ClC Chloride Channels in Epithelia: Recent Progress and Remaining Puzzles

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ClC chloride channels are widely expressed in epithelia. Recent insights into the roles of specific ClC channels have emerged from molecular and immunolocalization studies, mouse knockout models, and the linkage of mutations of these channels to the human hereditary diseases Bartter’s syndrome and Dent’s disease.

Epithelial cells maintain the composition of the plasma by a directional transport of ions, nonelectrolytes, and water. This unique ability requires the expression and orchestration of numerous transport proteins. Studies of the regulation and molecular identification of epithelial transport proteins have grown at an explosive rate during the past decade. The purpose of this review is to provide an overview of recent progress in our understanding of the role of one such group of proteins, the ClC family of voltage-gated chloride channels. Due to imposed limitations on the number of references, we are able to cite only a few primary sources and reviews concerning ClC channels and are unable to discuss results from the promising area of ClC channel expression in model organisms. For information on this field and more extensive references, we refer the reader to three recent excellent reviews of the ClC channel literature (4, 7, 16).

Chloride channels and epithelial functions

The transport properties of epithelial cells arise from the structural organization and polarization of their membranes. Epithelial cells are bound together as a continuous sheet by tight junctions that separate the cell membrane into two domains, apical and basolateral. These domains contain distinct transport proteins that can be regulated by hormones and other factors. Compared with voltage gating of channels in excitable membranes, this mechanism is generally thought to be a less important factor in governing epithelial membrane permeability. However, it has become increasingly clear that voltage-gated ClC channels play significant roles in epithelial cell physiology. Genetic diseases of epithelia, such as the renal disorders Bartter’s syndrome and Dent’s disease, have been associated with loss-of-function mutations of specific ClC channels (16). In addition, ClC channels are equipped to carry out several functions required of epithelial chloride channels: vectorial chloride movement, stabilization of membrane potentials, and regulation of cell volume.

A brief history of the ClC channel family

The ClC channel family was first identified by Jentsch and co-workers with the cloning of the so-called double-barreled voltage-gated ClC-0 channel from Torpedo electroplax (see Ref. 4). Presently, members of the ClC gene family are found in species as diverse as bacteria, yeast, plants, and invertebrate and vertebrate animals. To date, at least nine human ClC genes have been discovered. The homologies between the predicted amino acid sequences of ClC-0 and various ClC channels from other species are presented in Fig. 1A.

Interest in ClC channels has stemmed largely from their relevance to genetic diseases. Loss-of-function mutations of ClC channels have been directly linked to hereditary disorders, including both dominant and recessive forms of myotonia (ClC-1), renal salt-wasting disease (Bartter’s syndrome; ClC-Kb), and X-linked hereditary nephrolithiasis (Dent’s disease; ClC-5). A recent mouse knockout model of ClC-K1 also implicated multiple functions of this channel in diabetes insipidus. Although mutations of ClC-2 have not been reported, intense interest has also been focused on this channel because of its possible relevance to the genetic disease cystic fibrosis (CF). ClC-2 is present in most cells that express CF tranmembrane conductance regulator (CFTR), the chloride channel that is defective in CF and normally responsible for transepithelial chloride transport. For this reason, ClC-2 is considered a possible target for correcting chloride transport malfunctions in CF-affected epithelia.

The function and general structure of most ClC channels at present, incompletely understood. ClC protein monomers have predicted molecular masses ranging from 69 to 110 kDa. Hydropathy analyses indicate that they possess 13 hydrophobic domains (referred to as D1–D13, respectively). Approximately 10–12 of these domains are thought to span the cell membrane, with domain D13 and the COOH and NH₂ terminals of the protein located in the cytoplasm (3, 4, 7) (see Fig. 2). Recent analysis of two-dimensional crystals of a bacterial ClC channel homologue have confirmed that these channels are formed as dimers with two off-axis water-filled pores (9).

Although ClC channels are generally referred to as voltage-gated chloride channels, not all ClC channels are strictly voltage dependent and there is a lack of knowledge about the structural details of channel gating. Figure 1B summarizes basic ClC channel properties. As shown, nearly all functionally expressed ClC channels select for chloride over iodide. Note, however, that the selectivity mechanism has not as yet been correlated to specific structural features.
Distribution of ClC channels in epithelia

With the exception of ClC-1, which is predominantly expressed in skeletal muscle, all other human ClC channels have been detected in epithelial cells. Most of these channels are widely expressed, except for ClC-Ka and -Kb, which so far are thought to be found exclusively in the kidney and have an ~40% homology to the other epithelial ClCs. ClC-5 was also initially thought to be kidney specific; however, this channel has recently been detected in many tissues, including hepatocytes, retina, brain, vascular smooth muscle, and endothelial cells (see Ref. 20). In addition, there appear to be considerable species differences in the tissue distribution of ClC-5.

Epithelial cells, as well as neurons, blood cells, and skeletal and smooth muscle, can express more than one type of ClC protein, and it remains unclear how simultaneously expressed ClCs interact. Although some ClC channels such as ClC-0 have been shown to be homodimers, other ClC proteins (ClC-1 and ClC-2) form heteromeric channels with novel conductance properties when coexpressed in Xenopus oocytes (4). Whether endogenously expressed ClC channels normally form such heteromultimers remains an unanswered question.

Functional insights from specific ClC channels

ClC-2: Regulation and its role as a possible mediator of chloride secretion. ClC-2 is a ubiquitously expressed ClC channel that can be activated by hypotonicity, low extracellular Cl, and hyperpolarization and can be blocked by cadmium. Activated channels demonstrated linear instantaneous current-voltage relationships that, with longer test pulses, rectified inwardly in the steady state (see Ref. 4).

ClC-2 has been speculated to function as a swelling-activated chloride channel in epithelial cells. In neurons, it has been hypothesized to set intracellular chloride levels, thereby modulating membrane excitability. Although ClC-2 has been the focus of considerable interest from epithelial physiologists as a possible alternative pathway for chloride transport in CFC-affected epithelia, most current knowledge of its function and regulation has been gleaned from analyses using heterologous expression systems. Consequently, the necessity of considering its function in native epithelia has become increasingly clear.

To address this issue, several investigators have focused on characterizing chloride currents endogenous to epithelial cells by using molecular tools developed from knowledge of the primary structure of ClC-2. To understand whether this channel underlies swelling-activated or chloride secretory currents, these investigations have aimed to determine the distribution of ClC-2 and the regulation of its expression and conductance properties. Only a few salient findings will be considered in the following discussion.

Hyperpolarization-activated chloride currents with biophysical and pharmacological profiles similar to those of ClC-2 have been reported from whole cell recordings of numerous
native and cultured epithelial cells even before its cloning. Park et al. (10) used RT-PCR and Western blot analysis to demonstrate the presence of CIC-2 transcript and protein in rat parotid gland acinar cells, a system in which they previously had measured hyperpolarization-activated chloride currents. Transient overexpression of CIC-2 in HEK cells produced currents with similar kinetics, leading these investigators to infer its involvement in the native hyperpolarization-activated chloride current of parotid acinar cells.

Kajita et al. (5), following earlier studies of hyperpolarization-activated chloride currents in choroid plexus epithelium, presented Western blot evidence for the presence of CIC-2 in these cells. Interestingly, transfection with antisense RNA specific to CIC-2 not only reduced the detected levels of CIC-2 but also significantly attenuated the current. What remains puzzling are the apparent differences in the halide selectivity and sensitivity to external pH of native chloride currents in choroid plexus compared with heterologously expressed CIC-2 (4).

One potential answer to these discrepant features of hyperpolarization-activated chloride currents in epithelial cells is the possibility that these currents result from channels formed by heteromeric interactions between CIC-2 and coexisting CIC species (4). Heterogeneity could also result from alternative splicing, as recently shown for CIC-2 clones isolated from a guinea pig intestinal epithelial library (2). Clearly, further studies are required to test the likelihood of these possibilities as potential mechanisms of functional diversity.

Few studies have addressed the question of whether CIC-2 expression in epithelial cells is polarized, i.e., restricted to either the apical or basolateral membrane. Blaisdell et al. (1) investigated the role of CIC-2 in mediating transepithelial chloride transport across primary fetal lung epithelial monolayers, a chloride secretory epithelium. Using confocal microscopy of immunofluorescently labeled CIC-2, these investigators localized this protein near the apical membrane. In addition, they were able to stimulate the short-circuit current by acidification of the apical bathing solution and to block this increase with mucosal addition of cadmium, as predicted for the functional properties of CIC-2 present in the apical membrane. Moreover, the pH-evoked increases in the short-circuit current were comparable to current levels observed following stimulation of CFTR by cAMP in the same epithelia. These findings provide indirect evidence that CIC-2 can mediate chloride transport across the apical membranes of the airway epithelia. Further work is needed to determine whether this channel can compensate for defective chloride transport function in CF.

Further insights relevant to this issue come from Tarran et al. (14). In whole cell patch-clamp studies of ciliated airway epithelial cells from wild-type and CFTR knockout mice, these investigators found evidence that CFTR might regulate a basal chloride current similar to CIC-2. This current was detected in ~3% of CFTR knockout mice compared with 27% in normal mice. Increases in intracellular chloride levels shift the voltage dependence of CIC-2 gating to more depolarized potentials closer to the physiological range (reviewed in Ref. 4). Therefore, this apparent CFTR regulation of CIC-2 could be by a direct interaction with CFTR and/or by a change in the intracellular chloride concentration concomitant with cAMP-stimulated chloride secretion.

**ClC-K channels: role in renal function.** The ClC-K channels are the only group of CIC channels that are almost exclusively expressed in the kidney and urinary tract. For this reason, cloning of the closely related channels ClC-K1 and ClC-K2 from rat, and the similar human channels ClC-Ka and ClC-Kb, spurred much speculation regarding their roles in renal physiology and pathophysiology. These channels have a high degree of sequence identity within species (91% between ClC-Ka and ClC-Kb and 82% for ClC-K1 and ClC-K2). The lack of complete correspondence between human and rat ClC-K proteins initially hindered their comparison as cross-species orthologs. In addition, early attempts to functionally express ClC-K channels by using heterologous systems resulted in controversy. Recent studies offering insights into these remaining puzzles will be discussed here.

**ClC-Kb.** We will first discuss ClC-Kb since a breakthrough in the understanding of ClC-K channels first came through studies of this channel. In elegant genetic investigations, Simon et al. (13) unequivocally linked mutations in the gene encoding ClC-Kb with the human disease Bartter's syndrome type III. In the case of mutations in the Na+-K+-2Cl- cotransporter (NKCC2) and the ATP-sensitive K+ channel (ROMK; Kir1.1) that result, respectively, in Bartter's syndrome types I and II, mutations of ClC-Kb result in a phenotype that includes salt wasting, hypokalemic alkalosis, and hypotension (Fig. 3). Because Bartter's syndrome is characterized by hypotension, it follows that inhibition of ClC-Kb (as well as NKCC and ROMK) could have antihypertensive effects. Thus ClC-Kb represents a potential target for pharmacological treatment of hypertension.

Early efforts to express ClC-K channels in heterologous systems were met with variable success and hence controversy. Recently, Waldegger and Jentsch (17) used ClC-K constructs subcloned into a vector optimized for *Xenopus* oocyte expression and reconciled some of the earlier findings. In confirm-
K1 knockout (8) resulted in animals that were unable to con-suggest that ClC-Ka is homologous to ClC-K1. A mouse ClC-epithelial physiology is less well established. Recent studiessive agents. but also to test potential inhibitors of ClC-Kb as antihyperten-sion system is also required, not only to allow determination offunctional currents could be observed only with a chimericconstruct consisting largely of ClC-Kb but containing regions ofClC-K1 as well. The expressed currents clearly differed inbiophysical properties from those carried by ClC-K1, having a nonrectifying current-voltage relationship, a conductivitysequence of chloride > bromide > iodide. Recently, Li et al. (6) reported that transient expression of rat ClC-3 in mamma-lian cultured cells resulted in a current that, with respect to rectification and permselectivity, was nearly identical to those of ClC-4 or ClC-5. In the remainder of this paper, we will focus on ClC-3 and ClC-5 since presently little is known about the distribution or function of ClC-4 in epithelial tissues.

ClC-3. ClC-3 is widely distributed and highly conserved across species. Human and rat ClC-3 differ by only two amino acids. ClC-3 also has the highest homology of any ClC channel (~60%) to scClC-1 (gef1), a yeast protein that facilitates copper incorporation into a transmembrane iron transport protein (4). Although only one ClC-3 isoform has been found in humans, a second, shorter isoform that is apparently a splice variant exists in rats. Li et al. (6) observed plasma membrane currents associated with expression of the short isoform, but not the long isoform, by transient transfection of CHO-K1 cells. Although the reasons for this difference are unclear, the existence of two isoforms certainly will complicate further functional analysis of this protein.

Several groups have reported a predominantly intracellular localization of ClC-3. In yeast studies, the closely related scClC-1 (gef1) has been localized to a post-Golgi compartment (reviewed in Ref. 4). ClC-3 has been localized to the perinu-clear region in pigmented ciliary epithelial cells (18), renal A6 cells (for discussion, see Ref. 20), and retinal pigment epithelial cells (20), whereas in hepatocytes it has been localized to the canalicular membrane (see Ref. 6).

Despite its predominantly intracellular localization in many epithelia, ClC-3 has been proposed as a volume-activated membrane channel responsible for regulatory volume decrease (RVD; for review see Ref. 7). Specifically, ClC-3 has been reported to be activated by cell swelling, and mutation of a single amino acid abolished this regulation (reviewed in Ref. 7). However, subsequent evidence from several laboratories does not support this view. Although antisense inhibition of ClC-3 expression decreased swelling-activated currents in pigmented ciliary epithelium (18), these cells still exhibited significant vo-

**FIGURE 3.** Disruption of the genes encoding 3 proteins underlying sodium chloride reabsorption by the thick ascending limb of Henle (TALH) loop results in 3 different types of Bartter's syndrome. Mutations in CLCNKA, which encodes ClC-Kb, inhibit reabsorption of sodium chloride by blocking the basolateral exit pathway for chloride, thus leading to salt wasting and hypotension. NKCC, Na+/K+/2Cl⁻ cotransporter; ROMK, renal outer medullary K channel.

ClC-Ka. In contrast to ClC-Kb, the role of ClC-Ka in renal cell epithelial physiology is less well established. Recent studies suggest that ClC-Ka is homologous to ClC-K1. A mouse ClC-K1 knockout (8) resulted in animals that were unable to con-centrate urine, producing a phenotype of diabetes insipidus. In addition, the chloride conductance of the thin ascending limb was reduced. This pattern is distinct from the Bartter's phenotypelike divergent functional properties of heterologously expressed ClC-K1 and chimeric ClC-Kb/K1, these findings suggest that human ClC-Ka functions differently from ClC-Kb and may play a role in the urine-concentrating mechanism.

"Outwardly rectifying" ClC channels (ClC-3, -4, and -5). ClC-3, -4, and -5 channels comprise a common branch of the ClC family and have a predicted amino acid identity of ~80% (4). These channels, like the ClC-K channels, initially proved relatively difficult to express, and perhaps for this reason their properties were disputed. Recent transient expression studies in mammalian cells have provided convincing evidence that both ClC-4 and ClC-5 currents show extreme outward rectification, conducting only at potentials exceeding +20 mV and characterized by the halide selectivity sequence of chloride > bromide > iodide (see Ref. 16). In contrast, results for ClC-3 have been controversial, with some authors reporting a more hyperpolarized conductance with a selectivity of iodide > chloride. Recently, Li et al. (6) reported that transient expression of rat ClC-3 in mammalian cultured cells resulted in a current that, with respect to the sequence of chloride > bromide > iodide, is similar to that of ClC-4 or ClC-5. In the remainder of this paper, we will focus on ClC-3 and ClC-5 since presently little is known about the distribution or function of ClC-4 in epithelial tissues.

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ume-activated chloride currents and were able to volume regulate, i.e., the effects on RVD and currents were only partial, indicating that other genes are involved in RVD. In addition, transiently expressed CIC-3 currents had different properties from swelling-activated currents in CHO-K1 cells (6). For these reasons, the previously postulated role of CIC-3 in cell volume regulation presently remains unclear and controversial (see NOTE ADDED IN PROOF).

CIC-5. The detailed linkage analysis of CIC-5 to Dent’s disease qualifies this channel, from a genetic standpoint, as one of the most thoroughly studied epithelial CIC channels. Dent’s disease is a renal disorder characterized by hypercalciuria, kidney stone formation, and low-molecular-weight proteinuria. Expression of CIC-5 channels with Dent’s-related mutations in oocytes led to a loss of chloride conductance function. How the loss of a chloride conductance can lead to the above pathology remains a major puzzle. Two major groups have recently reported the successful engineering of CIC-5 knockout mice (11, 19). These models undoubtedly will prove useful in bridging the gap that presently exists between our understanding of the defective CIC-5 genotype and the resulting phenotype of Dent’s disease.

Although CIC-5 channels in humans initially were believed to be expressed exclusively in the kidney, recent studies reported the detection of CIC-5 transcript or protein in several other human cells and tissues, including airway epithelium, retinal pigment epithelium, and aortic endothelial and smooth muscle cells (for discussion, see Refs. 12 and 20). Since there are no reports of functional alterations in organs other than the kidney in Dent’s patients, other proteins or mechanisms presumably compensate for CIC-5 malfunction in these other cells.

Within the mammalian kidney, there is general agreement that CIC-5 is predominantly expressed in the proximal tubule (particularly the S3 segment) within intracellular vesicles, with some reports of expression in the apical membrane of cortical collecting duct intercalated cells. Several laboratories have colocalized CIC-5 with H+-ATPase in proximal tubule cells (for review, see Ref. 16). For this reason, it was proposed that CIC-5 mediates a chloride conductance that is necessary for endosomal acidification (16). As shown in Fig. 4A, the negative charge carried by chloride would compensate for the accumulation of positive charges within the endosomal lumen due to the transport of protons into this space. Disruption of CIC-5 would limit proton entry and, hence, acidification, inhibiting normal endocytosis in the proximal tubule and preventing reabsorption of protein from the ultrafiltrate. The model is attractive because it explains the proteinuria of Dent’s disease. However, on the basis of the present understanding of CIC-5 currents from functional expression studies, it has several shortcomings. First, CIC-5 channels do not appear to be optimally suited for the proposed shunting function. As noted above, when CIC-5 is expressed in the plasma membrane, there is a strong outward current, i.e., chloride moves from the extracellular fluid into the cytoplasm and the channel conducts relatively little current in the opposite direction. If a similar channel orientation occurs in the endosome, chloride would move into the cytoplasm, not into the endosomal lumen. Therefore the current is in the wrong direction to support efficient acidification, i.e., the movement opposite to the direction indicated in Fig. 4A. Second, acid solutions inhibit CIC-5 currents, therefore making it unlikely that chloride movements through CIC-5 channels would directly facilitate acidification as shown in Fig. 4A. Consequently, the role of CIC-5 proteins in endosomal function may be different or more complex than initially thought.

A complete understanding of the role of CIC-5 in endosomes clearly requires a better understanding of its potential trafficking between different membrane compartments. Evidence exists that, under certain conditions, H+-ATPase is vigorously recycled and trafficked between intracellular compartments and the plasma membrane. Indeed, targeting sequences have been identified for the yeast scCIC-1 protein (see Ref. 4). Similar studies of CIC-5 will be important, particularly to understand the pathophysiology of Dent’s disease.

Because of the high urine calcium levels seen in Dent’s disease, the relationship of CIC-5 to calcium homeostasis presents
another unsolved riddle. Recently, decreased ClC-5 mRNA and protein levels were observed in the renal cortices of vitamin D-deficient, thyroparathyroidectomized rats (12). Injection of these animals with parathyroid hormone (PTH) restored expression levels to normal. It is difficult to discern whether PTH directly upregulates ClC-5 channels or whether the effects are indirect. PTH is known to stimulate the trafficking of sodium phosphate transporters in renal cells by targeting these proteins for lysosomal degradation. Further studies using channels tagged as fluorescent fusion proteins to monitor trafficking of ClC-5 channels may prove helpful in this regard.

Recently, two separate groups have independently engineered knockout mouse by using a similar strategy of disrupting regions of the ClC-5 gene encoding functionally important channel structures and inserting a neomycin resistance cassette in place of these areas (11, 19). The phenotype generated by one of these models is astonishingly close to that manifested in Dent’s-affected individuals (19) and includes not only the low-molecular-weight proteinuria that also was observed in the other mouse (11) but also, importantly, the development of hypercalciuria, kidney calcium deposits, and bone abnormalities. Together, these two mouse models provide exceptional promise for future physiological studies that will be useful in solving the mysteries of how ClC-5 dysfunction leads to impaired endocytosis and the pathology of Dent’s disease.

Summary and remaining issues

ClC channels have emerged at the forefront of epithelial cell physiology, bringing together fields as diverse as genetic disease mechanisms, channel trafficking, epithelial membrane polarization, ion transport, endocytosis, and cell volume regulation. A number of important and exciting issues that span disciplines ranging from developmental and cell biology to structural biology remain unanswered. For example, do ClC channels regulate other epithelial transport proteins? Conversely, what proteins regulate and/or modulate ClC conductance properties and gating? In epithelial cells, are endogenously expressed ClC channels normally associated with regulatory subunits? Do ClC channels play a role in development, the cell cycle, cell differentiation, secretion, or oxidative metabolism? What is the molecular structure of ClC channels, and which regions comprise the pore and which determine channel gating? Considering that it has been only a decade since the primary structure of the very first ClC channel was determined and realizing the scope of the profound advances sparked by that initial work, we are confident that the eagerly awaited answers to these questions will soon be revealed.

NOTE ADDED IN PROOF

This point (see p. 165) has recently been clarified by studies of a ClC-3 knockout mouse that show no change in volume-regulated Cl\(^{-}\) currents (see Stroubawa SM et al., Neuron 29: 185–196, 2001).

We wish to recognize the multitude of colleagues and collaborators whose important work we were unable to cite in this short review. We also thank Dr. Merritt Maduke for her help and Drs. Ana Pajor, Steve Weinman, and Luis Reuss for reading an earlier version of this manuscript.

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