From Tonus to Tonicity: Physiology of CLC Chloride Channels

SIEGFRIED WALDEGGER and THOMAS J. JENTSCH
Center for Molecular Neurobiology (ZMNH), University of Hamburg, Germany.

Abstract. Chloride channels are involved in a multitude of physiologic processes ranging from basal cellular functions such as cell volume regulation and acidification of intracellular vesicles to more specialized mechanisms such as vectorial transepithelial transport and regulation of cellular excitability. This plethora of functions is accomplished by numerous functionally highly diverse chloride channels that are only partially identified at the molecular level. The CLC family of chloride channels comprises at present nine members in mammals that differ with respect to biophysical properties, cellular compartmentalization, and tissue distribution. Their common structural features include a predicted topology model with 10 to 12 transmembrane regions together with two C-terminal CBS domains. Loss of function mutations affecting three different members of the CLC channel family lead to three human inherited diseases: myotonia congenita, Dent’s disease, and Bartter’s syndrome. These diseases, together with the diabetes insipidus symptoms of a knockout mouse model, emphasize the physiologic relevance of this ion channel family.

Chloride is the predominant anion of the extracellular milieu. Various transporters and channels participate in the translocation process of chloride ions across both cellular plasma membranes and membranes of intracellular organelles. Although none of the hitherto identified chloride transporting proteins directly consumes ATP, several transporters gain advantage from the energy stored in transmembrane gradients of other ions to move chloride uphill against its electrochemical gradient. Coupling to the sodium gradient (maintained by the Na,K-ATPase) allows accumulation of chloride intracellularly above its equilibrium concentration. This concept is realized by NaCl- and NaK2Cl-cotransporters, of which several isoforms were identified at the molecular level. On the other hand—due to the inverse transmembrane distribution of potassium—a structurally related group of KCl-cotransporters (consisting of at least four members) is capable of lowering intracellular chloride concentration below equilibrium. Other transport proteins involved primarily in intracellular pH regulation may additionally affect the chloride gradient. This applies to Cl/HCO3 exchangers, which accumulate chloride within the cell during HCO3 secretion, and to Na-linked Cl/HCO3 exchangers, which extrude chloride ions while accumulating Na and HCO3.

Despite the influence of the aforementioned transport proteins on intracellular chloride concentration, the electrochemical gradient of chloride across the plasma membrane is generally not far from equilibrium. Thus, at the resting potential of cells, opening of chloride channels will neither lead to large excursions of the plasma membrane potential nor to dramatic changes in intracellular chloride concentration. The importance of passive chloride diffusion through chloride channels comes to the fore mainly when either the membrane potential or the chloride concentration gradient across the membrane severely deviate from the electrochemical equilibrium. This occurs for example during strong membrane potential depolarizations in the course of an action potential or during excessive accumulation of intracellular chloride driven by secondarily active transport processes. In the first case, potential-driven chloride influx through chloride channels contributes significantly to the repolarization of action potentials (as observed in skeletal muscle cells). In the latter, concentration-driven chloride efflux through chloride channels allows bulk flow of chloride as observed in chloride-secreting or -reabsorbing epithelia. In the central nervous system, the electrochemical gradient for chloride across neuronal plasma membranes determines the action of GABA and glycine, whose receptors constitute chloride channels that open upon ligand binding. Opening of these channels drives the membrane potential toward the reversal potential for chloride. Because the chloride reversal potential of a typical nerve cell is about −70 mV and therefore at some distance from the threshold (−55 mV) for generating an action potential, an inhibitory synaptic action is induced by GABA and glycine. Shifting the chloride reversal potential to more positive values—for example by intracellular chloride accumulation observed in some neurons during early stages of central nervous system development—turns the inhibitory into an excitatory action of these neurotransmitters. Moreover, additional physiologic examples for the importance of passive chloride diffusion through chloride channels are found in nearly all
mammalian cells. These examples include the role of chloride channels in the process of both cell volume regulation and acidification of intracellular organelles. Cell volume regulation by activation of chloride channels depends on the extrusion of chloride, which together with the concurrent movement of potassium as positively charged counterion reduces intracellular solute content. Due to the high water permeability of nearly all plasma membranes, changes in cell solute content have parallel effects on the volume of the cell (1). The supportive role of chloride channels in acidification of intracellular vesicles on the other hand is based on the movement of negative charges through these channels. This partially neutralizes the positive charge movement driven by the electrogenic proton ATPase, which otherwise would have to pump protons against a positive intravesicular potential. The resulting electroneutral transport of HCl allows higher vesicular pH gradients to be achieved (2).

In view of this whole host of functions, it is not surprising that numerous distinct chloride channels have been identified on the functional level based on electrophysiologic characteristics such as conductance, ion selectivity, and gating behavior, as well as pharmacologic sensitivities. This variety of functionally distinct chloride channels, however, is far from being mirrored in the number of hitherto cloned chloride channel genes. Up to now only three unambiguously accepted structural classes of such genes have been identified (3): ligand-gated chloride channels (GABA_A and glycine receptors), the cystic fibrosis transmembrane conductance regulator (CFTR) (a member of the ATP-binding cassette family of transport proteins), and the CLC family of chloride channels. The molecular correlates of several important functionally defined classes of chloride channels, as, for example, the outwardly rectifying chloride channel or chloride channels activated by calcium or cell swelling, are still controversial. This review will focus on molecular and physiologic aspects of the CLC chloride channel family, emphasizing the role of disturbed CLC channel function in human disease.

The CLC Chloride Channel Family: Common Structural Features

The initial cloning of the Torpedo electric organ chloride channel ClC-0 (4) was the crucial event for the discovery of a large gene family (Figure 1). There are now nine different CLC genes known in mammals (5), and it has been found that CLC genes are highly conserved during evolution with representatives in organisms as diverse as animals, yeast, plants, eubacteria, and archaeabacteria (5). The structure of this new channel family showed no homology to other previously cloned ion channels and did not share any comparable sequence motifs with other cloned chloride channels such as the ligand-gated chloride channels or CFTR. A detailed biochemical study of the transmembrane topology of CLC channels performed on the muscle chloride channel ClC-1 demonstrated the intracellular localization of the N- and C-termini of the protein (6). In accord with extended hydropathy analysis, a topology model was suggested with 10 to 12 transmembrane domains (Figure 1). The uncertainty in the number of membrane-spanning regions arose from a broad hydrophobic region at the C-terminal end of the transmembrane-spanning block that was difficult to analyze, and it is still unclear whether this so-called D9-D12 region spans the lipid bilayer 3 or 5 times. This limitation, together with the controversy about the topology of the D4 region suggested to be located extracellularly in one study (6) and to span the membrane in another study (7), points out that the transmembrane topology of CLC channels is not yet entirely resolved. Crystal structure determination of CLC channel proteins (presumably at first from bacteria), together with further topologic studies, will be necessary to clarify this point conclusively.

Apart from the well conserved transmembrane topology, all mammalian and most of the other CLC channel proteins share another structural feature: a tandem repeat of CBS domains in the cytoplasmic carboxy terminus (8). CBS motifs—named after their presence in cystathionine-β synthase—appear in

![Figure 1. The CLC family of chloride channels. (Top) The dendrogram indicates the degree of similarity between different human CLC gene products. Mammalian CLC channels are grouped into three branches. The first one includes ClC-1, ClC-2, and the ClC-K channel (ClC-0, the Torpedo channel, also belongs to that branch). The closely related ClC-3, -4, and -5 channels form the second branch. The third branch contains ClC-6 and ClC-7. (Bottom) The transmembrane topology model of CLC channels as proposed by Schmidt-Rose and Jentsch (6). N- and C-termini are located intracellularly. A glycosylation site in the extracellular loop between transmembrane regions D8 and D9 is indicated, as well as the two CBS domains at the intracellular carboxy terminus.](image-url)
many different proteins (9,10), but their function is still obscure. A mislocalization of the yeast CLC protein (gef1) (11) observed after mutations in these domains (12) suggested that CLC CBS domains may be involved in the sorting process. Since the deletion of the second CBS domain in CIC-1, however, still was compatible with channel function (13), there seems to be no absolute requirement for that domain.

In contrast to either ligand-gated anion channels or voltage-gated cation channels that consist of a single pore surrounded by several (5 and 4, respectively) homomeric or heteromeric subunits (14,15), CLC channels show a completely different architecture. Even before its molecular identification, the CIC-0 channel from the electroplax (the electric organ of the ray Torpedo) was shown to have a peculiar “double-barreled” structure with two apparently identical pores that gate independently from each other (“fast gates”), but which can additionally be gated simultaneously by another common gate (“slow gate”) (16,17). The following two observations cleared the way for the model of a “double-barreled” homodimeric structure with each pore formed by a single ClC-0 protein. First, the expression of the CIC-0 cDNA alone was sufficient to reproduce the “double-barreled” structure, excluding the need for an additional subunit (18). Second, the expression of hybrid channels by assembling wild-type subunits with mutated subunits determined the presence of two pores, the wild-type and the mutated pore, in one single channel (19,20). Recent experiments have shown that the same concept likewise applies to the skeletal muscle chloride channel CIC-1 (21,22) and thus may represent a general feature of CLC chloride channels. This model, however, was not passed unchallenged and an alternative model for CIC-1 was proposed with a single pore composed of two subunits (23). It appears rather unlikely though that the architecture of CIC-1 differs from that of CIC-0. The pore of CLC channels is as yet poorly defined. As expected for a channel in which probably just one protein subunit forms a pore, mutations in several regions of CLC channels led to changes in pore properties.

In the cation channel superfamily, well defined charged protein residues within a transmembrane domain (the S4 segment) sense the electric field over the membrane and lead to conformational changes (gating) that open or close the pore. However, the voltage-dependent gating of CLC channels may be based on a completely different mechanism. The gating of CIC-0 (24), CIC-1 (25), CIC-2 (25), and a CeCIC channel (26) (a voltage-gated chloride channel from the nematode Caenorhabditis elegans) depends on the permeating ion as the gating charge. For CIC-0, a model was proposed in which the presence of the anion at the cytoplasmic end of the pore facilitates channel opening (24). Because the anion moves along the electric field on the way through the pore to its binding site, gating becomes voltage-dependent and dependent on the ambient anion concentration. A refinement of this model suggested an external chloride binding site that moves together with the bound anion in the electric field and thus confers voltage sensitivity to the channel (27).

### Physiology and Pathophysiology of CLC Chloride Channels

**CIC-1, the Major Skeletal Muscle Chloride Channel, Is Mutated in Myotonia**

Skeletal muscle cells are large, multinucleated cells that result from the embryonic fusion of many myoblasts. It would be impossible for sarcolemmal action potentials to have any effect on the calcium stores in the center of a muscle cell, if it were not for the fact that the sarcolemma invaginates in form of the T-tubules to make contact with the calcium stores, i.e., the terminal cisternae of the sarcoplasmic reticulum. The long diffusion distances within the T-tubule system, however, hinder ion concentrations in efficiently equilibrating with the normal extracellular milieu and thus raise a problem: Repolarizing potassium currents would critically increase the intratubular potassium concentration after repeated action potentials and thereby depolarize the resting membrane potential. Beyond a certain limit, this would trigger new action potentials thus disturbing muscle relaxation after voluntary contraction. A distinct sarcolemmal chloride conductance normally resolves this problem by relieving the potassium conductance during action potential repolarization, thus preventing intratubular potassium accumulation. Because the intratubular (extracellular) chloride concentration by far exceeds intratubular potassium concentration, the effect of a decrease in intratubular chloride concentration during action potential repolarization can be ignored. Impaired muscle relaxation and muscle stiffness (myotonia) indeed is observed in several inherited diseases, which show a decrease in sarcoplasmic membrane chloride conductance (28,29).

Soon after expression cloning of CIC-0 from electrocytes (which can be imagined as modified polarized muscle cells) of the ray Torpedo, homology screening identified CIC-1 as a chloride channel gene expressed nearly exclusively in skeletal muscle (30). The perfect agreement between CIC-1 and the macroscopic skeletal muscle chloride conductance with regard to both developmentally regulated appearance of expression and electrophysiologic properties strongly suggested that CIC-1 is the molecular entity of this chloride conductance. This assumption was proven in the very same year by the observation of a mutated CIC-1 gene in the myotonic ADR mouse strain (31). The next year it was shown that mutations in the CIC-1 gene are also responsible for human myotonia congenita (32), which can be inherited as an autosomal recessive (Becker type) or as an autosomal dominant (Thomsen type) disease. Both forms are caused by mutations of CIC-1, the type of mutation determining the mode of inheritance. Mutations causing a total loss of CIC-1 function (e.g., severe truncations) lead to Becker type recessive disease, whereas all dominant mutations are missense mutations, which exert a dominant negative effect on CIC-1 channel function even of that encoded by the intact allele (almost always by shifting the voltage dependence of CIC-1 to very positive potentials) (33). This probably occurs via an effect on the common gate that controls both pores of the double-barreled channel.
ClC-2, a Hyperpolarization and Swelling Activated Chloride Channel with Unknown Function

ClC-2 is a nearly ubiquitously expressed chloride channel (34) that activates upon rather strong hyperpolarization of the membrane potential, cell swelling (35), and lowering of the extracellular pH (36,37). It was shown that this type of gating depends on an N-terminal peptide domain that was suggested to bind like a “ball” to the cytoplasmic loop between transmembrane regions D7 and D8 (35,37). In contrast to these detailed molecular insights into ClC-2 channel gating, information on the physiologic role of this chloride channel is still sparse. Hyperpolarization-activated currents similar to those observed in ClC-2-expressing Xenopus oocytes have been described in different cell lines with a comparable sensitivity to cell swelling (38,39). This might suggest a role of ClC-2 in cell volume regulation, although ClC-2 certainly does not represent cell swelling (38,39). This might suggest a role of ClC-2 in cell volume regulation, although ClC-2 certainly does not represent cell swelling (38,39). This might suggest a role of ClC-2 in cell volume regulation, although ClC-2 certainly does not represent cell swelling (38,39).
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Although the interspecies correlation of CIC-Ka/-Kb to CIC-K1/K2 on the basis of structural criteria is not feasible, on the basis of functional criteria derived from the described molecular genetic approaches we can now correlate CIC-Ka to CIC-K1 and CIC-Kb to CIC-K2. Loss of function mutations in the human CIC-Ka gene thus should give rise to diabetes insipidus-like symptoms, whereas the knockout of the CIC-K2 gene should provide an animal model for Bartter’s syndrome. Two highly exciting theories, which might be proven in the near future.

**CIC-3 and CIC-4 Are Closely Related to CIC-5, Another Renal Chloride Channel that Is Mutated in Dent’s Disease**

CIC-5 (61,62) belongs to a branch of the CLC family that also includes CIC-3 (63) and CIC-4 (64). Although only about 35% identical to the other branches, within this group they share a high degree of identity (approximately 80%). CIC-5 expression in *Xenopus* oocytes (62,65) or mammalian cells (65) induces strong outwardly rectifying chloride currents upon depolarization to membrane potentials more positive than about +20 mV. One would expect this odd voltage dependence to preclude a physiologic role of CIC-5 in most nonexcitable cells, unless there were additional (hitherto unidentified) subunits that could modify this voltage dependence to more physiologic values. Only recently was it possible to functionally express CIC-4 in *Xenopus* oocytes and mammalian cells (65). Its marked outward rectification and ion selectivity resembles CIC-5, as do its pH sensitivity and the effect of a mutation at the end of D3 (65). By contrast, functional expression of CIC-3 provided contradictory data. Although some studies failed to observe CIC-3-induced currents (62,65), other studies reported outwardly rectifying currents with an I > Cl selectivity (7,63,66,67). Moreover, CIC-3 was proposed to represent the molecular entity of the swelling activated chloride current (67), even though the described currents differed in rectification, single-channel conductance, and calcium selectivity from those reported earlier (66). Although supported by alterations of current characteristics after mutagenesis of a CIC-3 protein residue (67), in view of the other contradicting data this issue has to be clarified by additional studies.

The physiologic functions of CIC-3 and CIC-4, which show a broad expression pattern (63,64,68,69), still remain elusive. As in the case of CIC-Kb, however, an inherited kidney disease provided valuable insights into the functional role of CIC-5 that is predominantly expressed in this organ (61,62): Dent’s disease, which is caused by mutations in CIC-5 (70). Urinary loss of low molecular weight proteins is the most consistent abnormality of this X-linked kidney disorder. In addition, hypercalciuria is an early and common feature leading to secondary symptoms such as nephrocalcinosis and kidney stones that may culminate in renal failure. Many different CIC-5 mutations have been identified in Dent’s disease (70–74), most of them severely affecting CIC-5-induced chloride currents as determined in heterologous expression systems. This convincingly proves that Dent’s disease is due to disturbed CIC-5 channel function. The selective loss of small proteins points to a defect appeared after expression of a CIC-K2 splice variant lacking a complete transmembrane domain ([46]) and resemble endogenous oocyte currents elicited by several unrelated proteins ([57]). Together with the broad expression along the nephron, this circumstance would have rendered the enigma virtually unsolvable, to which native chloride current CIC-Kb/K2 can be correlated. Genetic evidence, however, answered this question: Mutations in the CIC-Kb gene cause Bartter’s syndrome (58). This inherited renal salt wasting disorder is characterized by a disturbed NaCl reabsorption in the TAL. This puts CIC-Kb into a functional relationship with the apical NaK2Cl cotransporter and the ROMK potassium channel, whose inactivation cause other variants of Bartter’s syndrome (59,60), suggesting a mechanism in which chloride is taken up apically by the cotransporter and exits basolaterally via CIC-Kb (Figure 2). The missing unambiguous electrophysiologic characterization, however, still hinders the correlation to native chloride currents described in the basolateral membrane of the TAL.

![Diagram](image-url)
in the proximal tubule, where filtered low molecular weight proteins are normally reabsorbed by endocytosis. Indeed, immunocytochemistry revealed that CIC-5 is located in subapical endosomes of the proximal tubule, where it colocalizes with the proton ATPase and with reabsorbed protein (75–77). Thus, it is reasonable to speculate that CIC-5 may allow chloride to enter the endosome and dissipate the positive charge generated during acidification by the proton ATPase. Impaired acidification of this intracellular compartment due to loss of function of CIC-5 could in turn affect endocytic processes, thus causing defective proximal tubular reabsorption of proteins (Figure 2). It is likely that other chloride channels, probably also from the CLC family, fulfill similar functions in other cells and organelles. A role for CLC channels in ionic homeostasis of intracellular organelles is now well established for the yeast CLC gef1p (12,78).

It is unclear how this process could explain the increased intestinal calcium absorption and elevated serum concentrations of active vitamin D in this disorder (79,80). In some studies, CIC-5 was shown to be expressed in the TAL of Henle’s loop (76,77), which is the major site of renal calcium reabsorption. However, calcium reabsorption in this nephron segment occurs paracellularly driven by a positive luminal potential that results from the transcellular net transport of 2 ions combined with 1 Na ion (Figure 2). A disturbance in this process readily explains the hypercalciuria observed in Bartter’s syndrome, but it does not explain it in Dent’s disease, particularly as NaCl reabsorption in the TAL is not affected. The mechanism leading to hypercalciuria thus is still obscure (81). Recently, CIC-5 expression in a mouse model was reduced by a ribozyme approach (82). Surprisingly, although there was no change in CIC-5 mRNA levels within the kidney, an approximately 70% decrease in the amount of CIC-5 protein was observed. The transgenic ribozyme-expressing animals showed no obvious phenotype, and their renal function obviously was normal except for a borderline hypercalciuria. A barely significant elevation of urinary calcium was observed in male, but not female, animals under high calcium diet during the first few months after birth. It may be expected that a total knockout of the CIC-5 gene leads to a more convincing phenotype.

In the collecting duct in addition to β-intercalated cells, expression of CIC-5 has also been reported in the acid-secreting α-intercalated cells (75,77), where it again largely colocalizes with the proton ATPase in intracellular vesicles and in the apical plasma membrane (Figure 2). Although it seems reasonable to assume a role in proton secretion also in these cells, the physiologic importance of this observation is uncertain because urinary acidification is normal in the majority of patients with Dent’s disease (83).

CIC-6 and CIC-7, Widely Expressed Chloride Channels with not yet Determined Function

The third known branch of the CLC gene family is represented by two CLC proteins that only share approximately 30% identity with the other CLC family members: CIC-6 and CIC-7 (84). Both proteins are expressed in nearly all tissues, and they appear early in mouse development. The genomic structure of both genes was investigated (85). As shown for CIC-6 (86), these proteins may be localized to intracellular compartments, thus explaining the failure to measure chloride currents in heterologous expression systems (47,57). Additional studies will be needed to elucidate the physiologic functions of these putative channel proteins.

Conclusion

The successive unveiling of the CLC chloride channel gene family yielded important insights into the variety of physiologic functions served by particular CLC channels. As impressively illustrated by several inherited diseases, the breadth of CLC channel functions extends from regulation of electrical excitability over pH regulation of intracellular compartments to transepithelial transport. Physiologic mechanisms as different as maintenance of normal muscle tonus or generation of the exceptional renal medullary tonicity depend on proper CLC channel function. Although in the past few years much has been learned about their structure, we are only beginning to understand how the different structural features of the CLC family members correspond to this diversity of functions. Moreover, there remain CLC channels with unclear physiologic importance. Both of these questions will give forthcoming studies strong impetus.

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