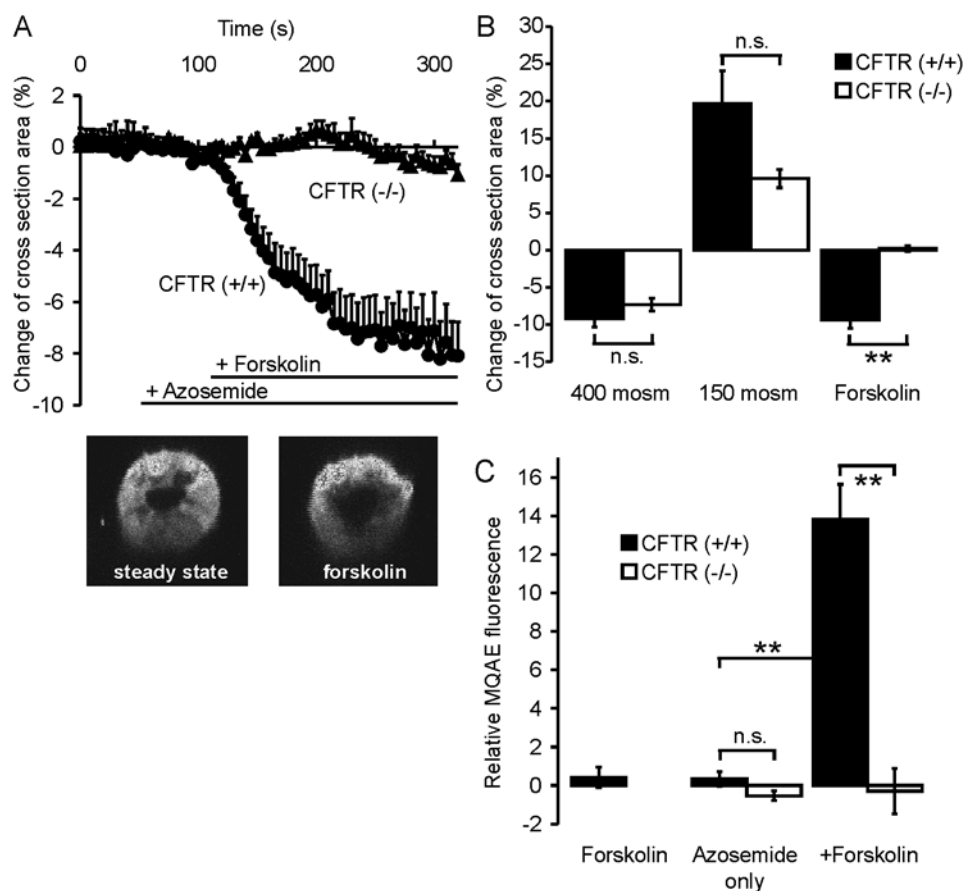
Figure 7. Changes in crypt volume and [Cl⁻]ᵢ in response to forskolin stimulation in CFTR (+/+) and (−/−) crypts

A, time course of changes in the crypt cross-sectional area. *B*, maximal change upon forskolin stimulation or incubation with non-isotonic solutions. The volume change after a hyperosmotic challenge was almost equal in CF and normal mice. Hyposmotic challenge produced a stronger change in crypt cross-section area in CFTR (+/+) mice. Upon forskolin stimulation, crypt cross-section area decreased by $9.4 \pm 1.2\%$ in CFTR (+/+) crypts, while there was no change in CFTR (−/−) crypts ($n = 10$ crypts from 6–7 mice in each group, $P < 0.01$). *C* shows MQAE fluorescence at $t = 10$ min, which was used to follow changes in [Cl⁻]ᵢ in CFTR (+/+) and CFTR (−/−) crypts. Azosemide alone caused no change in MQAE fluorescence in CF or normal mice. Subsequent forskolin stimulation caused a substantial increase in MQAE fluorescence ($13.8 \pm 0.34\%$) in CFTR (+/+) crypts, and no change in CFTR (−/−) crypts ($n = 5$–7 crypts from 3–5 mice in each group; **$P < 0.01$: Student's unpaired t test). While a portion of the increase in MQAE fluorescence in the normal crypts could be due to the fact that they shrink, the complete lack of change in signal intensity rules out a forskolin-induced change in [Cl⁻]ᵢ in the CF crypts.

using [^{3}H]bumetanide binding that the number of active Na^+–K^+–$2Cl^-$ cotransporters increased in CF nasal epithelial cells upon stimulation with a cAMP analogue, even though $^{36}Cl^-$ efflux did not alter (Haas *et al.* 1993). The different results of these two groups are possibly due to differences in the expression of the Na^+–K^+–$2Cl^-$ cotransporter in these cells, since Slotki *et al.* (1993) reported extremely low Na^+–K^+–$2Cl^-$ cotransporter activity in CF-P1-derived artificial chromosome (PAC) cells, irrespective of CFTR expression. Another possibility is differences in the expression of non-CFTR Cl^- channels in different cell types, which are also dependent on Na^+–K^+–$2Cl^-$ cotransport for Cl^- supply and may regulate its activity and/or expression. A third possibility is that epithelial cells from CF patients have residual CFTR expression levels that may be sufficient to prevent the downregulation of NKCC1 expression.

In contrast to biliary, bronchial and pancreatic epithelia, the intestines of CFTR null mutant mice do not display functional evidence of major non-CFTR anion conductances, and there is no functional CFTR protein expression in these mice (Ratcliff *et al.* 1993). We therefore chose this tissue from CFTR (−/−) mice to investigate NKCC1 expression and Na^+–K^+–$2Cl^-$ cotransporter regulation in the absence or presence of CFTR expression and function. To measure the expression and activity of NKCC1 in the same cellular system, we isolated colonic crypts from the proximal colon of a CFTR null mutant mouse strain and their normal wild-type littermates. CFTR (−/−) and (+/+) crypts were morphologically indistinguishable. NKCC1 expression levels were only slightly reduced in crypts from CFTR (−/−) mice. NKCC1 activity, however, was reduced in CFTR (−/−) crypts

compared to crypts of normal littermates. The decrease in transport rates was more than the decrease in expression levels, but not nearly as much as described for CF-PAC cells (Shumaker & Soleimani, 1999). One possible explanation for the increase in NKCC1 expression level in the CFTR-retransfected cell line compared to the CF-PAC cell line could be a difference in growth rate and/or differentiation status between the two, leading to differences in expression levels for the ion transport proteins involved in vectorial ion transport. Of note is that the same group has also found an upregulation of expression in the CFTR-retransfected cell line compared to the CF-PAC cell line for the Na^+–HCO_3^- cotransporter NBC1 and the apical anion exchanger DRA (Shumaker *et al.* 1999; Wheat *et al.* 2000). In native murine epithelia with a complete lack of CFTR expression, we did not find major differences in the expression levels of any of these transport proteins (Bachmann *et al.* 2003; also B. Riederer & U. Seidler, unpublished results). Thus, in the native intestine, factors other than CFTR are able to maintain a high expression of these anion transporters.

Even in the presence of normal NKCC1 expression and transport rates, cAMP-dependent activation could be defective in the absence of CFTR expression. In CFTR-deficient epithelia, several other ion-transport processes appear to be deregulated (Schwiebert *et al.* 1999), and it appears not so much the basal activity, but the cAMP-dependent regulation that is defective. Thus, in CFTR-deficient tissues or cells, the epithelial Na^+ channel ENaC is not inhibited by a rise in cAMP (Stutts *et al.* 1995), the outwardly rectifying depolarization-induced Cl^- channel (ORDIC) is not activated by cAMP (Jovov *et al.* 1995), electroneutral Na^+ absorption in the jejunum is not
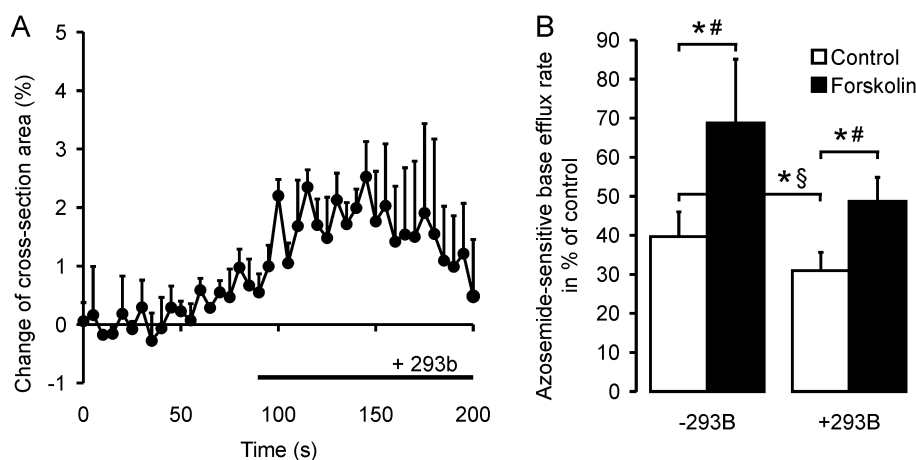
Figure 8. Effect of Hoe 293b, an inhibitor of cAMP-activated K^+ channels, on cotransporter activity

A, the volume effect of Hoe 293b alone, which is a slight swelling of the crypt (2.03 ± 0.3 %, *n* = 5 crypts from 3 mice, n.s.: Student's *t* test), measured confocally as the change of cross-section area. The basal NKCC activity is decreased in the presence of Hoe 293b (*B*), but NKCC1 (*B*) is activated by a rise in intracellular cAMP upon forskolin stimulation in both cases (*n* = 6–8 crypts from 3–5 mice in each group; * *P* < 0.05: § Student's unpaired *t* test, # Student's paired *t* test).

inhibited by cAMP (Clarke & Harline, 1996) and VIP-dependent K^+ channel opening is not observed (Loussouarn *et al.* 1996).

We therefore investigated the effect of raising intracellular cAMP with forskolin on the $Na^+-K^+-2Cl^-$ cotransporter in the cells that form the colonic crypts of CFTR $(-/-)$ and normal mice. As already shown for rat colonic crypts (Heitzmann *et al.* 2000), an increase in NKCC1 transport rate occurred upon stimulation of normal crypts with forskolin. Significantly, the same degree of activation was observed in CFTR $(-/-)$ crypts, although the time to achieve maximal stimulation was slower than in normal crypts, suggesting that the decrease in basal activity observed in the CFTR $(-/-)$ crypts is largely due to post-transcriptional events. These results demonstrate clearly that the absence of CFTR expression does not result in a general cellular defect in the cAMP-dependent regulation of the $Na^+-K^+-2Cl^-$ cotransporter.

For NKCC activation, evidence from a number of epithelial and non-epithelial cell types argues for complex and possibly multiple regulatory mechanisms, among them a fall in cytosolic Cl^- activity, transient cell shrinkage, phosphorylation of the cotransporter or other phosphorylation events (Haas *et al.* 1993, 1995; Matthews *et al.* 1994; Lytle & Forbush, 1996; Lytle, 1997, 1998; Heitzmann *et al.* 2000). Recently, volume-sensitive protein kinases have been discovered, and it has been suggested that they are the link between cAMP-mediated secretion and phosphorylation of basolateral anion transporters (Lytle, 1997; Roig *et al.* 2000). Since we have observed no forskolin-induced anion secretion in the isolated colon of these mice in the Ussing chamber, we would not have expected a secretion-related volume or $[Cl^-]_i$ decrease in CFTR $(-/-)$ crypts. It was feasible, however, that the activation of basolateral K^+ channels, or unknown transport events, could result in cell shrinkage and/or a decrease in $[Cl^-]_i$ even in CFTR-deficient crypts. To address this, the crypt cross-sectional area was measured by confocal microscopy (Heitzmann *et al.* 2000). Addition of forskolin in the presence of azosemide in CFTR $(+/+)$ crypts resulted in a marked decrease in this parameter, but no change was observed in CFTR $(-/-)$ crypts. Although this already makes a cellular Cl^- loss unlikely, we nevertheless assessed changes in $[Cl^-]_i$ using the fluorescent dye MQAE. Again, in CFTR $(+/+)$ crypts in the presence of azosemide there was a marked increase in MQAE fluorescence, indicative of cellular Cl^- loss, whereas this increase did not occur in CFTR $(-/-)$ crypts. Finally, we studied whether NKCC1 activation still occurred after blockade of the cAMP-dependent, chromanol-sensitive K^+ channels in the colon. Although

Hoe 293b decreased basal $Na^+-K^+-2Cl^-$ cotransporter activity, activation by forskolin still occurred.

The stimulation of $Na^+-K^+-2Cl^-$ cotransport activity in CFTR $(-/-)$ cells in the absence of changes in cell volume and $[Cl^-]_i$ show that cell shrinkage is not required for NKCC1 activation. In contrast, an increase in intracellular cAMP clearly activates the $Na^+-K^+-2Cl^-$ cotransporter in a direct fashion, supporting the results of Haas *et al.* (1993). One such activation pathway could be a protein kinase A (PKA)-mediated phosphorylation of the protein, since NKCC1 contains highly conserved PKA consensus sites in their cytoplasmic domains. However, it is not yet known whether these consensus sites are indeed phosphorylated by PKA, whether NKCC1 phosphorylation results in changes in transport activity, or what additional cAMP-dependent events are necessary to fully activate the transporter.

Thus, it appears that cAMP-dependent NKCC1 activation does not require either altered chemical gradients or secretion-associated events such as shrinkage or ionic disequilibrium. The data imply that receptor-mediated signal transduction events activate both the apical and basolateral ion transport pathways required for the initiation and maintenance of the secretory process. This is consistent with the observation of Heitzmann *et al.* (2000) in rat and our work with murine colonic crypts, that forskolin-associated shrinkage is small in the absence of $Na^+-K^+-2Cl^-$ cotransport inhibition. The situation is similar to that observed by us in rabbit parietal cells, where Ca^{2+}-dependent and cAMP-dependent secretagogues, although both resulting in acid production and cellular shrinkage with rapid volume recovery, resulted in a differential activation of the Na^+-H^+ exchanger isoforms NHE1 and NHE4 (Bachmann *et al.* 1998). This suggests strongly that secretagogue-induced Na^+-H^+ exchange activation in these cells is not simply secondary to secretion-associated shrinkage, but is induced directly by the secretagogue-associated cellular signalling pathways.

In summary, our results demonstrate normal NKCC1 expression, slightly reduced basal $Na^+-K^+-2Cl^-$ cotransporter rates, and fully preserved cAMP-dependent activation in native colonic crypts from CFTR-deficient mice. Since our functional data from the CFTR-knockout mouse colon are in agreement with those from nasal epithelial cells of CF patients, a severe dysfunction of this anion importer is unlikely to contribute to the secretory defect in CF epithelia. Furthermore, the cAMP-dependent activation of NKCC in CFTR $(-/-)$ crypts occurred in the absence of cellular volume and $[Cl^-]_i$ loss, ruling out the latter as the final common pathway for NKCC1 activation.

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