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Severe Defects in Absorptive Ion Transport in Distal Colons of Mice that Lack ClC-2 Channels

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Abstract

Background & Aims—The fluid secretion model predicts that intestinal obstruction disorders can be alleviated by promoting epithelial Cl^- secretion. The cAMP-activated anion channel CFTR mediates Cl^--dependent fluid secretion in the intestine. Although the role of the ClC-2 channel has not been determined in the intestine, this voltage-gated Cl^- channel might compensate for the secretory defects observed in patients with cystic fibrosis and other chronic constipation disorders. We investigated whether mice that lack ClC-2 channels (*Clcn2*$^{-/-}$) have defects in intestinal ion transport.

Methods—Immunolocalization and immunoblot analyses were used to determine the cellular localization and the amount of ClC-2 expressed in mouse early (EDC) and late distal colon (LDC). Colon sheets from wildtype and *Clcn2*$^{-/-}$ littermates were mounted in Ussing chambers to determine transepithelial bioelectrical parameters and Na^+, K^+ and Cl^- flux.

Results—Expression of ClC-2 was higher in the basolateral membrane of surface cells in the EDC, compared to the LDC, with little expression in crypts. Neither cAMP nor Ca^{2+}-induced secretion of Cl^- was affected in the EDC or LDC of *Clcn2*$^{-/-}$ mice, whereas the amiloride-sensitive short circuit current (ISC) was increased approximately 3-fold in *Clcn2*$^{-/-}$ EDC, compared to that of control littermates. Conversely, electroneutral Na^+, K^+ and Cl^- absorption were dramatically reduced in colons of *Clcn2*$^{-/-}$ mice.

Conclusions—Basolateral ClC-2 channels are required for colonic electroneutral absorption of NaCl and KCl. The increase in the amiloride-sensitive ISC in *Clcn2*$^{-/-}$ mice revealed a compensatory mechanism that is activated in the colons of mice that lack the ClC-2 channel.

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Keywords

NaCl absorption; KCl absorption; fluid secretory defects; mouse model; intestinal epithelial cells

Introduction

Cystic fibrosis (CF) is the most common genetic disease in Caucasians, occurring in approximately one out of 3,200 live births [1]. CF is caused by mutations in the *CFTR* gene, which encodes an apical cAMP-activated anion channel in epithelial tissues. CF disease has been linked to Cl^-, bicarbonate and fluid secretory defects in several organs, such as exocrine glands, gastrointestinal tract and airways [1–3]. It has been reasoned that activation of an alternative epithelial Cl^- efflux pathway might alleviate the secretion defect in CF. Mouse airway and exocrine glands possess a Ca^{2+}-activated fluid secretory process independent of CFTR [4, 5] that is thought to rely on apical Ca^{2+}-activated Cl^- channels (CaCC) to promote Cl^- efflux into the luminal space. The CaCC in many epithelia is encoded by the *Tmem16A* gene, a member of the newly discovered TMEM16 family of Ca^{2+}-activated Cl^- channels [6–9]. Although it has been suggested that apical Ca^{2+}-activated Cl^- channels are directly involved in intestinal Cl^- secretion [10], other studies failed to demonstrate a connection between Ca^{2+}-activated Cl^- channels and intestinal secretion [11, 12]. It appears that activation of the basolateral Ca^{2+}-activated K^+ channel KCNN4 during Ca^{2+}-activated intestinal secretion hyperpolarizes intestinal epithelial cells, and consequently, generates a greater driving force for CFTR-mediated Cl^- efflux [12]. Indeed, if CFTR is not first activated, a rise in intracellular Ca^{2+} does not cause Cl^- secretion [11, 13, 14].

It has been proposed that ClC-2, one of the four plasma membrane-associated Cl^- channels in the mammalian CLC family [15], may substitute for CFTR in CF airway cells [16]. ClC-2 has also been suggested to mediate Cl^- secretion in the mouse small intestine [17], where it was reported to localize to the apical pole and tight junction complex. Conversely, ClC-2 channels are not expressed in the crypt cells of guinea pig distal colon, but in the basolateral membrane of the NaCl absorbing surface epithelium. A Cl^- conductance with ClC-2-like rectification and selectivity properties was detected in surface epithelium cells, consistent with the basolateral subcellular distribution of this channel [18–20]. It has been argued that transepithelial transport defects are responsible for blindness and male infertility in *Clcn2* knockout mice [21, 22]. Moreover, double *Clcn2* and *CFTR* knockout mice do not exhibit a worsening of the CF phenotype, indirectly suggesting that ClC-2 does not contribute to fluid secretion [23]. This is consistent with the basolateral localization of ClC-2 channels in mouse intestine [24].

NaCl absorption in the intestinal tract occurs by either an electrogenic or electroneutral mechanism depending on the gut segment and species [25]. In the mouse colon, the bulk of NaCl absorption occurs across the luminal membrane via the electroneutral Na^+/H^+ and Cl^-/HCO_3^- exchangers [25]. Na^+ is subsequently extruded to the serosal side by the basolateral Na^+-K^+ ATPase, while a basolateral K^+ channel recycles K^+ ions to the serosa. Finally, a basolateral Cl^- efflux pathway completes the molecular machinery involved in electroneutral NaCl absorption. The molecular identity of the basolateral Cl^- efflux pathway has not yet been elucidated, but it has been suggested that basolateral ClC-2 channels may mediate this Cl^- efflux to the serosal side [19].

The aim of this study was to determine the physiological roles of ClC-2 in mouse colonic epithelium. Immunohistochemical localization and immunoblotting studies found that ClC-2 is differentially expressed along the distal colon, i.e. the amount of plasma membrane ClC-2

channels in surface cells was less in the late than the early distal colon. Consistent with this distinction, mouse distal colon was subdivided into an early (EDC) and late (LDC) distal colon for functional analysis. As noted in a previous report [23], we found that mice lacking ClC-2 channels did not display impaired electrogenic Cl^- secretion, consistent with CFTR being the primary, if not the only, channel responsible for Cl^- secretion in the colon. Amiloride-sensitive I_{SC}, an index of epithelial Na^+ channel ENaC activity, increased ~3 fold in the *Clcn2*$^{-/-}$ EDC compared to wildtype littermates. In addition to the increased ENaC activity in the *Clcn2*$^{-/-}$ EDC, unidirectional Na^+, K^+ and Cl^- fluxes studies showed that electroneutral absorption of NaCl and KCl was severely impaired in the *Clcn2*$^{-/-}$ EDC. Taken together, our results demonstrate that ClC-2 channels are involved in Cl^- absorption rather than Cl^- secretion in the distal colon. Consequently, ClC-2 specific blockers might be effective agents for treating constipation by reducing NaCl and water absorption in the colon.

Materials and Methods

General procedures

Clcn2$^{-/-}$ mice were generated as previously described [22]. *Clcn2*$^{-/-}$ and wildtype littermates of 2–6 months old were used in experimental procedures approved by the NIDCR Animal Care and Use Committee, University of Rochester Animal Resources Committee and CECs IACUC. Reagents were from Sigma-Aldrich unless otherwise indicated.

Intestinal tissue isolation and Ussing chamber experiments

Mice were anesthetized with CO_2 gas and killed by exsanguination via cardiac puncture. A segment of distal colon about 2 cm in length was excised and divided in two pieces. We defined these segments as EDC and LDC, respectively. The EDC and LDC segments (each 1 cm length) were located 1.5 cm and 0.5 cm from the anus, respectively. Segments were rinsed with 0.9% NaCl and cut open lengthwise, and then the muscular layer was partially stripped by scraping with a glass microscope slide to create a mucosal sheet [26]. The resulting mucosal sheet was mounted on a tissue-holding slide (0.1 cm^2) and placed in an Ussing chamber (Physiologic Instruments). Silicone grease was used to prevent edge damage. The solution bathing the apical and basolateral hemi-chambers consisted of (in mM): 115 NaCl, 5 KCl, 25 $NaHCO_3$, 1 $CaCl_2$ and 2 $MgCl_2$, at pH 7.4. Indomethacin (5 μM) was added to the serosal side to prevent endogenous cAMP production via prostaglandin E_2 release [13, 14]. Solutions were gassed with 95% O_2/5% CO_2. The temperature was kept at 37°C using a water jacket system. Current pulses were generated with pClamp 8 software (Molecular Devices) and the voltage and current signals were acquired using a digidata 1320A interface (Molecular Devices). V_{TE} was measured in the current clamp mode using an EVC4000 amplifier (World Precision Instruments). At 8 s intervals, a 10 μA current pulse was injected. R_{TE} was calculated from the voltage deflection created by the current injection (ΔV_{TE}). The equivalent I_{SC} (expressed as $\mu Eq*h^{-1}cm^{-2}$) was calculated from V_{TE} and R_{TE} according to Ohm's law.

Na^+, K^+ and Cl^- fluxes in isolated colonic strips

Na^+ and Cl^- fluxes were measured using $^{22}Na^+$ and $^{36}Cl^-$ as tracers. Colonic tissue was prepared and mounted in an Ussing chamber as described above. Voltage clamp recordings were acquired using a VCC MC2 amplifier and AD converter (Physiologic Instruments). R_{TE} was calculated by clamping the potential to ±10 mV at 15 s intervals. Radioisotopes were added 30 minutes after equilibration. Mucosal to serosal fluxes were measured [27] by supplementing the bicarbonate buffered solution in the apical side with 2 μCi of $^{22}Na^+$ (PerkinElmer) and 2 μCi of $^{36}Cl^-$ (American Radiolabeled Chemicals). For serosal to mucosal flux experiments 4 μCi of $^{22}Na^+$ and 4 μCi of $^{36}Cl^-$ were added to the serosal

chamber. An aliquot of the side containing the radioisotopes was taken at the beginning of each experiment, followed by four aliquots from the opposite side at 30 min intervals. To inhibit ENaC-mediated Na^+ uptake, 10 μM amiloride was used on the apical side and 1 μM tetrodotoxin on the basolateral side to inhibit any remaining enteric neuronal activity. Radioactivities were measured in a Tri-Carb 2100 TR Liquid Scintillation Analyzer (PerkinElmer) and Cobra Auto-Gamma counter (PerkinElmer). The measured beta counts were corrected for the $^{22}Na^+$ contribution by calculating and applying a beta spill-over factor calculated with pure $^{22}Na^+$ isotope standard to obtain $^{36}Cl^-$ activity. $^{22}Na^+$ radioactivity was taken directly from gamma-counting readings [28].

For mucosa to serosa K^+ flux measurements, serosal K^+ was replaced by Rb^+. For serosa to mucosa K^+ flux measurements, mucosal K^+ was replaced by Rb^+. 250 μL aliquots were taken every 30 minutes, diluted 20 times in a solution containing 500 ppm (mg/L) CsCl and K^+ concentrations were measured by atomic absorption spectrometry using a K-Na lamp (AAnalyst 200 Atomic Absorption spectrophotometer; PerkinElmer). Calibrations for potassium were performed by linear regression analysis using 0.25, 0.5 and 1 ppm as standards. To calculate net fluxes, tissues used for mucosa to serosa and serosa to mucosa experiments with similar R_{TE} were paired. Fluxes are expressed as $\mu Eq*h^{-1}cm^{-2}$.

Tissue processing and immunohistochemistry studies

Immunolocalization studies on EDC and LDC segments were performed as described previously [24]. A rabbit polyclonal antibody (1:100 dilution) directed to the C-terminus of ClC-2 (Alomone) was used.

Western blot analysis

EDC and LDC colonic cells were isolated by adding a Ca^{2+}-free solution into the luminal space as described previously [18], except that the incubation step with Ca^{2+}-free solution was increased from 3 minutes to 15 minutes. Colon cells were pooled from wildtype or *Clcn2*$^{-/-}$ mice (two females and two males per group) prior to biotinylation [29]. EDC and LCD cell lysates and plasma membrane fractions (50 μg/lane) were used for analysis as described elsewhere [29] using the same antibody as for immunolocalization studies (1:200 dilution). Densitometric analysis of band intensities was performed using Adobe Photoshop CS3 (Adobe Systems Incorporated).

qPCR studies

Total RNA from wildtype and *Clcn2* $^{-/-}$ mice (two females and two males per group) was isolated using RNeasy columns (Qiagen). Two μg of total RNA was transcribed to cDNA using Maxima® First Strand cDNA Synthesis kit (Fermentas). Expression levels of α-*ENaC*, β-*ENaC*, γ-*ENaC* and *Clcn2* were evaluated by analysis of relative gene expression using SYBR Green based quantitative PCR (qPCR) by ACGT, Inc. (Wheeling, IL). Expression levels of the target genes were compared to those of three housekeeping genes (GAPDH, β-actin and cyclophilin) in pooled samples. The qPCR primer sets used in this study are provided in supplemental table 1.

Quantification of plasma aldosterone

Mice were anesthetized by intraperitoneal chloral hydrate injection (400 mg/kg) and the blood was collected by cardiac puncture. Blood samples from wildtype and *Clcn2* $^{-/-}$ littermate mice (three females and three males per group) were centrifuged at 3000 rpm for 3 minutes and serum samples were stored at −86 °C until further analysis. Aldosterone serum levels were determined from pooled samples by radioimmunoassay by Ani Lytics Incorporated (Gaithersburg, MD).

Results

Differences in electrogenic Na^+ absorption along the distal colon of wildtype and *Clcn2*$^{-/-}$ mice

Two segments of distal colon per mouse, EDC and LDC, were mounted in Ussing chambers and their bioelectric properties compared. In the wildtype mouse, LDC displayed a robust amiloride-sensitive I_{SC}, whereas the amiloride-sensitive I_{SC} in the EDC was markedly lower (Figure 1A). Similar measurements using tissues from *Clcn2*$^{-/-}$ mice suggest that the amiloride-sensitive I_{SC} was enhanced in *Clcn2*$^{-/-}$ EDC compared to wildtype EDC, whilst that of LDC did not seem changed (Figure 1B). A summary of these results (Figure 1C) shows a statistically significant ~3-fold increase in I_{SC} of EDC from *Clcn2*$^{-/-}$ animals, but not LDC. Figure 1D shows that the R_{TE} was also less in the EDC compared to the LDC of wildtype animals. The R_{TE} was increased in the EDC of mice lacking ClC-2 compared to their controls littermates (Figure 1D).

Cl^- Secretion is not affected in the mouse distal colon lacking ClC-2 channels

In view of the differences in electrogenic Na^+ absorption in the colon of wildtype and *Clcn2*$^{-/-}$ animals, we designed experiments to test whether wildtype EDC and LDC display differences in electrogenic Cl^- secretion, and whether these are affected upon *Clcn2* gene ablation. It has been demonstrated that colonic Cl^- secretion can be activated by an intracellular increase in cAMP or Ca^{2+} [25]. cAMP and Ca^{2+} induced Cl^- secretion mechanisms are both critically dependent on the presence of active apical CFTR channels. Because CFTR channels are gated by an increase in intracellular cAMP, Ca^{2+}-mediated Cl^- secretion requires pre-activation of the CFTR channel by cAMP [13]. Considering the dependence of the Ca^{2+}-mediated process on intracellular cAMP, Ussing chambers experiments to examine Cl^- secretion in EDC and LDC used a sequential cAMP and then Ca^{2+} mobilization protocol. cAMP-dependent secretion was induced by addition of 10 μM forskolin and 100 μM IBMX to the serosal side. In order to quantify Ca^{2+}-induced Cl^- current, cAMP-activated Cl^- secretion was then abolished by blocking the basolateral cAMP-activated KCNQ1/KCNE3 K^+ channel with 10 μM serosal chromanol 293B, which does not affect CFTR channels. Ca^{2+}-mediated secretion was then stimulated by serosal addition of 50 μM carbachol.

Figure 2 shows the changes in the V_{TE} elicited by addition of secretagogues to the serosal aspect of EDC from wildtype and *Clcn2*$^{-/-}$ mice. 10 μM amiloride was present in the luminal solution throughout the experiment to block the ENaC-mediated Na^+ absorption. The V_{TE} became more negative upon addition of secretagogues (luminal side respect to serosal side), consistent with an increased Cl^- secretion into the lumen. As seen in Figures 2A and 2B, EDC from wildtype and *Clcn2*$^{-/-}$ animals displayed both cAMP- and Ca^{2+}-mediated Cl^- secretion. Neither cAMP- nor Ca^{2+}-dependent Cl^- secretion was affected in EDC segments in the *Clcn2*$^{-/-}$ mice compared to wildtype littermates. Table 1 summarizes the values obtained for cAMP- and Ca^{2+}-mediated Cl^- colonic secretion in wildtype and *Clcn2*$^{-/-}$ mice EDC and similar experiments performed in LDC. It was found that cAMP-dependent secretion was significantly higher in LDC than in EDC, but neither cAMP- nor Ca^{2+}-dependent secretion was affected by *Clcn2* gene ablation. Similar results were obtained when chromanol 293B was omitted and carbachol was added after stimulation with 10 μM forskolin and 100 μM IBMX (not shown).

Serum aldosterone and ENaC expression levels in wildtype and *Clcn2*$^{-/-}$ mice

ENaC channels are expressed in the apical membrane of absorptive colonocytes. ENaC channels are heteromultimers composed of α, β and γ subunits and their expression is modulated by the steroidal hormone aldosterone [30]. Because the amiloride-sensitive I_{SC} was

enhanced in the EDC of mice lacking ClC-2, we tested whether the serum aldosterone level was also increased in the *Clcn2*$^{-/-}$ mice. However, serum aldosterone was unaffected in *Clcn2*$^{-/-}$ mice (wildtype = 1276 ± 264 pg/mL (n=6); *Clcn2*$^{-/-}$ = 1031 ± 95 pg/mL (n=6); $p > 0.4$, *t* test). Additionally, qPCR analysis showed that there were no significant differences in the expression levels of α, β and γ ENaC subunit transcripts in the EDC from wildtype and *Clcn2*$^{-/-}$ mice (Supplementary Table 2).

Electroneutral absorption of NaCl and KCl is markedly decreased in the early distal colon of mice lacking ClC-2 channels

The enhanced amiloride-sensitive I_{SC} in the *Clcn2*$^{-/-}$ EDC may reveal the activation of an electrogenic mechanism that could compensate for the loss of a ClC-2 channel-dependent electroneutral Cl^- absorption. To test the hypothesis that electroneutral Cl^- absorption is dependent upon ClC-2 channels, we measured unidirectional Na^+ and Cl^- fluxes in EDC sheets mounted in Ussing chambers maintained under short circuit conditions (V_{TE}=0 mV). Consistent with ClC-2 channels playing a physiological role in electroneutral Cl^- absorption by the EDC, Figure 3 shows that mucosa to serosa fluxes (J_{MS}) for both Na^+ and Cl^- were significantly decreased in the *Clcn2*$^{-/-}$ EDC compared to that of wildtype littermates. In contrast, serosa to mucosa fluxes (J_{SM}) for both Na^+ and Cl^- were enhanced in *Clcn2*$^{-/-}$ EDC. Consequently, Na^+ and Cl^- net fluxes ($J_{Net}=J_{MS}-J_{SM}$) showed that a substantial electroneutral NaCl absorption takes place in wildtype EDC, whereas this absorptive process was essentially abolished in the *Clcn2*$^{-/-}$ EDC (Figures 3A and 3B).

As chloride fluxes across the EDC were higher than sodium fluxes we explored the possibility that an additional Na^+-independent electroneutral Cl^- flux might be present. One such flux could correspond to a K^+ absorptive process mediated by apical membrane H^+/K^+ exchange and basolateral K^+ exit through KCl cotransport and/or K^+ channels [25], where Cl^- efflux through ClC-2 might be involved.

To test this possibility we studied potassium fluxes in the EDC. Our measurements indicate that there is indeed a significant mucosa to serosa movement of K^+ across wildtype EDC that was severely decreased in the epithelium from *Clcn2*$^{-/-}$ animals (Figure 3C). This absorptive K^+ flux was Cl^--dependent, since it was significantly lowered when Cl^- was reduced from 135 mM to 10 mM by replacement with gluconate in the wildtype EDC. J_{MS} K^+ was decreased from 3.6 ± 0.7 $\mu Eq*h^{-1}cm^{-2}$ (n=5) in control to 2.1 ± 0.3 $\mu Eq*h^{-1}cm^{-2}$ (n=5) in low Cl^- conditions ($p<0.05$, t test).

ClC-2 expression varies along the mouse distal colon

The impairment of electroneutral NaCl and KCl absorption in the *Clcn2*$^{-/-}$ EDC suggests that ClC-2 channels act as a basolateral Cl^- efflux pathway in electroneutral Cl^- absorption. It would be reasonable to expect that changes in tissue abundance of ClC-2 might correlate with the observed rates in electroneutral transepithelial Cl^- transport. To test this possibility, ClC-2 immunolocalization experiments were performed. Figure 4 shows that the ClC-2-associated immunoreactivity was confined to the surface epithelium of both EDC and LDC (left panels) with no apparent staining in the crypt epithelium.

At the subcellular level, we found pronounced differences between the EDC and LDC in the distribution of ClC-2 channels: most of the ClC-2-associated immunostaining was present at the basolateral pole of surface cells in the EDC (*left panel*, Figure 5A), whereas immunostaining was mostly intracellular in the LDC (*right panel*, Figure 5A). Further, densitometric analysis of western blot assays performed on biotinylated plasma membrane proteins showed that the amount of ClC-2 protein in EDC was ~2 fold higher than in LDC (*left panel*, Figure 5B). In contrast, densitometric analysis of western blots from whole cell

lysates confirmed that ClC-2 channel protein expression in EDC was comparable to that in the LDC (*right panel*, Figure 5B). Consistent with this latter observation, qPCR analysis of *Clcn2* transcripts showed that there was no difference between EDC and LDC expression (Supplementary table 3) when normalized to three different housekeeping genes (β-actin, GAPDH and cyclophilin).

There was a strong β-actin immunoreactive signal in whole cell lysates, while no β-actin immunoreactivity was detected in the biotinylated proteins associated with the plasma membranes (Supplemental Figure 1). The lack of immunoreactivity in the biotinylated fraction indicates that there is little contamination of the plasma membrane preparation by intracellular proteins. The specificity of the ClC-2 antibody in both immunolocalization (Figure 4) and western blot (Figure 5) assays was confirmed by the absence of immunoreactivity in tissue and protein samples from the *Clcn2*$^{-/-}$ colon.

Conclusions

Colonic epithelium displays both absorptive and secretory functions. Transcellular Cl^- fluxes are involved in both of these physiological processes. NaCl and water secretion takes place mainly in the crypts in response to secretagogues [31]. CFTR Cl^- channel activity constitutes the rate-limiting step in intestinal fluid secretion. The importance of the CFTR channel to intestinal fluid secretion can be inferred from the severe intestinal obstructive disorders associated with CF, such as meconium ileus in newborns and intestinal obstruction in CF patients at later ages [31]. Variability in the severity of the intestinal phenotype [10] could be due to compensatory changes in the expression of other channels or transporters. In that regard, it has long been postulated that an alternative apical Cl^- pathway could compensate for the epithelial hyposecretory defects associated with the CF phenotype. Schwiebert et al. postulated that ClC-2 Cl^- channels might compensate for the lack of functional CFTR channels in CF airway epithelium [16]. Gyömörey et al. also suggested a secretory role for ClC-2 channels in the mouse small intestine [17]. Moreover, it has been postulated that lubiprostone, a prostaglandin E_1-derived compound used for chronic constipation treatment, induces Cl^- secretion by directly activating ClC-2 channels [32]. However, recent work by Bijvelds et al. clearly shows that intestinal Cl^- secretion induced by lubiprostone requires CFTR channels [33], supporting previous observations where lubiprostone was linked to activation of prostanoid receptors which increases intracellular cAMP levels [34].

Mice lacking ClC-2 channels do not show apparent intestinal defects, although the role of ClC-2 channels in intestinal ion transport has not been clearly established [21–23]. In the present study, we found that neither cAMP- nor Ca^{2+}-mediated Cl^- secretion depends on ClC-2 expression in mouse EDC and LDC epithelia. These results are similar to those reported previously in the distal colon of *Clcn2*$^{-/-}$ mice [23]. It is important to note that this last study also characterized Cl^- secretion in double mutant mice lacking ClC-2 channels and expressing the ΔF508 CFTR mutation. Surprisingly, the double targeted mice survived better than the CFTR$^{\Delta F508/\Delta F508}$ mice, apparently by partially reversing the obstructive intestinal defect displayed by the CFTR$^{\Delta F508/\Delta F508}$ mice [23]. This observation is consistent with ClC-2 in the colon playing a major role in NaCl and fluid absorption, but not fluid secretion.

Colonic surface epithelium plays a major role in NaCl and fluid absorption. It is generally accepted that secretion and absorption in the intestine are spatially separated along the surface-crypt axis. Absorptive processes are located in surface epithelial cells, whereas fluid secretion takes place in the crypts [35], although there is evidence that some NaCl absorption might also occur in colonic crypts [36]. We found that ClC-2 channels are expressed in the colonic surface epithelium, consistent with ClC-2 playing a role in Cl^- absorption. Indeed,

our Na^+ and Cl^- net flux measurements demonstrate that electroneutral NaCl absorption is essentially eliminated in the colon of *Clcn2*$^{-/-}$ mice. We also found that electroneutral net Na^+ absorptive flux is substantially higher than the amiloride-sensitive I_{SC} in the wildtype colon (>10 fold), supporting the concept of electroneutral NaCl absorption as the main mechanism by which Na^+ and Cl^- ions are reabsorbed in the EDC.

Cl^- J_{MS} fluxes were higher than Na^+ J_{MS} fluxes, suggesting that there is an additional electroneutral pathway for Cl^- entry. Indeed, we found that a Cl^--dependent K^+ absorption process takes place in the EDC and is severely affected in the *Clcn2*$^{-/-}$ mice. Together, it seems that ClC-2 channels act as a basolateral Cl^- efflux pathway in the mouse EDC. However, absorptive Na^+, K^+ and Cl^- fluxes (J_{MS}) are not abolished in the mouse lacking ClC-2 channels, suggesting that there is an alternative basolateral Cl^--efflux pathway. Basolateral KCC1 K^+-Cl^- cotransporters, which are involved in K^+ absorption in the rat distal colon [37], may also contribute to Cl^- efflux across the basolateral membrane in mouse colonic surface cells.

We also found that Na^+ and Cl^- J_{SM} fluxes were enhanced in the *Clcn2*$^{-/-}$ EDC, probably because an alternative Cl^- secretion pathway was unmasked upon *Clcn2* gene disruption. NaCl absorption and secretion are regulated by signals arising from endocrine, autonomic nervous system and immune system [38]. Other regulatory mechanisms may involve protein-protein interactions. For example, CFTR has been linked to the regulation of ENaC [39] and NHE3 [40], which are apical membrane proteins that mediate Na^+ absorption. Moreover, it has been suggested that KCNN4 K^+ channels inhibit KCNMA1 K^+ channels by a mechanism involving protein-protein interaction [41]. By analogy with these data, one might speculate that the observed increase in Na^+ and Cl^- J_{SM} fluxes in the *Clcn2*$^{-/-}$ EDC are related to the removal of a regulatory effect of ClC-2 on cellular or paracellular transport proteins. The higher R_{TE} observed in *Clcn2*$^{-/-}$ EDC and small intestine [42] and the interaction of ClC-2 with other proteins [43] support this hypothesis. Furthermore, ClC-2 complexes with HSP90 to regulate channel activity [43] and ClC-2 is found in lipid-rich domains where it appears to cluster with other proteins [44].

The enhanced amiloride-sensitive I_{SC} displayed by the EDC lacking ClC-2 resembles the compensatory mechanism observed in the distal colon of mouse deficient in the apical Na^+/H^+ exchanger NHE3. *Nhe3*$^{-/-}$ mice displayed more functional ENaC channels in the distal colon, in an apparent attempt to compensate for the absence of the primary apical NHE3-associated Na^+ influx pathway [45]. The enhanced ENaC activity observed in the EDC of mice lacking ClC-2 did not correlate with an increase in aldosterone levels or expression of ENaC transcripts. Thus, enhanced ENaC activity might be associated with increased targeting of intracellular ENaC to the membrane or to an increase in open probability of the channels already residing there. Whether ClC-2 can substitute for the CFTR channel in CF intestine ultimately depends on the cellular and subcellular location of the ClC-2 channel. If ClC-2 channels play a role in intestinal Cl^- secretion, then an apical localization in the secretory crypt cells is expected. In contrast, our studies revealed that the ClC-2-associated immunostaining in the mouse distal colon was confined to the surface epithelium in both EDC and LDC. At the subcellular level, most of the ClC-2-associated immunolabeling was present at the basolateral membrane of the EDC, whereas the immunostaining appears to be mostly intracellular in the LDC. The diffuse intracellular pattern observed in the LDC resembles the intracellular pattern for ClC-2 in human sigmoid colon [20]. Western blots of biotinylated plasma membrane proteins from EDC and LDC confirmed that ClC-2 protein in the plasma membrane of EDC was ~2-fold higher compared to LDC. On the other hand, the amount of ClC-2 protein from whole cell lysates was similar between EDC and LDC. Further qPCR analysis of *Clcn2* transcripts in EDC and LDC confirmed that there is no difference in the expression levels of *Clcn2* between EDC and LDC. We also found that

cAMP-activated Cl^- secretion and amiloride-sensitive I_{SC} are higher in LDC compared to EDC, demonstrating that secretory and absorptive functions differ along the distal colon.

In summary, our results strongly support an important role for ClC-2 in colonic absorption rather than secretion. The absence of diarrhea in the *Clcn2*$^{-/-}$ mouse contrasts with the observation in *Nhe3*$^{-/-}$ animals. This might be due the more restricted localization of ClC-2 along the intestinal tract [24]. Controlling ClC-2 function by the use of specific drugs might be of importance for intestinal disorders of fluid transport. On the other hand, the possible role of ClC-2 as regulator of other Cl^- and Na^+ permeation pathways by direct protein-protein interactions is an open field for future research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CFTR	cystic fibrosis transmembrane conductance regulator gene
C*lcn2*	ClC-2 gene
EDC	early distal colon
LDC	late distal colon
I_{SC}	short circuit current
R_{TE}	transepithelial resistance
V_{TE}	transepithelial potential difference

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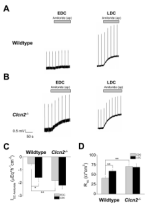

Figure 1. Amiloride-sensitive I_{SC} and R_{TE} in EDC and LDC

Representative current clamp experiments performed in EDC and LDC from wildtype (**Panel A**) and *Clcn2*$^{-/-}$ (**Panel B**) epithelial sheets showing transepithelial potentials before and after addition of 10 μM amiloride to the mucosal side. **C.** Calculated amiloride-sensitive I_{SC} in EDC and LDC from wildtype and *Clcn2*$^{-/-}$ mice. **D.** Transepithelial resistance values for EDC and LDC from wildtype and *Clcn2*$^{-/-}$ mice before addition of amiloride. Indomethacin (5 μM) was present in the serosal side. Values are given as mean ± SEM from n=10 per experimental condition, except for *Clcn2*$^{-/-}$ EDC (n=9). (*), $p<0.05$, *t* test. (**), $p<0.01$, *t* test between different and same genotypes, respectively.

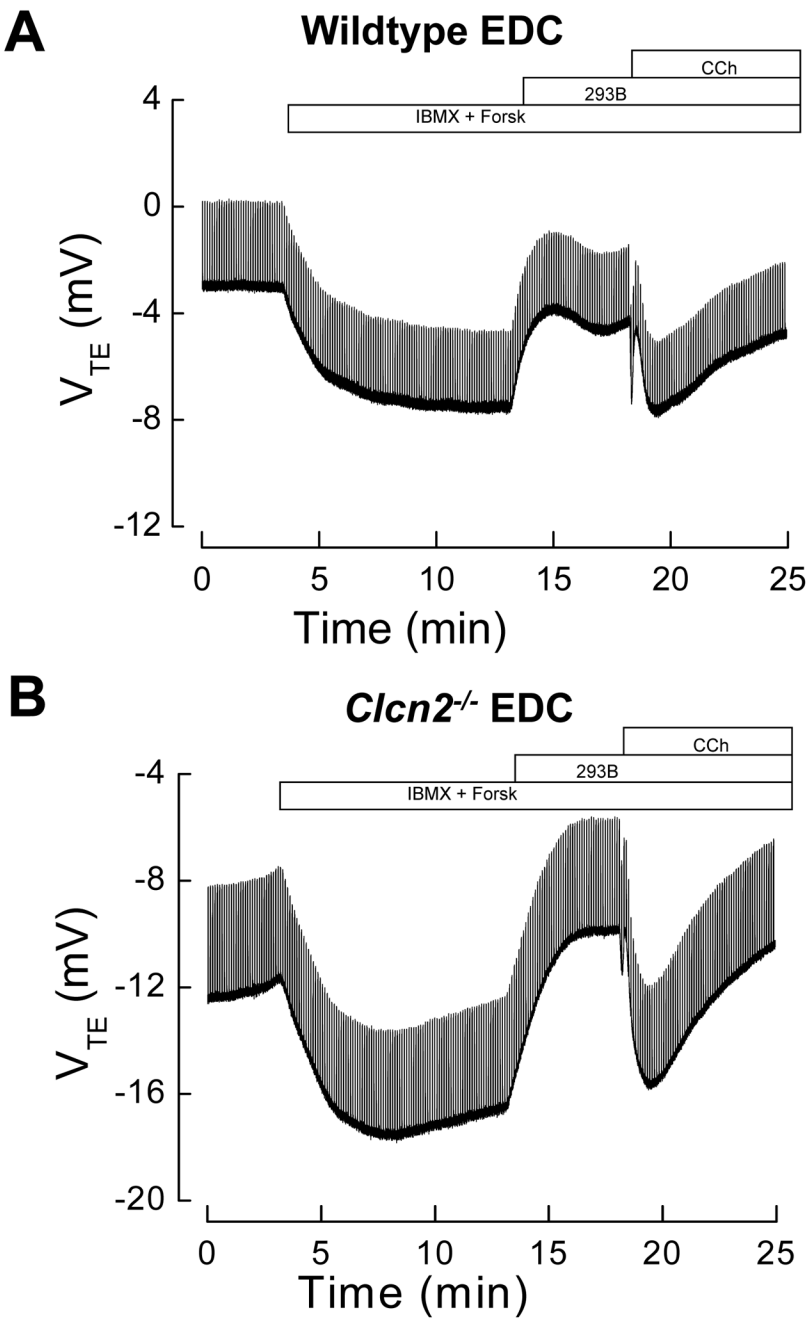

Figure 2. cAMP- and Ca^{2+}-activated Cl^- secretion in EDC from wildtype and *Clcn2*$^{-/-}$ mice

Cl^- secretion was elicited by stimulating an increase in cAMP (100 μM IBMX +10 μM Forsk) and Ca^{2+} (50 μM CCh) in wildtype EDC (**Panel A**, n=10) and *Clcn2*$^{-/-}$ EDC (**Panel B**, n=9). Before addition of CCh, 10 μM chromanol 293B was added to the serosal side to block basolateral cAMP-activated K^+ channels, and consequently, cAMP-induced Cl^- secretion. Indomethacin (5 μM) was present in the serosal side. Representative experiments are shown in panels A and B.

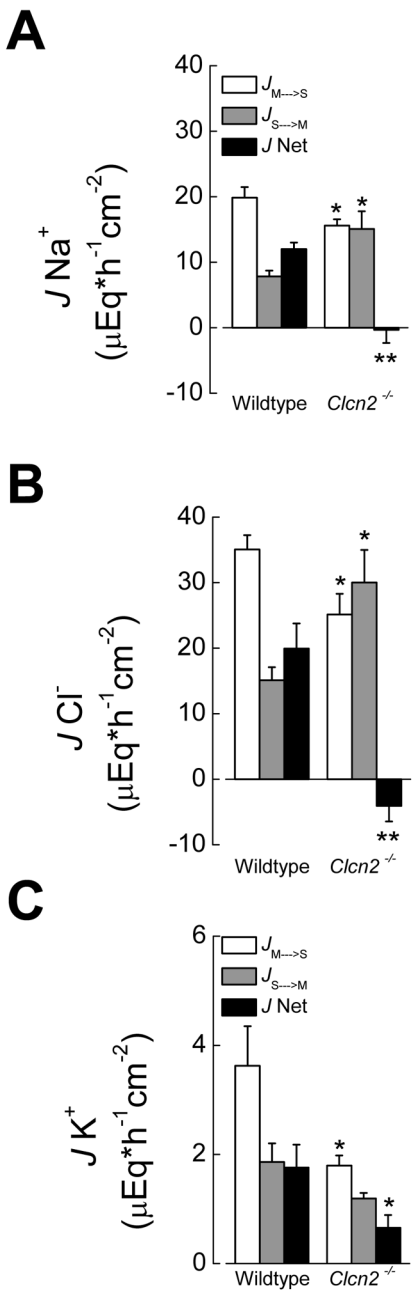

Figure 3. Unidirectional and net Na^+, Cl^- and K^+ fluxes in wildtype and *Clcn2*$^{-/-}$ EDC

J_{MS}, J_{SM} *and* J_{net} for Na^+ **(A),** Cl^- **(B)** and K^+ **(C)** fluxes studies performed under short circuit conditions. Values are given as mean ± SEM; comparison of the same condition between wildtype and *Clcn2*$^{-/-}$ mice where (*), $p<0.05$, and (**), $p<0.01$, *t* test. Amiloride (10 μM) was present in the apical side and 1 μM tetrodotoxin in the basolateral side.

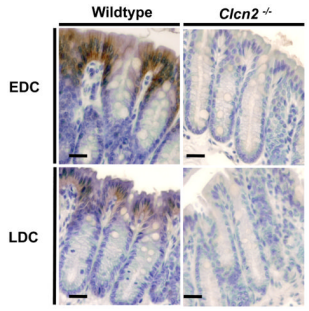

Figure 4. ClC-2 channels are expressed in the mouse distal colon surface epithelium
Immunolocalization studies performed in EDC (upper panels) and LDC (lower panels) from wildtype (left panels) and *Clcn2*$^{-/-}$ (right panels) mice. Bars = 50 μm.

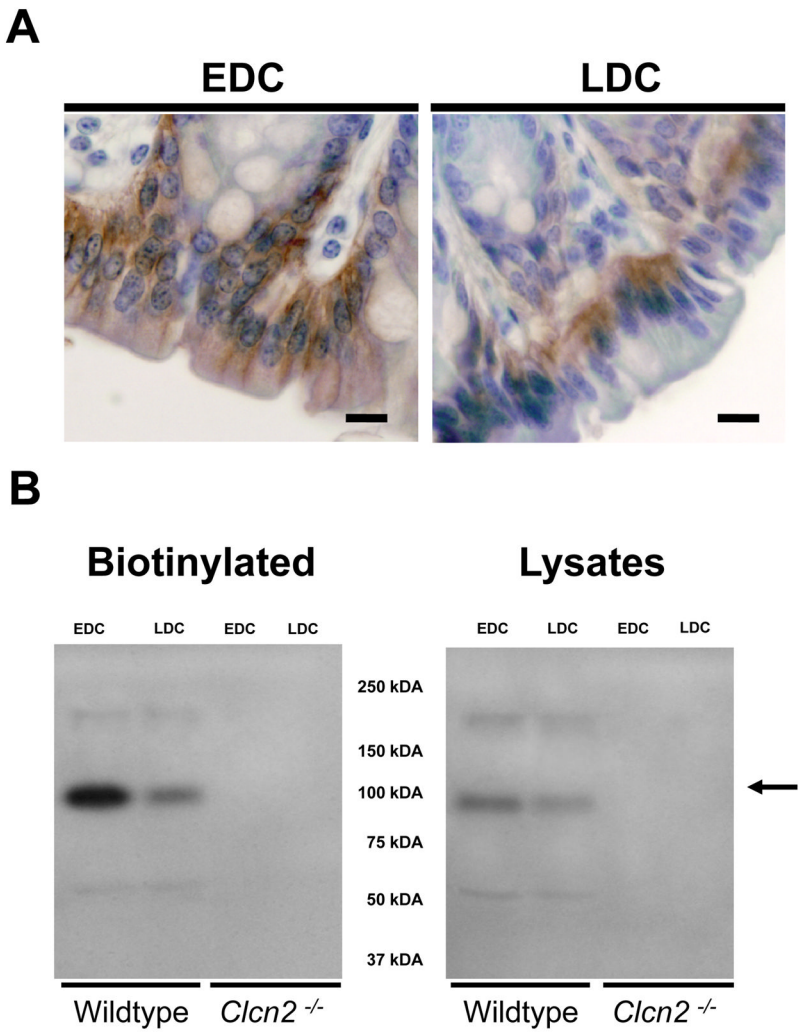

Figure 5. Differential cellular expression of ClC-2 along the mouse distal colon

A. Higher magnification of the surface epithelium from wildtype EDC (left panel) and LDC (right panel). Bars = 20 μm. **B.** Western blot assays for ClC-2 protein abundance using biotinylated plasma membrane (left panel) and whole cell lysates (right panel) in the EDC and LDC from wildtype and *Clcn2*$^{-/-}$ mice.

Table 1

	Wildtype		Clcn2−/−	
	EDC	**LDC**	**EDC**	**LDC**
Basal I_{SC} ($\mu Eq*h^{-1}cm^{-2}$)	−7.0 ± 1.3 (n=10)	−4.7 ± 0.8 (n=10)	−9.7 ± 0.9 (n=9)	−5.1 ± 1.0** (n=10)
Δ $I_{IBMX+Forsk}$ ($\mu Eq*h^{-1}cm^{-2}$)	−6.0 ± 0.5 (n=10)	−8.7 ± 0.6** (n=10)	−6.2 ± 1.5 (n=9)	−11.6 ± 3.4 (n=10)
Δ I_{293B} ($\mu Eq*h^{-1}cm^{-2}$)	5.3 ± 0.9 (n=10)	5.8 ± 0.7 (n=10)	6.3 ± 1.1 (n=9)	7.5 ± 2.4 (n=10)
Δ I_{CCh} ($\mu Eq*h^{-1}cm^{-2}$)	−9.5 ± 1.9 (n=10)	−11.2 ± 1.0 (n=10)	−7.1 ± 1.7 (n=9)	−12.3 ± 3.6 (n=10)

ΔI_{SC} values for Ussing chamber experiments. Summary of Ussing chamber experiments performed on EDC and LDC from wildtype and *Clcn2*$^{-/-}$ mice. Data are means ± SEM (**), p <0.01 between EDC and LDC in the same genotype, respectively (*t* test).