ClC-2 regulates mucosal barrier function associated with structural changes to the villus and epithelial tight junction

Prashant K. Nighot and Anthony T. Blikslager
Department of Clinical Sciences, North Carolina State University, Raleigh, North Carolina

Submitted 23 December 2009; accepted in final form 19 May 2010

Nighot PK, Blikslager AT. ClC-2 regulates mucosal barrier function associated with structural changes to the villus and epithelial tight junction. Am J Physiol Gastrointest Liver Physiol 299: G449–G456, 2010. First published May 20, 2010; doi:10.1152/ajpgi.00520.2009.—We have previously shown an important role of the chloride channel ClC-2 in orchestrating repair of tight junctions in ischemia-injured mucosa. In this study, we examined the role of ClC-2 in regulating barrier function of normal murine intestinal mucosa. Ex vivo, ClC-2−/− ileal mucosa mounted in Ussing chambers had significantly higher transepithelial electrical resistance (TER) and reduced [3H]mannitol mucosal-to-serosal flux compared with wild-type (WT) mouse mucosa. We also noted that ileum from ClC-2−/− mice had a significantly reduced in vivo [3H]mannitol blood-to-lumen clearance compared with WT animals. By scanning electron microscopy, flat leaflike villi were found to have tapering, rounded apical tips in ClC-2−/− mucosa. By transmission electron microscopy, the apical intercellular tight junctions in ClC-2−/− intestine revealed lateral membranes that were less well defined but closely aligned compared with electron-dense and closely apposed tight junctions in WT mucosa. The width of apical tight junctions was significantly reduced in ClC-2−/− intestine. Such an alteration in tight junction ultrastructure was also noted in the testicular tissue from CIC-2−/− mice. The CIC-2−/− intestinal mucosa had reduced expression of phospho-myosin light chain (MLC), and inhibition of myosin light chain kinase (MLCK) in WT mucosa partially increased TER toward the TER in CIC-2−/− intestine. Contrary to our prior work on the reparative role of ClC-2 in injured mucosa, this study indicates that CIC-2 reduces barrier function in normal mucosa. The mechanisms underlying these differing roles are not entirely clear, although ultrastructural morphology of tight junctions and MLCK appear to be important to the function of CIC-2 in normal mucosa.

myosin light chain kinase; chloride channel; mannitol flux

THE INTESTINAL EPITHELIUM provides a vital interface between the host and the luminal environment, and at this strategic location it serves physiologically complex but highly regulated functions. These functions are mucosal transport and maintenance of barrier function. The loss of intestinal barrier function contributes to the pathophysiology of important intestinal diseases such as inflammatory bowel disease, celiac disease, ischemic disease, and graft-versus-host disease (32). The apical intercellular tight junctions are largely responsible for barrier function (3). Tight junctions consist of an array of membrane-spanning proteins (e.g., occludin and claudins) linked by cytoplasmic plaque proteins to the cytoskeleton. Tight junctions are dynamic structures that can be regulated during solute absorption to alter barrier function (34–36). Besides controlling paracellular permeability, tight junctions are also considered to be a signaling platform for cellular processes including cell polarity and differentiation (30). The groups of proteins that functionally interact with tight junction components include actin cytoskeletal elements, kinases and phosphatases, and intracellular trafficking apparatus.

Recently, ion transporters and ion channels have also been shown to interact with tight junctions. For example, Na⁺-K⁺-ATPase, the sodium-glucose cotransporter SGLT-1, sodium/hydrogen exchangers (NHEs), and the chloride channels cystic fibrosis transmembrane conductance regulator (CFTR) and ClC-2 have been shown to regulate tight junction function (27). Previously, we demonstrated evidence for an important role of ClC-2 in the recovery of epithelial barrier function by orchestrating repair of apical tight junctions (19, 20, 26). Specifically, we showed that ClC-2-deficient mice have impaired epithelial barrier recovery that is independent of epithelial restitution (26). However, during these studies, we also noticed a reduction in barrier function in uninjured CIC-2−/− tissues compared with wild-type (WT) tissues, suggesting differing functions of ClC-2 in normal versus injured tissues.

The ClC-2 channel is expressed in a variety of mammalian secretory epithelia and has been implicated in native chloride transport (2, 9, 12, 22, 31). A member of the CIC family of anion channels, ClC-2 is activated upon hyperpolarization, acidic extracellular pH, and osmotic cell swelling (11, 14, 31). ClC-2 channels serve organ- and tissue-specific functional roles, including inhibitory GABA responses in neurons and gastric chloride secretion (25, 29). ClC-2-deficient mice suffer from progressive retinal and testicular degeneration and recently have been shown to develop leukoencephalopathy in the form of widespread progressive vacuolation in the white matter of the brain and spinal cord without alteration in seizure thresholds (5, 6).

In murine small intestine, ClC-2 protein is predominantly expressed at the apical tight junction, and this localization is thought to be advantageous for innate ClC-2 activity as well as its regulatory interactions with signaling molecules in the tight junction complex (12, 15, 22, 26). However, the regulatory role of ClC-2 within the tight junction is not fully understood, and while it appears to be crucial for mucosal junctional repair, the increase in barrier function in the absence of ClC-2 in preliminary studies is intriguing. Therefore, the objective of the present experiments was to investigate the role of ClC-2 in uninjured murine intestinal mucosa.

METHODS

Experimental animals. Studies were approved by the North Carolina State University Institutional Animal Care and Use Committee. Breeding pairs of heterozygous mice (ClC-2+/−) were a kind gift of Dr. James E. Melvin (University of Rochester, Rochester, NY). ClC-2-null (ClC-2−/−) and WT (ClC-2+/+) mice 2 wk or ~3 mo of age were used. The generation of ClC-2 knockout (KO) mice is described elsewhere (24). All mice were maintained on a standard diet. The experimental protocol was approved by the institutional animal care and use committee.

Address for reprint requests and other correspondence: A. T. Blikslager, Dept. of Clinical Sciences, North Carolina State Univ., 4700 Hillsborough St., Raleigh, NC 27606 (e-mail: anthony_blikslager@ncsu.edu).
laboratory diet. The progeny mice were genotyped by PCR using primers specific for amplification of either intact or disrupted murine ClC-2 alleles. For amplification of WT and disrupted alleles, two discriminating forward primers (WT ClC-2: 5’-ATG TAT GCC CGG TAC ACT CAG GAA CTC-3’; disrupted ClC-2: 5’-CCT GAA AGG TGC CAC TCC CAT TGT CC-3’) and a reverse primer (5’-ACA CCC AGG TCG CCC AAA TCT GG-3’) were used simultaneously in PCR to yield a WT ClC-2 product of 200 bp and a disrupted ClC-2 product of 300 bp. PCR was carried out with a Bio-Rad iCycler and a 25-µl reaction volume of Promega GoTaq Green Mastermix, according to the manufacturer’s instructions. The final concentration of primers was 2.5 µM each for the WT and disrupted ClC-2 forward primers and 5 µM for the reverse primer. PCR was performed at the following temperature profiles: initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s. The total number of cycles was 35, followed by a final extension for 2 min at 72°C. PCR products were separated by agarose gel electrophoresis and visualized under UV light after staining with ethidium bromide.

**Ussing chamber experiments.** Ileal tissues were harvested immediately after euthanasia, cut longitudinally, and placed on 0.12-cm²-aperture Ussing chambers (21). Tissues were bathed on the serosal and mucosal sides with Ringer solution. The serosal bathing solution contained 10 mM glucose, which was osmotically balanced on the mucosal side with 10 mM mannitol. Bathing solutions were oxygenated (95% O₂-5% CO₂) and circulated in water-jacketed reservoirs by lead citrate, and examined with a transmission electron microscope (Phillips/FEICO model 208s; Hillsboro, OR). For scanning electron microscopy, tissues were excised and placed in McDowell and Trump 4F:1G fixative at 1°C for 1 h, followed by 6 h in 10% neutral buffered saline (pH 7.4), followed by 0.2 ml of buffered saline containing [³H]mannitol (10 µCi/ml) in the tail vein. Time zero and 1.5-h blood samples (0.3 ml) were collected via retroorbital bleeding into heparinized vials. After euthanasia, perfusates were collected from intestinal loops. The plasma and intestinal perfusate samples were assessed for β-emission with a scintillation beta counter, and the [³H]mannitol clearance was calculated as described previously (21, 26).

**Histological and electron microscopic examination.** Tissues from ileal intestinal segments were collected in 10% neutral buffered formalin for histological evaluation. Tissues were sectioned (5 µm) and stained with hematoxylin and eosin. For electron microscopy, ileal tissues were fixed in McDowell and Trump 4F:1G fixative and processed for transmission electron microscopy by standard techniques (10). In brief, after two rinses in 0.1 M sodium phosphate buffer (pH 7.2), samples were placed in 1% osmium tetroxide in the same buffer for 1 h at room temperature. Samples were rinsed twice in distilled water and dehydrated in an ethanol series culminating in two changes of 100% acetone. Tissues were then placed in a mixture of Spurr resin and acetone for 30 min, followed by 2 h in 100% resin with two changes. Finally, samples were placed in fresh 100% resin in molds and polymerized at 70°C for 8 h to 3 days. Semithin (0.25–0.5 µm) sections were cut with glass knives and stained with 1% toluidine blue-O in 1% sodium borate. Ultrathin (70–90 nm) sections were cut with a diamond knife, stained with methanolic uranyl acetate followed by lead citrate, and examined with a transmission electron microscope (Phillips/FEICO model 208s; Hillsboro, OR). For scanning electron microscopy, tissues were excised and placed in McDowell and Trump 4F:1G (1) fixative for a minimum of 1 h at 4°C. After two rinses in 0.1 M sodium phosphate buffer (pH 7.2), samples were dehydrated in a graded ethanol series to 100% ethanol, at which time they were critical point dried with liquid CO₂. Finally, they were mounted on specimen stubs with colloidal silver, sputter-coated with gold-palladium, and examined with a JOEL JSM-6360LV scanning electron microscope. Transmission electron microscopic examination and imaging were carried out in a blinded fashion by an individual not directly associated with the study (Abbey G. Wood, Coordinator, Laboratory for Advanced Electron and Light Optical Methods, North Carolina State University). Sections were taken from n ≥ 4 mice, and three separate measurements were taken across the width of each tight junction. These measurements were averaged before developing a single width measurement for each tight junction. For morphometric analysis, the images were processed through Sigmascan Pro 5.0 (Systat Software).

**Transcellular fluxes of horseradish peroxidase.** In additional Ussing chamber experiments, transcellular transport was assessed by the mucosal-to-serosal flux of horseradish peroxidase (HRP, type II, Sigma Chemical) (4, 7). After a 15-min equilibration period, 4.5 × 10⁻⁵ M HRP was added to the mucosal bathing solution. Subsequently, two consecutive 1-h flux periods were conducted, at the end of which 0.5-ml samples were collected from the serosal bathing solution. The flux of intact HRP across the intestine was determined by a kinetic assay using 0.003% H₂O₂ and 0.009% o-dianisidine di-HCl (Sigma Chemical) as described previously (4, 7).

**In vivo blood-to-lumen [³H]mannitol clearance.** Experimental surgery, preparation of intestinal segments, and blood-to-lumen [³H]mannitol flux were performed as reported in our previous studies (21, 26). Briefly, anesthetized mice were subjected to midline laparotomy. The ileal segments were ligated with 4-0 silk and injected with 0.25 ml of buffered saline (pH 7.4), followed by 0.2 ml of buffered saline containing [³H]mannitol (10 µCi/ml) into the tail vein. Time zero and 1.5-h blood samples (0.3 ml) were collected via retroorbital bleeding into heparinized vials. After euthanasia, perfusates were collected from intestinal loops. The plasma and intestinal perfusate samples were assessed for β-emission with a scintillation beta counter, and the [³H]mannitol clearance was calculated as described previously (21, 26).

**Immunofluorescence and confocal microscopy.** Intestinal tissues were embedded in OCT medium (Tissue Tek, Sakura, Torrance, CA), and visualized by confocal microscopy (Zeiss LSM 410 confocal microscope; Carl Zeiss, Thornwood, NY). All images were acquired in the absence of primary and secondary antibodies, and isotype-matched controls were included in each experiment. For each experimental condition, three representative mice were used.

**Immunofluorescence and confocal microscopy.** Intestinal tissues were embedded in OCT medium (Tissue Tek, Sakura, Torrance, CA), and visualized by confocal microscopy (Zeiss LSM 410 confocal microscope; Carl Zeiss, Thornwood, NY). All images were acquired in the absence of primary and secondary antibodies, and isotype-matched controls were included in each experiment. For each experimental condition, three representative mice were used.
frozen, sectioned at 5 μm, and stored at −80°C until use. The sections were thawed, fixed in cold acetone, and blocked with normal goat serum. The sections were incubated with mouse anti-occludin, mouse anti-claudin-1, and -2, mouse anti-zonula occludens-1 (ZO-1) (Zymed, San Francisco, CA), and/or polyclonal ClC-2 (Alpha Diagnostics, San Antonio, TX) or rabbit phospho-MLC2 (Cell Signaling Technology, Danvers, MA) antibodies diluted in 2% normal goat serum. When used, phalloidin-Alexa Fluor 546 conjugate and TO-PRO-3 (Invitrogen) antibodies diluted in 5% normal goat serum. When used, a Nikon Eclipse 2000E inverted microscope equipped with the Nikon C1 confocal laser scanning system.

Statistical analysis. Data are reported as means ± SE. Whenever needed, data were analyzed by using an ANOVA for repeated measures (Sigmasstat). A Tukey’s test was used to determine differences between treatments after ANOVA (P < 0.05).

RESULTS

Barrier function in ClC-2−/− small intestine. Barrier function of the ClC-2−/− small intestine was studied ex vivo by mounting the ileal portion of the small intestine in Ussing chambers and by in vivo blood-to-lumen clearance of the paracellular probe mannitol. In Ussing chamber studies, the TER in the ClC-2−/− small intestine was significantly increased compared with the WT small intestine (96.00 ± 15.13 and 50.51 ± 6.87 Ω·cm² in ClC-2−/− and WT small intestine, respectively; P < 0.01) (Fig. 1A). Since TER in the small intestine is principally attributed to paracellular permeability, mucosal-to-serosal fluxes of [3H]mannitol were conducted to study paracellular permeability. Consistent with increases in TER, mucosal-to-serosal [3H]mannitol flux was significantly reduced in the ClC-2−/− small intestine (0.11 ± 0.02 and 0.55 ± 0.11 μm·cm⁻²·h⁻¹ in ClC-2−/− and WT small intestine, respectively; P < 0.01) (Fig. 1B). Similarly, serosal-to-mucosal [3H]mannitol fluxes were also found to be significantly reduced in ClC-2−/− small intestine compared with WT small intestine (data not shown). In vivo intestinal permeability was assessed by blood-to-lumen clearance of [3H]mannitol for a period of 1.5 h, as described in METHODS. In ClC-2−/− small intestine, in vivo blood-to-lumen clearance of [3H]mannitol was found to be significantly lower compared with WT small intestine (0.17 ± 0.02 and 0.06 ± 0.01 mll/h × 100 g for WT and ClC-2−/− small intestine, respectively, P < 0.01).
after 1.5 h; \( P < 0.01 \) (Fig. 1D). Thus both in vivo and ex vivo data indicated that the ClC-2/−/− small intestine has enhanced barrier function compared with WT small intestine. In additional experiments, transcellular passage of HRP was studied in the WT and ClC-2 KO intestine (Fig. 1C). No significant differences were found in mucosal-to-serosal fluxes of HRP in WT versus ClC-2 KO mice, suggesting that the difference in TER is attributable principally to paracellular resistance.

Morphological examination of ClC-2/−/− small intestine. There are no previous reports on morphological changes in the intestine of ClC-2/−/− mice. We found no differences in the villous height, villous width, number of villi per unit length, and depth of crypts in ClC-2/−/− mouse mucosa at the histological level. To find out whether there were any ultrastructural changes related to the altered barrier function in ClC-2/−/− small intestine, we carried out electron microscopy. By scanning electron microscopy, the shape of ileal villi of ClC-2/−/− intestine was notably different from WT mucosa (Fig. 2). In particular, the leaflike ClC-2/−/− villi appeared to have more transverse furrows and tapering, rounded tips compared with the flattened apical tips in WT villi. This morphological alteration in ClC-2/−/− intestine made the base of the villi more readily visible when viewed from the apical surface in the ClC-2/−/− intestine, unlike WT intestine (Fig. 2A). Consequently, the ratio of measurements of length to breadth on the apical surface of villi differed significantly between ClC-2/−/− and WT intestine (3.37 ± 0.08 and 2.01 ± 0.05 for ClC-2/−/− and WT villi, respectively; \( P < 0.001 \) (Fig. 2B). By transmission electron microscopy, ClC-2/−/− and WT enterocytes had comparable appearance of the brush border (microvilli) and cell size, but the lateral intercellular spaces were found to be dilated in ClC-2/−/− intestine (Fig. 3).

Furthermore, in ClC-2/−/− intestine, the apical intercellular tight junctions revealed lateral membranes that were less well defined and poorly apposed compared with dense, well-defined, and closely apposed lateral membranes in the tight
junctions of WT intestine (Fig. 4A). Morphometric assessment of the tight junctions revealed that the width of apical tight junctions was reduced by ~50% in CIC-2−/− intestine (6.8 ± 0.33 and 13.84 ± 1.04 nm for CIC-2−/− and WT intestine, respectively; \( P < 0.001 \)) (Fig. 4B).

Analysis of tight junction protein and myosin light chain in CIC-2−/− intestine. In light of the alterations in barrier function and ultrastructural morphology of tight junctions in CIC-2−/− intestine, we carried out analyses of the tight junction integral proteins occludin, claudin-1, and claudin-2 (a pore-forming protein) and the cytoplasmic plaque protein ZO-1 by immunoblotting and immunolocalization. The total expression of occludin, claudin-1, claudin-2, and ZO-1 in whole mucosal lysates was not different in CIC-2−/− mucosa compared with WT mucosa (data not shown). We did not find significant differences in the expression of occludin, claudin-1, or claudin-2 in the detergent-insoluble fractions collected from WT and CIC-2−/− intestine (data not shown). In addition, the immunolocalization of all the above proteins was found to be comparable in WT and CIC-2−/− intestine by confocal immunofluorescence (data not shown).

Barrier function is known to be regulated by myosin II regulatory light chain (MLC) phosphorylation (32, 35). In view of the altered barrier function in CIC-2−/− mice, we questioned the possible role of this protein. In addition, we had identified an association of MLCK with CIC-2 by mass spectroscopy (data not shown). Confocal immunofluorescence revealed the presence of phospho-MLC at the tight junction complex of WT intestinal epithelium. This phospho-MLC immunofluorescence was seen at the tip as well as the base of the villi (Fig. 5, A–D). Alternatively, in CIC-2−/− intestine, immunofluorescence of phospho-MLC appeared to be reduced compared with WT intestine (Fig. 5, E and F). In light of the reduced presence of phospho-MLC in the CIC-2−/− intestine, we tested whether inhibition of MLCK would raise barrier function in the WT intestine to levels comparable to CIC-2−/− intestine. WT and CIC-2−/− intestine were mounted in Ussing chambers in the presence of a selective MLCK peptide inhibitor. Inhibition of MLCK in WT intestine led to a significant increase in TER over untreated control tissue. This percent increase was significantly greater that that observed in CIC-2−/− intestine (~30% and ~12% increase in TER in MLCK-inhibited WT and CIC-2−/− tissues over untreated tissues; Fig. 6A; \( P < 0.01 \)). By Western blot analyses of the intestinal tissues collected at the end of the Ussing chamber experiments, relative expression of phospho-MLC compared with total MLC was found to be lower in control CIC-2−/− intestine compared with control WT intestine (Fig. 6A). Furthermore, treatment with the MLCK inhibitor peptide 18 resulted in reduction in phospho-MLC expression in both WT and CIC-2−/− intestine (Fig. 6A). Similarly, mucosal-to-serosal mannitol flux was reduced by 44.12 ± 2.11% after MLCK inhibition in WT intestine compared with 28.78 ± 3.60% reduction in CIC-2−/− intestine (Fig. 6B; \( P < 0.05 \)).

Effect of pharmacological inhibition of CIC-2 on small intestinal electrophysiology. In further experiments, we wanted to examine whether pharmacological inhibition of CIC-2 would also result in increases in TER. WT and CIC-2−/− ileal mucosae were mounted on Ussing chambers and treated with the CIC-2 inhibitor ZnCl₂ (300 μM). After 1.5 and 3 h of continuous treatment, WT untreated control intestine revealed a significantly lower TER compared with WT intestine treated with ZnCl₂ (Fig. 7; \( P < 0.05 \)). Unlike WT small intestine, ZnCl₂ treatment did not significantly alter TER in CIC-2−/− small intestine.

Tight junction morphology and CIC-2−/− phenotype. Among the major phenotypes reported in CIC-2−/− mice are testicular and retinal degeneration at an early age (6). In view of the alteration in tight junction morphology in the intestine, we were interested in knowing whether similar changes could be detected in other organs in which CIC-2−/− phenotypes are reported. Therefore, we examined tight junction ultrastructure in the testis of CIC-2−/− mice at 2 wk of age. Examination of seminiferous tubules revealed mild disorganization of germinal epithelium and a complete lack of lumina in the seminiferous tubules of CIC-2−/− mice compared with WT mice (data not shown). By transmission electron microscopy, the tight junctions between Sertoli cells in the seminiferous tubules of WT testes had well-defined, apposed lateral membranes; however, in CIC-2−/− mice, the Sertoli cell tight junctions had lateral membranes that were closely aligned (Fig. 8). In addition, the small intestine of these 2-wk-old pups showed alterations in the
significantly elevated barrier function compared with WT junction, is the overriding factor in normal tissue, whereas P

In the present study, we report an important role of ClC-2 in intestinal mucosal barrier function. In both in vivo and ex vivo experiments, we found that ClC-2/− intestine had significantly elevated barrier function compared with WT small intestine in terms of elevated TER and reduced paracellular permeability to [3H]mannitol. This is in contrast to prior studies in which we showed a beneficial effect of ClC-2 on recovery of small intestinal barrier function in ischemia-injured jejunum. The mechanisms for these differences are not clear, although it is possible that the Cl− secretory function of ClC-2, which would be expected to increase conductance of the tight junction, is the overriding factor in normal tissue, whereas injured tissues may use ClC-2 as a protein platform that contributes to reconstruction of tight junctions in injured tissues, and this effect has a more prominent role in barrier function in injured tissue. Nonetheless, this is highly speculative at this time until further studies can be performed to compare the function of normal and injured intestine.

The correlation between TER and paracellular permeability to mannitol has been documented previously (16, 17). Interestingly, there were no notable histological differences between WT and ClC-2/− small intestine. However, scanning electron microscopy revealed altered villous morphology in ClC-2/− small intestine. The villi in ClC-2/− small intestine had round apical tips compared with flat WT villi. The mechanism by which these alterations affect barrier function is unclear, although they suggest that the villi and other ultrastructural components of the mucosa, most notably the tight junctions, are responsible for the mechanism of the effect of ClC-2 on barrier function. In line with this notion, and because ClC-2/− intestine had reduced paracellular permeability, we focused on the ultrastructure of apical tight junctions. Close examination by transmission electron microscopy indicated that the tight junctions of ClC-2/− small intestine appeared to be poorly defined. Alternatively, WT tight junctions were closely apposed, and the structure of these junctions appeared to be well defined. Furthermore, morphometric observations revealed that the width of tight junctions in ClC-2/− small intestine was ~50% reduced compared with WT tight junctions. These ultrastructural changes in the tight junction appear to be responsible for the altered barrier function in ClC-2/− small intestine. There are not many reports exploring the ultrastructure of tight junctions and its relation to alterations in paracellular permeability, but a dynamic relation between tight junction membrane contact points and tight junction permeability in K+ repletion experiments in cultured human retinal pigment epithelial cells has been reported previously (28).

An interesting observation concerning the influence of ClC-2 on tight junction morphology can be drawn from the work of Bosl et al. (6), where ClC-2/− mice were reported to have round apical tips compared with flat WT villi. The mechanisms for these differences are not clear, although it is possible that the Cl− secretory function of ClC-2, which would be expected to increase conductance of the tight junction, is the overriding factor in normal tissue, whereas ClC-2 on barrier function. In line with this notion, and because ClC-2/− intestine had reduced paracellular permeability, we focused on the ultrastructure of apical tight junctions. Close examination by transmission electron microscopy indicated that the tight junctions of ClC-2/− small intestine appeared to be poorly defined. Alternatively, WT tight junctions were closely apposed, and the structure of these junctions appeared to be well defined. Furthermore, morphometric observations revealed that the width of tight junctions in ClC-2/− small intestine was ~50% reduced compared with WT tight junctions. These ultrastructural changes in the tight junction appear to be responsible for the altered barrier function in ClC-2/− small intestine. There are not many reports exploring the ultrastructure of tight junctions and its relation to alterations in paracellular permeability, but a dynamic relation between tight junction membrane contact points and tight junction permeability in K+ repletion experiments in cultured human retinal pigment epithelial cells has been reported previously (28).

An interesting observation concerning the influence of ClC-2 on tight junction morphology can be drawn from the work of Bosl et al. (6), where ClC-2/− mice were reported to have round apical tips compared with flat WT villi. The mechanisms for these differences are not clear, although it is possible that the Cl− secretory function of ClC-2, which would be expected to increase conductance of the tight junction, is the overriding factor in normal tissue, whereas...
to have testicular and retinal degeneration. Both tissues have epithelium that forms a blood-organ barrier, suggesting a role of ClC-2 in ionic homeostasis. In testicular tissues, ClC-2 is expressed on the membrane of Sertoli cells that supports germ cells and mediates inwardly rectifying Cl⁻ currents (6). We wanted to determine whether, similar to the tight junctions in ClC-2−/− intestinal epithelium, the morphology of tight junctions between the Sertoli cells in ClC-2−/− testis is altered. Indeed, at 2 wk of age, when testicular degeneration starts in ClC-2−/− mice, the tight junctions between Sertoli cells were found to have very closely aligned lateral membranes compared with WT testis. Besides forming a blood-testis barrier, the tight junctions between Sertoli cells dynamically regulate movement of germ cells. More specifically, they must open up to allow the passage of spermatogonia from the basement membrane of seminiferous tubule to the adluminal surface (23). Thus it is possible that functional and structural alteration in the tight junctions, in the absence of ClC-2, might be a primary or contributing reason for testicular degeneration and absence of mature sperm cells in the adluminal compartment of seminiferous tubules.

In further work, we investigated the composition of tight junctions in ClC-2−/− small intestine by analyzing select proteins, namely occludin, claudin-1, claudin-2, and ZO-1, by Western blotting and immunofluorescence. Although there were no differences in the total expression and immunolocalization of these select proteins in the WT and ClC-2−/− intestine, these techniques may not have been adequate to detect small alterations. On the other hand, ClC-2−/− intestine was found to have reduced expression of phospho-MLC. MLCK-mediated phosphorylation of MLC is known to induce tight junction permeability, and inhibition of MLCK with a cell-permeant peptide inhibitor reduces tight junction permeability under physiological conditions as well as within a pathophysiological setting (8, 32, 37). In accordance with the reduced expression of phospho-MLC in ClC-2−/− intestine, WT intestine displayed comparatively larger increases in TER and reductions in mannitol fluxes in response to MLCK inhibition. Compared with approximately twofold differences in baseline TER from ClC-2−/− intestine (Fig. 1A), inhibition of MLCK in WT intestine resulted in ~30% increase in TER, negating some but not all of the differences in the TER. The mechanistic link between ClC-2 and phosphorylation of MLC is not clear at this point. Phosphorylation of MLC leading to subsequent contraction of actin-myosin bundles has been shown to be crucial for apical Cl⁻ efflux resulting from cAMP-mediated activation (13). Although the importance of cytoskeletal remodeling in response to intracellular Cl⁻ concentrations has been demonstrated previously (18), this information is largely attributable to cAMP-activated CFTR. Previous in vitro studies have shown that disruption of the actin cytoskeleton activates channel activity of ClC-2 based on an inhibitory interaction of actin with the NH₂ terminus of ClC-2 (1).

We investigated whether pharmacological inhibition of ClC-2 mimics the changes in intestinal barrier function. The results of this series of experiments revealed increases in barrier function in response to pharmacological inhibition of ClC-2 with ZnCl₂. Based on these experiments, although the role of the secretory activity of ClC-2 in barrier modulation could not be ruled out, it is possible that pharmacological inhibition of ClC-2 might also inhibit its interaction with other signaling molecules.

Overall, this study demonstrates functional and morphological alterations in the tight junction barrier of the intestinal mucosa of ClC-2−/− mice. In a previous study focused on postischemic barrier recovery we reported impaired barrier recovery and repair of tight junctions in ClC-2−/− mice (26). In view of our present findings of reduced baseline paracellular permeability and altered tight junction morphology in ClC-2−/− mice, the role of ClC-2 in mediating barrier function appears complex, and different between normal and injured mucosa. Moreover, it is not known, despite higher transepithelial resistance, whether the ClC-2−/− intestine is more susceptible to the injury. Although the basis of ClC-2 mediation of barrier function remains unclear, the link with MLCK demonstrates active signaling mechanisms involved in the role of ClC-2 in intestinal barrier function. Further studies are needed to elucidate interactions of ClC-2 with signaling and structural molecules at the tight junction to fully explain our findings.

ACKNOWLEDGMENTS

The authors thank Dr. Jerrold Turner, Dept. of Pathology, University of Chicago, for his advice. The technical assistance provided by the coordination of the Laboratory for Advanced Electron and Light Optical Methods (LAE-LOM), Abbey G. Wood, Laboratory Animal Resources, North Carolina State University, and Sung H. Lee is appreciated.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
REFERENCES


