ClC-2 in guinea pig colon: mRNA, immunolabeling, and functional evidence for surface epithelium localization

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Received 26 April 2002; accepted in final form 23 May 2002

Catalán, Marcelo, Isabel Cornejo, Carlos D. Figueroa, Marí a Isabel Niemeyer, Francisco V. Sepúlveda, and L. Pablo Cid. ClC-2 in guinea pig colon: mRNA, immunolabeling, and functional evidence for surface epithelium localization. Am J Physiol Gastrointest Liver Physiol 283: G1004–G1013, 2002; 10.1152/ajpgi.00158.2002.—The principal function of the colon in fluid homeostasis is the absorption of NaCl and water. Apical membrane Na+/H+ channels, Na+/HCO3− and Cl-/HCO3− exchangers, have all been postulated to mediate NaCl entry into colonocytes. The identity of the basolateral exit pathway for Cl− is unknown. We have previously demonstrated the presence of the ClC-2 transcript in the guinea pig intestine. Now we explore in more detail, the previously demonstrated presence of the ClC-2 transcript in the guinea pig intestine. Now we explore in more detail, the

SMALL AND LARGE INTESTINES are sites of abundant fluid transport in mammalian species. In humans, ~10 liters/day of fluid are absorbed in these two segments of gut, most of it being absorbed in the small bowel with ~1,500 ml crossing the ileocecal valve. The colon extracts most of this fluid, leaving only 100–200 ml of water per day. The colon is also capable of increasing its absorptive capacity two- to threefold when the small bowel losses are increased. The result of ion transport is excretion of a stool containing <5 mM Na+, 2 mM Cl−, and 9 mM K+. This highly efficient transport of large amounts of salt and water is due to polarized colonic epithelial cells equipped with a number of ion channels, carriers, and pumps, located either on the luminal or basolateral aspects of the membrane. Several of these have been identified at the molecular level (26).

It has generally been accepted that secretion and absorption in the colon are spatially separated in the intestine surface crypt axis. Absorptive processes are located in surface epithelial cells, whereas the secretion would be a property of crypt epithelial cell (20). This model might require some modification, because there is recent evidence that NaCl absorption might also occur in colonic crypts (16). Crypt cells express abundant CFTR chloride channels located in the apical membrane of the colonocytes. This constitutes the rate-limiting step in the transepithelial translocation of sodium chloride under the action of secretagogues (17).

In distal colon epithelium, depending on species or salt status, the transport of NaCl is due to electroneutral absorption by luminal Na+/H+ and Cl-/HCO3− exchangers or electrogenic absorption via luminal epithelial Na+ channels (ENaC). In both cases, the participation of a chloride channel located in the basolateral membrane has been postulated (26). The only basolateral chloride conductances characterized so far in colonic epithelial cells are a 25–30 pS volume-sensitive Cl− channel rat colonic crypts (11) and an outwardly rectifying DIDS-sensitive Cl− channel of 20–90 pS conductance in mouse crypts (29). No molecular counterpart has been proposed for these channels.

ClC-2 belongs to the most numerous family of chloride channel proteins discovered so far. This ClC family consists of nine different mammalian members with sequence identity varying between 30 and 90% (21). ClC-2 is widely expressed in mammalian tissues, but its physiological role has not yet been ascertained. It has been suggested to be important in certain neurons, where ClC-2 is thought to be implicated in the control
of intracellular chloride to regulate the effects the GABA<sub>A</sub> receptor action (38). CIC-2 has also been identified in the human colonic T<sub>84</sub> epithelial cell line (5) where it was proposed to participate in fluid secretion not associated with the cAMP-dependent CFTR chloride channel (14). Similar speculations have been raised for other epithelial cells (4, 33, 35), but a function in transepithelial transport has not been clearly defined. Studies of CIC-2-deficient mice have recently shown a severe degeneration of the retina and the testes, which might be related to a deficient control of their ion environment by the blood-testes and blood-retina barriers (3).

CIC-2 shows low activity under resting conditions but opens slowly on hyperpolarization (39). When expressed in amphibian oocytes, CIC-2 can be activated by hypotonic cell swelling (18). This is consistent with a role in regulatory volume adjustments, but evidence against such function has also been reported (1). It is of great interest to understand the possible physiological role of CIC-2 and particularly its function in determining the membrane conductance of epithelial and other cells. The possibility of altering the gating of CIC-2 is relevant given its potential as an alternative to CFTR in the impaired secretory state of cystic fibrosis patients (30, 35).

We (6) previously demonstrated the presence of the CIC-2 transcript in the guinea pig small intestine and colon and speculated that it might play a role in trans-epithelial transport. Other groups have explored the possible function of CIC-2 in the epithelium of the gastrointestinal tract. Sherry et al. (36) reported the presence of CIC-2 in the canalicular membrane of gastric parietal cells where it would play a role in HCl secretion. Lipecka et al. (27) have used immunohistochemical techniques to suggest that the location of CIC-2 is predominantly basolateral in rat colon, but intracellular in human intestine, where it is seen mainly at a cytosolic supranuclear region. Gümörey et al. (19) have used murine small intestine to show that CIC-2 is expressed at the tight junction between epithelial cells where it colocalizes with zonula occludens-1 protein. Despite this unexpected finding, functional data suggest that a CIC-2-like channel activity contributes to Cl<sup>-</sup> secretion, compatible with a function as apical membrane conductance. A similar tight junctional location for CIC-2 has been reported by Mohammad-Panah et al. (30) in Caco-2 cells. In this colonic carcinoma cell line, they demonstrate hypotonicity-induced anion secretion (measured with I<sup>-</sup>) which is partially inhibited using a CIC-2 antisense approach.

The aim of this work is to explore, in more detail, the presence of CIC-2 in the colon by in situ hibridization and immunohistochemistry and to obtain evidence of its function by electrophysiological means. The results indicate that CIC-2 mRNA is preferentially expressed in surface epithelium of guinea pig distal colon and is absent from the crypt compartment. A similar result was obtained by immunocytochemistry that, in addition, suggests the CIC-2 is absent from the apical aspect of colonocytes but expressed at the basolateral membrane. Isolation of surface epithelial cells followed by patch-clamp study shows currents with the hallmark of recombinant guinea pig (gp)CIC-2. This includes Cl<sup>-</sup> > I<sup>-</sup> permeability, sensitivity to Cd<sup>2+</sup> and kinetics of opening and closing similar to that seen with recombinant gpCIC-2. Cellular location and subcellular distribution of CIC-2 in guinea pig colon suggest a function for this channel in the NaCl absorption process.

**MATERIALS AND METHODS**

**Cell and tissue isolation.** Male guinea pigs obtained from the Instituto de Salud Pública (Santiago, Chile) and weighing 200–400 g were used throughout the study. All experiments were done according to international regulations for animal care and were approved by the Bioethics Committee of the Centro de Estudios Científicos, Valdivia, Chile. Surface colonocytes were isolated by a modification of previously published methods (10). Food was withheld for 48 h before animals were killed by using ketamine overdose. A segment of distal colon ~8 cm in length was rinsed with warmed 0.9% sodium saline. The segment was filled with solution A containing (in mM) 44 K<sub>2</sub>HPO<sub>4</sub>, 7 K<sub>2</sub>SO<sub>4</sub>, 10 sodium citrate, 180 glucose, and 10 HEPES, pH 7.4. The segment was tied at both ends under moderate pressure and incubated in solution A at 37°C for 10 min. The intraluminal solution was then changed by solution B (in mM): 44 K<sub>2</sub>HPO<sub>4</sub>, 7 K<sub>2</sub>SO<sub>4</sub>, 10 Na<sub>2</sub>EDTA, 180 glucose, 0.5 DTT, and 10 mM HEPES, pH 7.4 and incubated for 3 min at 37°C. Surface epithelial cells were released by gentle mechanical disruption and recovered from the luminal solution. After isolation, the colonocytes were washed and suspended in solution A and maintained at 4°C. It has been demonstrated that the cell fraction used here is devoid of dividing cells as judged by bromodeoxiuridine labeling (10). In addition, the cells used for patch-clamp retained the characteristic “figure-of-eight” morphology of highly differentiated surface cells that is also observed in villus cells from small intestine (31). No release of crypts, which are easily identifiable morphologically, occurred during the rather mild procedure used here.

**In situ hibridization.** Nonradioactive in situ hybridization was carried out using digoxigenin-labeled riboprobes. Two pairs of antisense and sense probes of 250 and 300 bp, respectively, were synthesized from gpCIC-2-3’- and 5’-untranslated ends (GenBank accession no. AF113529). After plasmid linearization, in vitro transcription was performed with T7 and T3 RNA polymerases according to a previously published protocol (9). Cryosections (7-μm-thick) obtained from colon segments were fixed in 4% paraformaldehyde for 30 min at room temperature. The endogenous peroxidase was inactivated with H<sub>2</sub>O<sub>2</sub> at 0.3% in methanol. Subsequently, the sections were permeabized with 0.2% Triton X-100 in PBS and acetylated using 0.1 M triethanolamine, pH 8.0, plus 0.25% acetic anhydride. After 3-h prehybridization, the sections were incubated at 65°C overnight in a solution containing 50% formamide and a final mix of probes concentration of 0.2 ng/μl. After the hybridization, the samples were treated with 40 μg/ml RNAase at 37°C for 45 min. After washing with 2× SSC and 0.1× SSC for 15 min at 65°C twice each, the endogenous biotin activity was blocked (Biotin blocking reagent; DAKO). The slides were then incubated with an horseradish peroxidase-coupled anti-digoxigenin antibody diluted 1:100 for 45 min at room temperature. Sections were incubated with biotinyl-tyramide and with...
streptavidin-horseradish peroxidase solution following the manufacturer’s instructions (GenPoint kit; DAKO). Peroxidase was developed using a dianinobenzidine-substrate-chromogen system, and methyl green was used for counterstaining.

**Immunohistochemistry.** Samples obtained from guinea pig colon were fixed in Bouin’s fluid or periodate-lysine paraformaldehyde at room temperature for 24–48 h (28). The tissue blocks were dehydrated in a graded series of ethanol and embedded in Histosect (Merck). Sections (5-μm-thick) were mounted on glass slides previously coated with polylysine (Sigma-Aldrich). Tissue sections were dewaxed with xylene, rehydrated through a graded series of ethanol and treated with absolute methanol and 1% hydrogen peroxide to block endogenous pseudoperoxidase activity. After being rinsed several times in 0.05 M Tris-HCl buffer, pH 7.8, the sections were incubated overnight with an anti-peptide antibody raised in rabbits against the synthetic peptide RSRRHGLPRGTSPSDDDKC corresponding to residues 888–906 of rat CIC-2 (1:100 to 1:400) (Alomone Labs, Israel). Once incubation was completed, the sections were rinsed three times for 5 min each with 0.05 M Tris-HCl buffer and incubated with a biotinylated anti-rabbit antibody and streptavidin-peroxidase (Kit LSAB +; DAKO), for 15 min each. Peroxidase was developed for 5 min using a commercial liquid dianinobenzidine-substrate-chromogen system (DAKO). All incubations were carried out at 22°C in a water bath that was used as a moist chamber. When immunostaining was completed, the sections were rinsed with distilled water and contrasted with Harris hematoxylin for 30 s. Finally, the sections were dehydrated in ethanol, cleared with xylene, and mounted using Canada balsam. In addition, acetone-fixed frozen sections were also used, and the bound anti-CIC-2 antibody was detected by a fluorescein-labeled anti-rabbit (Fab)”2 immunoglobulin (DAKO). Sections were analyzed by laser confocal microscopy. Controls of the immunostaining procedure included omission of the specific antibody, replacement by nonimmune rabbit serum and incubation with the specific antibody in the presence of an excess of the same peptide used for immunization (25 to 50 μg/ml).

**Electrophysiological studies.** The experiments using fresh isolated colonocytes were performed in cells at room temperature in 35-mm diameter polysyline-treated plastic petri dishes mounted directly on the stage of an inverted microscope. The bath solution contained (in mM) 140 NaCl, 2 CaCl₂, 1 MgCl₂, 22 sucrose, 10 HEPES pH 7.4. Alternatively, a 16 mM Cl⁻ solution was made by equimolar replacement with gluconate or Cl⁻. The pipette solution (35 mM Cl⁻) contained (in mM) 100 Na gluconate, 33 CsCl, 1 MgCl₂, 2 EGTA, 1 ATP, and 10 HEPES, pH 7.4. The gpCIC-2 plasmid used in the electrophysiological studies is in an expression vector under the control of the cytomegalovirus promoter (pCR3.1; Invitrogen). HEK-293 cells used for transient transfections were grown in DMEM/F-12 supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂ humidified incubator. At 60–80% confluence, the cells were cotransfected with 1.5 μg of total expression plasmids for gpCIC-2 and α3H in a 3:1 ratio using Lipofectamine Plus (Life Technologies). Expression of CD-8 antigen was used as a means to identify effectively transfected cells within the dish (22). After 24–48 h, the cells were incubated briefly with microspheres coated with an antibody against the CD8 antigen (Dynabeads). The experiments were performed in bead-decorated cells at room temperature. Solutions were as described for isolated colonocytes experiments.

Standard whole cell patch-clamp recordings were performed as described elsewhere (12) using an amplifier (model EPC-7; List Medical, Darmstadt, Germany). The bath was grounded via an agar bridge. Patch-clamp pipettes were made from thin borosilicate (hard) glass capillary tubing with an outside diameter of 1.5 or 1.7 mm (Clark Electromedical, Reading, UK) using a P-97 puller (Sutter Instruments). The pipettes had a resistance of 3–5 MΩ. Voltage and current signals from the amplifier were digitized using a computer equipped with a Digidata 1200 (Axon Instruments) AD/DA interface. The voltage pulse generator and analysis programs were from Axon Instruments. Unless otherwise stated, when giving trains of pulses, an interval of 60 or 80 s between pulses was left at the holding potential to allow for complete current deactivation. Time courses for current (i) activation and deactivation were fit to double exponential plus a constant term equation of the form i(t) = a₁ exp(−t/τ₁) + a₂ exp(−t/τ₂) + c, where a₁, a₂, and c are current amplitudes, and τ₁ and τ₂ are time constants.

Generally, a single representative experiment is shown when similar results have been obtained in at least three separate experiments. Pooled data are given as means ± SE, and statistical differences were assessed by Student’s t-test.

**RESULTS**

Distribution of gpCIC-2 transcript in distal colon epithelium. The location of CIC-2 transcript distal colon epithelium was determined by in situ hybridization with digoxigenin-labeled riboprobes. Figure 1, A and B shows distal colon cryosections hybridizing antisense and sense gpCIC-2 riboprobes, respectively. Prominent staining with the antisense probe was seen only on surface epithelium, whereas there was no apparent staining over crypts or nonepithelial tissue (Fig. 1A). No background staining was observed in control sections hybridized with the sense probe (Fig. 1B). Figure 1, C and D, shows higher magnification pictures confirming the presence of prominent staining of surface epithelium colonocytes (Fig. 1C) with absence of staining over crypt epithelial cells (Fig. 1D). Similar results were obtained with proximal colon cryosections (not shown).

Location of gpCIC-2 by immunohistochemistry. The distribution of CIC-2 in guinea pig distal colon was studied by immunoperoxidase and immunofluorescence. Figure 2B shows a low magnification view of a distal colon section reacted with anti-CIC-2 antibody and revealed with a peroxidase reaction. There was strong immunostaining over surface epithelium with no staining over the crypt region. In contrast with the transcript distribution, there was also staining present in the muscularis mucosae. Figure 2, A and C, shows higher magnification views of surface cells in which subcellular distribution of CIC-2 was examined. There was diffuse immunolabeling over what appears to be the cytosolic compartment, with a defined staining delineating the limit between the cells and also at the basal pole of surface colonocytes (arrows in Fig. 2, A and C). The label was absent from nuclei and also from the apical border of the cells (arrows in Fig. 2D). A control section demonstrates that preincubation with the antigenic peptide blocked immunolabeling (Fig. 2E). No immunostaining was observed in goblet cells in the vicinity of the colonocytes.
Immunolabeling experiments were also carried out detecting the antibody with an FITC-labeled secondary antibody and viewed on a confocal microscope. Figure 3A shows the distribution of label over surface epithelium of distal colon. Figure 3B is a bright field image of the same section. The staining was predominantly at the limit between epithelial cells and absent from the apical aspect of the colonocytes. Control experiments in which the antibody was preabsorbed with the antigenic peptide blocked all basolateral immunostaining seen between colonocytes leaving only a faint background fluorescence (Fig. 3C). Similar results were obtained.
omitting the primary antibody or using rabbit preimmune serum (not shown).

**Hiperpolarization-activated chloride currents in isolated colonocytes.** The evidence presented above suggests that ClC-2 is expressed in surface epithelial cells in guinea pig distal colon. To verify whether currents consistent with this expression were present in these cells, surface colonocytes were isolated by established methods (10) and assayed by patch-clamp. Initial experiments without ATP in the pipette gave occasional hyperpolarization-activated currents that were consistently found after inclusion of the nucleotide. Figure 4B shows a family of currents elicited in an isolated colonocyte by the voltage protocol shown in Fig. 4A. As described before for recombinant gpClC-2, there were small currents at positive or moderately negative potentials, but larger currents activated slowly with strong hyperpolarization. In 20 separate cells, the mean current observed at $-140$ mV was $-520 \pm 87$ pA. A postpulse to 40 mV shows typical slow deactivation of tail currents. Figure 4C shows that replacement by gluconate of all but 16 mM Cl in the bathing medium had little effect on inward currents. This replacement, however, substantially decreased outward currents observed at positive main pulses and tail currents, consistent with them being due to Cl influx. Figure 4D shows currents in high and low Cl obtained after activation to $-140$ mV in response to a 100-ms voltage ramp taking the potential to 40 mV. The relationship was almost linear with a reversal potential of approximately $-40$ mV [equilibrium potential for chloride ($E_{Cl}$) = $-37$ mV]. On partial replacement of Cl with gluconate, there was a displacement of the reversal potential to a more positive value and a general decrease in current with a tendency to inward rectification. This result is similar to that obtained with recombinant gpClC-2 as illustrated in the current-voltage relations shown in Fig. 4E.

The slow activation of anion current observed in isolated colonocytes on hyperpolarization is reminiscent of the behavior of the recombinant gpClC-2 (6). To make a quantitative comparison, the time dependence of activation of colonocyte current at the most extreme hyperpolarizations used, namely $-140$ and $-160$ mV, was analyzed by fitting current activation to two exponentials plus a time-independent value. The fit to this model was reasonable, and the constants obtained are shown in Fig. 5A together with equivalent data obtained for recombinant gpClC-2 expressed in HEK-293 cells. The time constants were voltage dependent, becoming faster at the most hyperpolarized voltage used. There was no significant difference between these and the corresponding activation time constants for the recombinant channel. On return to a depolarized voltage after activation, deactivation consistent with channel closure took place (see tail currents in Fig. 4). The time course of deactivation was also analyzed by a two-exponential decay model. The constants obtained are given in Fig. 5B and compared with those of recombinant channels measured under the same conditions. The slow time constant for deactivation of colonocyte Cl$^-$ current was somewhat faster than that of gpClC-2 but not significantly different from that of the splice variant gpClC-2A77–86. This last variant is known to

![Fig. 3. ClC-2 distribution in guinea pig colon studied by immunofluorescence. A: FITC labeling of ClC-2. B: brightfield image of the same field. C: control in which the antigenic peptide was used in a preabsorption step. D: brightfield image. Calibration bars for A and B apply also to C and D.](image)
be well represented in colonic epithelium (6). In contrast, the fast time constant for colonocyte current deactivation was markedly faster than that of the two types of recombinant channels.

ClC-2 is known to be inhibited by Cd²⁺/H₁₁₀₀₁ (7) and has a characteristic anion selectivity with a Cl⁻/I⁻ permeability sequence, as has been shown with rat ClC-2. (15, 39). Figure 6B shows an Cl⁻/I⁻ replacement experiment in a guinea pig colonocyte. The expected, slowly activating current was elicited by a pulse to −125 mV, and a tail was current-recorded at 40 mV. In I⁻-rich medium, the tail current anion flux was markedly diminished, consistent with a low permeation for this anion. The inward current was also inhibited in I⁻-replaced medium, a finding consistent with channel blockade. Figure 6D shows currents in Cl⁻- and I⁻-rich medium obtained after activation to −125 mV in response to a 100-ms voltage ramp taking the potential to 40 mV. On partial replacement of Cl⁻ with I⁻, there was a displacement of the reversal potential to a more positive value consistent with PCl⁻/Pᵢ⁻, where P is permeability. This behavior of isolated colonocytes is similar to that seen with recombinant gpClC-2. An example of such an experiment is shown in Fig. 6G. The results are similar in all respects except in the magnitude of the current that was 10-fold higher in the overexpression system.

DISCUSSION

The colonic epithelium has secretory and absorptive functions (26). Absorptive transport consists mainly of short-chain fatty acid transport and NaCl absorption accompanied by water, thus allowing for only minor losses of water in the feces, depending on the species. Secretory processes are set into action by the so-called secretagogues that through second messengers, such as cAMP or Ca²⁺, promote the secretion of NaCl and water. The step governing the rate of secretion is the output of Cl⁻ from epithelial cells, where it is accumulated above electrochemical equilibrium. The permeability pathway for Cl⁻ exit is provided by CFTR, a ClC activated by phosphorylation (17). CFTR is defective in cystic fibrosis, thus leading to reduced salt and fluid secretion. There is, therefore, a great deal of interest in finding possible alternative pathways for Cl⁻ exit that could be activated to replace the defective conductance. A candidate that has been repeatedly proposed to play such a role is the inwardly rectifying ClC-2. Currents that could presumably be associated with ClC-2 expression have been detected in T₈₄ and Caco-2 cells (14, 30), and in the Caco-2 line, it has been proposed to mediate anion secretion not associated with CFTR function. ClC-2-like currents have been reported in...
other cells capable of fluid secretion such as pancreatic acinar cells (4), parotid acinar cells (33), and mandibular duct gland cells (25), but their function in trans-epithelial transport has not been clearly defined. Assigning currents to a molecular counterpart, however, can be difficult as has been shown recently for hyperpolarization-activated Cl\textsuperscript{−}/H\textsuperscript{11002} currents of choroid plexus that have some resemblance with ClC-2 except for an I\textsuperscript{−}/H\textsuperscript{11002}/H\textsuperscript{11022}/Cl\textsuperscript{−}/H\textsuperscript{11002} permeability (23). These have now been reported to remain unchanged in a ClC-2 knockout mouse and cannot, therefore, be attributed to this channel (37). In contrast, loss of hyperpolarization-activated Cl\textsuperscript{−}/H\textsuperscript{11002} current in salivary acinar cells from Clcn2 knockout mice has firmly established the correspondence of the current and ClC-2 (32).

Absorption of NaCl and water in colonic epithelium can be electrogenic or electroneutral (26). The first is mediated by ENaC located in the apical membrane and providing the entry step for Na\textsuperscript{+} that is followed by pump-mediated efflux at the basolateral membrane. For electroneutral absorption, the entry step is provided by parallel exchangers of Na\textsuperscript{+}/H\textsuperscript{+} and Cl\textsuperscript{−}/HCO\textsubscript{3}−. The presence of a Cl\textsuperscript{−} conductive pathway in the basolateral membrane has been proposed for both electrogenic and neutral NaCl transport, as shown in the models discussed by Kunzelmann and Mall (26) in Figs. 2 and 3 of their review. The only Cl\textsuperscript{−} conductance that has been described in basolateral membranes of colonocytes is a volume-regulated, outwardly rectified conductance of isolated crypts (11). Outwardly rectified channels with I\textsuperscript{−} > Cl\textsuperscript{−} permeability of small intestinal villus enterocytes have been proposed to be basolateral and involved in Cl\textsuperscript{−} absorption (31). The molecular identity of the channels is unknown.

In the present work, we present data demonstrating the presence of ClC-2 in the colonic epithelium both at the transcript and protein level. In addition, we demonstrate a functional activity in isolated colonocytes consistent with the presence of active ClC-2 in the epithelium. We argue that ClC-2 might be a good candidate to be involved in NaCl absorption across the epithelium by providing the required exit pathway at the basolateral membrane of absorptive cells.

NaCl absorption and secretion in colonic epithelium has classically been assumed to be the property of separate compartments. The surface epithelium would be mainly absorptive, whereas the crypts would be the site of secretion (26). This contention had a rather convincing functional demonstration in now classical experiments showing secretion of fluid emerging from crypt mouths (41). Such a compartmentalization also agrees well with the tissue distribution of the mem-

![Fig. 5. Comparison of activation kinetics of Cl\textsuperscript{−} currents of isolated colonocytes compared with those arising from gpClC-2 overexpression. A: currents of the type shown in Fig. 4B at two hyperpolarizing pulses were analyzed as described in the text to provide exponential time constants (τ) for two components of activation (n = 7 cells). These are compared with equivalent data obtained in experiments with gpClC-2 expressed in HEK-293 cells (n = 11 cells). B: comparison of time course of deactivation of colonocyte Cl\textsuperscript{−} current with that of the currents generated by overexpression of gpClC-2 and gpClC-2Δ77–86 in HEK-293 cells. The time constants of a two-exponential fit to the deactivation at 40 mV after a main pulse to −140 mV are shown. The respective n values for means ± SE calculation were 6, 22, and 16 cells. Significant differences with respect to the colonocyte constants are indicated. Student’s t-test probability for the significance of the differences indicated were <0.5% for a, b, and c.](http://ajpgi.physiology.org/)

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brane transport systems known to contribute to these processes. The Na\(^+\)/H\(^+\) exchanger is typically associated with apical membranes of epithelia, and in the colon, it is expressed in surface epithelial cells (2). The ENaC is also expressed in the apical membrane of surface colonocytes (13). CFTR, the phosphorylation-regulated Cl\(^-\) channel, is mainly expressed in the crypt epithelium (40). Despite this evidence, controversy remains as to this strict separation of functions. There are reports of NaCl absorption in the crypts (16) and of secretion in surface epithelium (24). In the present paper, we demonstrate that ClC-2 transcript is exclusively expressed in the surface epithelium. If present in the crypt, ClC-2 is only in the upper reaches of these glands. The same distribution is observed when using an anti-ClC-2 antibody in immunohistochemical analyses. The immunoreactivity is present in the surface epithelium as a diffuse labeling within the cells and as a more defined label delineating the basolateral aspect of colonocytes. Previous intestinal immunohistochemical studies show intracellular, but not nuclear, staining and a suggested basolateral location for ClC-2 in rat colon, but only intracellular in human large intestine (27). Studies with murine small intestine show that ClC-2 expressed at the tight junction between epithelial cells exclusively (19). Our results are inconsistent with an apical membrane location, which would rule out a role for ClC-2 in a secretory process. We cannot be sure about the diffuse cytoplasmic immunolabeling observed here. If it reflects ClC-2 expression, one could speculate that cytoplasmic immunolabeling reveals an intracellular pool of channels that might be targeted to the membrane under appropriate stimulus. A similar speculation has been proposed to explain the intracellular location of ClC-2 in the human colon (27).

Clear immunolabel delineation of the basolateral aspect of the cells observed in the present work could be due to the presence of the channel protein in that membrane domain. If this interpretation were correct, it would lead to the prediction that functional channel activity compatible with ClC-2 expression should be observed in colonocytes. We tested this by direct comparison of ion currents in surface epithelial cells isolated from guinea pig distal colon and those generated by transfection of the recombinant channel into HEK-293 cells.

Fig. 6. Effect of I\(^-\) replacement and Cd\(^{2+}\) addition on Cl\(^-\) currents of isolated colonocytes compared with those arising from gpClC-2 overexpression. A: voltage protocol. B: currents obtained in a colonocyte before and after replacing all but 16 mM Cl\(^-\) in the bathing medium by I\(^-\). C: currents obtained in a colonocyte before the addition of and after the replacement of 200 \(\mu\)M Cd\(^{2+}\) to the bathing medium. D: example of a I\(^-\) for Cl\(^-\) replacement experiment. Current was elicited by a 600 ms pulse to \(-125\) mV followed by a ramp taking the voltage to 40 mV in 100 ms. A similar experiment with gpClC-2 expressed in a HEK-293 cell is shown in F. E: example of a Cd\(^{2+}\) addition experiment in a colonocyte. Current was elicited as in D. A similar experiment with gpClC-2 expressed in an HEK-293 cell is shown in G.
Whole cell patch-clamp studies in surface epithelial cells from guinea pig distal colon demonstrated the presence of Cl\(^{-}\)-currents that activated slowly in response to hyperpolarization. This gave rise to inwardly rectifying currents strongly reminiscent of recombinant gpClC-2. Unlike what is seen with the recombinant channel, small outward Cl\(^{-}\)-current was also observed, perhaps suggesting a degree of contribution of channels other than CIC-2. Comparison of the kinetics of activation of Cl\(^{-}\)-colonic current with that generated by gpClC-2 expression, however, gives an astonishing agreement. This strongly suggests that, at least at these hyperpolarized potentials, the contribution of a CIC-2 current is predominant. For deactivation, the kinetics is closer to that seen for a splice variant of gpClC-2 (gpClC-2Δ77–89), which is characterized by faster deactivation kinetics. Interestingly, this variant is best represented in guinea pig colonic epithelium (6). Experiments of partial replacement of extracellular Cl\(^{-}\)-with gluconate in colonocytes and in cells overexpressing gpClC-2 are also in agreement. In addition to the difference in permeability, there is evidence for modulation of the gating by the Cl\(^{-}\)-concentration suggested by the observed decrease in inward current in both types of experiments.

Two other pieces of evidence suggest that the Cl\(^{-}\)-currents recorded in isolated colonocytes are the expression of CIC-2 channels present in their membranes. The first is their sensitivity to relatively low concentration of Cd\(^{2+}\), which has been demonstrated in rat CIC-2 (7). This divalent cation is without any effect on the widely distributed Cl\(^{-}\)-channels activated by increases in cell volume (1). Cd\(^{2+}\) blocks the Cl\(^{-}\)-currents of guinea pig colonocytes in a very similar fashion as it does the overexpressed gpClC-2. Perhaps more importantly, CIC-2 has a permeability sequence in which P\(_{\text{Cl}}\) is greater than P\(_{\text{I}}\) (15, 39). As shown here for gpClC-2, I\(^{-}\)-also inhibits the current markedly. These characteristics are reproduced in the Cl\(^{-}\)-currents in colonocytes. The functional data, together with the transcript and protein analyses are, therefore, consistent with the presence of active CIC-2 channels in surface epithelial cells from distal colon.

Is the conductance mediated by CIC-2 compatible with electroneutral Cl\(^{-}\)-absorption? To answer this question would require knowledge of the transepithelial flux of Cl\(^{-}\)-mediated via the operation of the Na\(^{+}/H^{+}\) and Cl\(^{-}/HCO_{3}^{-}\) exchangers. This information is not available directly, but it could be surmised from work in which the mucosa-to-serosa Cl\(^{-}\)-flux has been measured in guinea pig colon (8). Under resting conditions the colon epithelium shows a small secretory Cl\(^{-}\)-flux, which is increased fivefold by theophylline. This occurs on the background of a relatively constant mucosa-to-serosa flux of ~3 μeq·cm\(^{-2}\)·h\(^{-1}\), which we may assume to be due to electroneutral Cl\(^{-}\)-absorption in this short-circuited epithelium. CIC-2 current at ~60 mV, deemed a physiological potential, is ~40 pA in the cell illustrated in Fig. 4B, and in five separate experiments, it was 49 ± 21 pA. If one assumes that there are 2 × 10\(^{6}\) cells per cm\(^{2}\), with surface CIC-2-expressing cells forming virtually a planar continuous epithelial layer, a flux of ~4 μeq·cm\(^{-2}\)·h\(^{-1}\) is obtained. This is in agreement with measured mucosa-to-serosa Cl\(^{-}\)-flux and would suggest that CIC-2 conductance might be enough to account for absorption of this anion.

The CIC-2-like current described here in surface colonocytes has not been studied in guinea pig crypts. It does not appear, however, to be present in rat or mouse crypt colonocytes. As reviewed by Schultheiss and Diener (34), rat crypt cells possess a slightly outwardly rectified basolateral Cl\(^{-}\)-conductance, besides that regulated by cAMP and that corresponding to CFTR. Rectification properties, single channel conductance, and anion selectivity separate this anion permeability pathway from CIC-2. Outwardly rectified Cl\(^{-}\)-channels are the only anion channels detected in a study of the basolateral membrane of mouse colonic crypts (29). Again selectivity and other properties are clearly different from those of CIC-2.

In summary, we demonstrate that CIC-2 mRNA and protein are located in the surface epithelium of the distal colon. Immunolocalization reveals that, in addition to some intracellular labeling, CIC-2 is present in the basolateral membranes but absent from the apical pole of colonocytes. Isolated surface epithelial cells exhibited hyperpolarization-activated chloride currents showing voltage-dependence, a Cl\(^{-}\)- > I\(^{-}\)-permeability and Cd\(^{2+}\)-sensitivity indistinguishable from those measured in parallel experiments with recombinant gpClC-2. The presence of active CIC-2-mediated currents in surface colonic epithelium, coupled to a basolateral location for CIC-2 in the distal colon, suggests a role for CIC-2 channel as the basolateral membrane exit pathway for Cl\(^{-}\}-as part of the NaCl absorption process. Confirmation or otherwise of these speculations will require the study of transepithelial Cl\(^{-}\)-fluxes and their sensitivity to CIC-2 inhibitors and regulation by changes in cell volume and extracellular pH.

We are grateful to Inés Siegmund, Pamela Ehrenfeld, and José Sarmiento for generous help during this work.

This study was funded by Fondecyt Grants 1990939 and 1000622 and an Equipment Grant from Fundación Andes. Support to the Centro de Estudios Científicos (CECS) from Empresas CMPC is also acknowledged. F. V. Sepúlveda was an International Research Scholar of the Howard Hughes Medical Institute and a Fellow of the J. S. Guggenheim Foundation. CECS is a Millennium Science Institute.

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