# 8 Chloride Transporting CLC Proteins[1]

Michael Pusch

## 8.1 Introduction

In the early 1980s, Chris Miller and colleagues described a curious "double-barreled" chloride channel from the electric organ of *Torpedo* fish reconstituted in planar lipid bilayers (Miller and White, 1980). Single-channel openings occurred in "bursts" separated by long closures. A single burst was characterized by the presence of two open conductance levels of equal size and the gating (i.e., openings and closings) during a burst could be almost perfectly described as a superposition of two identical and independent conductances that switched between open and closed states with voltage-dependent rates $\alpha$ and $\beta$ (Hanke and Miller, 1983) (Fig. 8.1).

$$\mathbf{c} \underset{\beta}{\overset{\alpha}{\longleftrightarrow}} \mathbf{o}$$

These relatively fast openings and closing events gave rise to the name "fast gate" for these gating transitions during a burst. But how could it be excluded that these events just represent the presence of two identical channels in the bilayer? It was the presence of the long inter-burst closed events, during which no channel activity was observed, that demonstrated that the two "protopores" were tied together in a molecular complex, and could be inactivated by the so-called slow gate. From these results Miller formulated the "double-barreled" model according to which the channel consisted of two physically distinct, identical protopores, each with a proper fast gate and an additional common gate that acts simultaneously on both protopores (Miller and White, 1984) (Fig. 8.1).

For a relatively long time this double-barreled $Cl^-$ channel remained a somewhat unique curiosity with little physiological relevance. This situation changed dramatically with the molecular cloning of the *Torpedo* channel by Jentsch and co-workers (Jentsch et al., 1990) in 1990, and immediately afterwards with the identification of mammalian homologues. Numerous novel physiological functions of the various CLC homologues were discovered (Jentsch et al., 2005; Pusch and Jentsch, 2005). We now know that CLC proteins are a large structurally defined family of $Cl^-$ ion channels and $Cl^-/H^+$ antiporters with nine distinct genes in mammals. The

[1] Portions reprinted, with permission, from Pusch, M., and T.J. Jentsch. 2005. Unique structure and function of chloride transporting CLC proteins. IEEE Trans. Nanobioscience 4:49–57.

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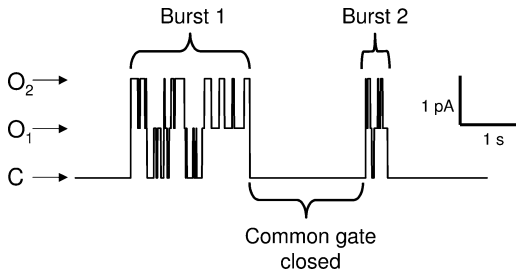


**Fig. 8.1** The double-barreled ClC-0 channel. A simulated single channel trace of ClC-0 is shown in which three conductance levels (C = closed; $O_1$, $O_2$ open conductance levels) are seen. Activity occurs in bursts and during a burst the probability of observing one of the conductance levels is distributed binomial as if activity arises from two independent and identical pores. The slow common gate acts on both pores simultaneously.

membrane-embedded part of CLC proteins bears no obvious similarity to any other class of membrane proteins, while the cytoplasmic C-terminus of most eukaryotic and some prokaryotic CLCs contains two regions with homology to cystathionine beta synthase (CBS) domains that are found in other proteins as well. Different members serve a broad range of physiological roles including stabilization of the membrane potential, transepithelial ion transport, and vesicular acidification. Their physiological importance is underscored by the causative involvement in at least four different human genetic diseases. The homodimeric architecture with two physically separate ion conduction pathways that was anticipated from functional studies of the *Torpedo* homologue ClC-0 was fully confirmed by solving the crystal structure of prokaryotic CLC homologues. The determination of the crystal structure of bacterial homologues marked at breakthrough for the structure-function analysis of these proteins (Dutzler et al., 2002, 2003). The structure revealed a complex fold of 18 α-helices per subunit with at least two $Cl^-$ ions bound in the center of each protopore. A critical glutamic acid residue was identified whose side-chain seems to occupy a third $Cl^-$ ion binding site in the closed state and that moves away to allow $Cl^-$ binding.

A big surprise was then the recent discovery that the bacterial CLC homologue that was used for crystallization is not a $Cl^-$ ion channel but a $Cl^-/H^+$ antiporter (Accardi and Miller, 2004). Subsequently, the mammalian CLC-4 and CLC-5 proteins were shown to be $Cl^-/H^+$ exchangers as well, and not $Cl^-$ ion channels, as previously assumed (Picollo and Pusch, 2005; Scheel et al., 2005). Thus, CLC proteins are either high-throughput voltage- and substrate-gated $Cl^-$ ion channels or strictly coupled, low throughput secondary active electrogenic $Cl^-/H^+$ transporters. It will be interesting to find out, how the same basic molecular architecture allows for these seemingly different functions. Also, the physiological and pathophysiological role of $Cl^-/H^+$ exchange activity is still an unexplored territory. In the present chapter, the history and the present knowledge about CLC chloride channels and transporters will be covered including the recent developments.

## 8.2 Overview Over the Family of CLC Proteins

Expression cloning was used to isolate the first member of the CLC family, the *Torpedo* channel ClC-0 (Jentsch et al., 1990). This channel is highly expressed in the electric organ of electric rays. It localizes to the noninnervated membrane of the electrocyte, where it serves to stabilize the voltage across that membrane and to pass large currents that are generated by the depolarizing influx of sodium through acetylcholine receptor channels located in the opposite, innervated membrane.

The sequence of ClC-0 (Jentsch et al., 1990) revealed a protein of about 90 kDa with a large hydrophobic core that was predicted to be able to span the membrane at least 10 times and a large predicted cytoplasmic tail. The channel properties of the $Cl^-$ current measured in oocytes after expressing the single cDNA coding for the ClC-0 protein were virtually identical to those described for the native, in bilayers reconstituted *Torpedo* channel (Bauer et al., 1991). In particular, the channel showed the typical double-barreled behavior (see Fig. 8.1). This showed that no additional subunits are required to form a completely and functional ClC-0 channel. Of note, the protein sequence of ClC-0 showed no significant homology to any other known ion channels, including the cAMP (cyclic adenosine monophosphate)-activated $Cl^-$ channel called cystic fibrosis transmembrane conductance regulator (CFTR) and the ligand-gated $GABA_A$ (gamma amino butyric acid) and glycine receptor $Cl^-$ channels. Thus, ClC-0 represented an entirely new channel class.

The isolation of ClC-0 opened the gate for cloning an entire family of CLC chloride channels by homology. Eventually, CLC homologues were found in all phyla. In mammals, the CLC family comprises nine distinct members. Based on sequence similarity, they can be grouped into three branches (Fig. 8.2). Members of the first

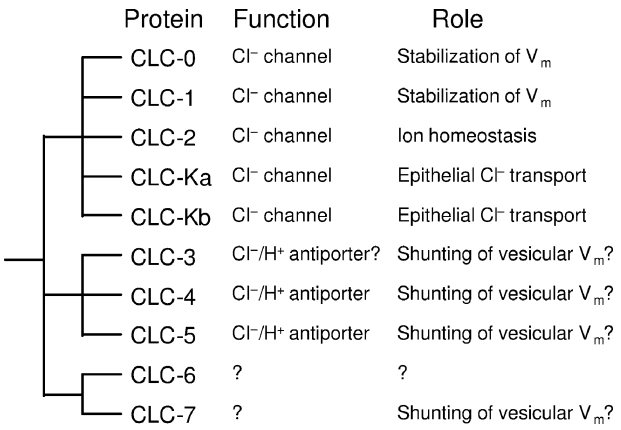


**Fig. 8.2** The CLC family of $Cl^-$ channels and $Cl^-/H^+$ transporters. The schematic dendrogram highlights the sequence similarity among the three groups in which the nine human CLC homologues can be classified. The *Torpedo* channel ClC-0 is included in the figure because it serves as an important model channel for functional studies. The *E. coli* homologue, ClC-ec1 (not shown in the figure), is most closely related to the ClC-3–ClC-5 branch.

branch (ClC-1,-2, -Ka, and -Kb) perform their function in the plasma membrane. Also, the *Torpedo* channel ClC-0 belongs to this class. In contrast, the members of the two other branches (comprising ClC-3, -4, -5, and ClC-6 and -7, respectively) are predominantly found in membranes of intracellular vesicles, in particular the endosomal–lysosomal pathway. Despite their predominant intracellular localization in vivo, ClC-3, ClC-4, and ClC-5 mediated currents can be measured in the plasma membrane in heterologous expression systems like *Xenopus* oocytes or transfected cells (Steinmeyer et al., 1995; Friedrich et al., 1999; Li et al., 2000). These three proteins, ClC-3, ClC-4, and ClC-5, share about 80% sequence identity, and have very similar functional properties. In contrast, no reproducible currents have been reported for ClC-6 and ClC-7 (Brandt and Jentsch, 1995; Buyse et al., 1997; Jentsch et al., 2002) and consequently, nothing is known about the biophysical properties of ClC-6 and ClC-7.

The diverse and important physiological functions of mammalian CLC proteins were impressively demonstrated by the pathologies resulting from their mutational inactivation in human genetic diseases, in mouse models, and in other representative model organisms, like *Caenorhabditis elegans* (Schriever et al., 1999; Strange, 2003) or *Arabidopis thaliana* (Hechenberger et al., 1996; Barbier-Brygoo et al., 2000). CLC proteins are also present in many prokaryotes (Mindell and Maduke, 2001), and so far the only structural data available are actually from bacterial CLCs (Mindell et al., 2001; Dutzler et al., 2002, 2003). At striking difference to what had been expected a priori, the bacterial CLCs whose crystal structure had been determined, turned out to be secondary active $Cl^-/H^+$ exchangers, and definitely not $Cl^-$ ion channels (Accardi and Miller, 2004). We now know that also some mammalian CLCs are $Cl^-/H^+$ exchangers (Picollo et al., 2004; Scheel et al., 2005). However, it is not known if any prokaryotic CLC behaves as a $Cl^-$ channel. Yet, the molecular and functional diversity in the microbe world is probably much larger than that anticipated from our human-centered viewpoint (Kung and Blount, 2004). Nevertheless, the current chapter focuses mainly on mammalian CLC proteins, with the *Torpedo* ClC-0 as the model CLC $Cl^-$ channel.

The electric organ of *Torpedo* is derived from skeletal muscle, and skeletal muscle is known to possess a large background chloride conductance (Bryant and Morales-Aguilera, 1971; Bretag, 1987). In fact, the first CLC homologue to be cloned after the *Torpedo* channel was ClC-1, the major skeletal muscle chloride channel (Steinmeyer et al., 1991b). Similar to ClC-0, its function is to stabilize the plasma membrane voltage of skeletal muscle fibers. Its disruption in a natural mouse mutant (Steinmeyer et al., 1991a) and mutations in humans (Koch et al., 1992; George et al., 1993; Pusch, 2002) and defects in ClC-1 splicing (Charlet et al., 2002; Mankodi et al., 2002) lead to myotonia, a form of muscle stiffness. The $Cl^-$ equilibrium potential in skeletal muscle is close to the $K^+$ equilibrium potential, and the lack of repolarizing current through ClC-1 leads to muscle hyperexcitability. Thus, stimuli that normally elicit just one action potential give rise to trains of action potentials, so-called myotonic runs (Adrian and Bryant, 1974), resulting in impairment of muscle relaxation. Interestingly, myotonia in humans can be inherited as a recessive or a

dominant trait. Many recessive myotonic mutations, like early stop codons, lead to a complete loss of function of the affected allele. The fact that heterozygous carriers of such mutations do not show myotonia demonstrates that a 50% gene-dosage is not sufficient to cause myotonia. In dominant myotonia, the mutant protein can still associate with wild-type (WT) ClC-1 subunits to form dimeric channels. Frequently, dominant mutations exert a dominant negative effect by changing the voltage-dependence of the dimer through the common gate (Pusch et al., 1995b; Saviane et al., 1999). As expected, dominant myotonia is clinically less severe as 25% of the channels are composed entirely of WT subunits in heterozygous patients.

In contrast to ClC-1, which is nearly exclusively expressed in skeletal muscle, ClC-2 is broadly, if not ubiquitously expressed (Thiemann et al., 1992). Its currents may be activated by hyperpolarization, cell swelling, and moderate extracellular acidification (Gründer et al., 1992; Jordt and Jentsch, 1997; Arreola et al., 2002). Many functions were attributed to ClC-2. Those roles, however, were not confirmed by a ClC-2 knockout (KO) mouse, which unexpectedly revealed testicular and retinal degeneration that were attributed to defects in transepithelial transport across Sertoli cells and the retinal pigment epithelium, respectively (Bösl et al., 2001; Nehrke et al., 2002). Although ClC-2 might play some role in regulating the intracellular chloride in neurons (Staley et al., 1996) and thereby affect inhibitory neurotransmission, ClC-2 KO mice lacked signs of epilepsy (Bösl et al., 2001). In contrast to these clear results from KO mice, in humans, heterozygous ClC-2 mutations were found to be associated with epilepsy in a few families (Haug et al., 2003). However, several key findings of the functional analysis of these mutations (Haug et al., 2003) could not be reproduced (Niemeyer et al., 2004). Additional families with mutations that have a more clear-cut functional effect are probably necessary to firmly establish the gene coding for ClC-2 as being associated with epilepsy. Thus, while ClC-2 is expressed in neurons, its precise role in these cells is not clear. Currents that are probably mediated by ClC-2 are also observed in astrocytes (Ferroni et al., 1997) and immunocytochemistry localized ClC-2 to astrocytic endfeet (Sik et al., 2000). It is thus conceivable that ClC-2 is involved in the $Cl^-$ ion homeostasis mediated by astrocytes.

For some time a controversy existed regarding the polarized expression of ClC-2 in epithelial cells. Transepithelial, i.e., vectorial ion and water transport depends critically on the expression of the specific transporter and channel proteins on either the apical, i.e., lumen or "outside-world" side, or the basolateral, or "blood facing" side of the epithelial cells. These different membrane compartments are separated by tight junctions and the correct targeting of membrane proteins depends on specific signal sequences that are recognized in the biosynthetic and/or the recycling pathways that deliver the membrane proteins via the fusion of small vesicles (Muth and Caplan, 2003). Several studies had reported an apical localization of ClC-2 (Blaisdell et al., 2000; Gyömörey et al., 2000) while others favored a basolateral localization (Lipecka et al., 2002; Catalán et al., 2004). Assuming an apical localization it was proposed that activation of endogenous ClC-2 channels might provide an alternative $Cl^-$ conductance pathway in epithelia of patients with

cystic fibrosis (Thiemann et al., 1992; Schwiebert et al., 1998), a genetic disease caused by the dysfunction of another, non-CLC $Cl^-$ channel called CFTR (Riordan et al., 1989). However, results from double KO mice lacking both, CFTR and ClC-2, demonstrated that this is not a valid hypothesis (Zdebik et al., 2004). In agreement with this negative result, it has recently been unequivocally demonstrated that ClC-2 is localized basolaterally in intestinal epithelia (Peña-Münzenmayer et al., 2005). Interestingly, in this study a putative di-leucine motif in the C-terminal cytoplasmic CBS2 domain was identified as being important for the correct targeting of ClC-2 (Peña-Münzenmayer et al., 2005).

ClC-2 has also been proposed to be an apical $Cl^-$ channel that is important for gastric acid secretion (see Jentsch et al., 2005 for discussion), but also this role could not be confirmed (Hori et al., 2004).

Thus, while ClC-2 is broadly expressed, its elimination in KO mice leads to a very specific and limited phenotype in testis and retina (Bösl et al., 2001). However, its precise cellular role in these tissues and in all other tissues where its function can be demonstrated, is not yet clearly established.

ClC-Ka and ClC-Kb are two highly homologous channels that are both expressed in certain epithelial cells of the kidney, as well as in epithelia of the inner ear (Uchida et al., 1993; Kieferle et al., 1994; Estévez et al., 2001). It is now known that these plasma membrane $Cl^-$ channels need a small accessory β-subunit, barttin, for their transport to the plasma membrane (Estévez et al., 2001). Both channels are important for transepithelial transport in kidney and in the inner ear. Mutational inactivation of ClC-Kb in humans leads to Bartter syndrome, a disease associated with severe renal salt wasting, because this channel plays an important role in NaCl reabsorption in a certain nephron segment (the thick ascending limb, TAL) (Simon et al., 1997). In this segment a NaK2Cl-cotransporter takes up $Na^+$, $K^+$, and $Cl^-$ ions at the apical membrane, in an electroneutral manner. Potassium ions are "recycled" back to the tubular lumen through the ROMK $K^+$ channel, generating a negative intracellular membrane potential, helping to extrude $Cl^-$ ions at the basolateral membrane through the CLC-Kb/barttin channel. $Na^+$ is extruded through the Na–K–ATPase at the basolateral membrane, that provides the energy for this vectorial NaCl transport. Interestingly, also mutations in the apical NaK2Cl co-transporter and mutations in the apical $K^+$ channel ROMK lead to Bartter's syndrome, with slightly different phenotypes (Simon et al., 1996a,b). The disruption of ClC-K1 in mice (probably equivalent to ClC-Ka in humans) leads to renal water loss as its expression in the thin limb is important for the establishment of a high osmolarity in kidney medulla (Matsumura et al., 1999). The "counter-current system" in the kidney serves to retain water and to produce urine of high osmolarity in situations of limited water supply (Greger and Windhorst, 1996) and, in particular, a large $Cl^-$ conductance in the thin ascending limb of the loop of Henle appears to be necessary, a conductance possibly mediated by ClC-Ka/barttin (Matsumura et al., 1999). The precise localization of the channel (only apical or apical and basolateral) is, however, not yet clearly resolved (Uchida et al., 1995; Vandewalle et al., 1997). It must be said that the precise role of CLC-Ka in the human kidney is, still, not fully resolved. It may be that the murine

CLC-K1 channel is not the exact functional homologue of the human CLC-Ka. Indeed, the two human CLC-K homologues are more closely related to each other than each one is two either murine CLC-K (Kieferle et al., 1994). The two CLC-K genes are localized very close to each other on chromosome 1, raising the possibility that they arose from a recent gene-duplication, after the separation of the human and mice lineages. Alternatively, the evolution of the two human genes might have been associated with convergent recombination events, reducing the sequence difference between them, and resulting in slightly different localization and function compared to the respective murine genes.

In humans, the loss of barttin, the common β-subunit of ClC-Ka and ClC-Kb, leads to sensineural deafness in addition to renal salt loss (Birkenhäger et al., 2001). This disease is also called type IV Bartter's syndrome, while mutations in CLC-Kb lead to type III Bartter's syndrome. The hearing loss in type IV Bartter's syndrome has been attributed to a defect in potassium secretion by inner ear epithelia. In these cells, ClC-Ka/barttin and ClC-Kb/barttin are needed for the basolateral recycling of chloride that is taken up by a basolateral sodium–potassium-two-chloride cotransporter (Estévez et al., 2001). The basolateral localization of ClC-Kb and ClC-Ka in the inner ear suggests that both channels might be localized exclusively in the basolateral membrane also in the kidney. Loss of barttin impairs the function of both ClC-Ka and ClC-Kb. Consequently, the renal disease phenotype in type IV Bartter's syndrome is more severe than that for patients having mutations only in ClC-Kb. However, many mechanistic aspects of the interaction between ClC-K channels and barttin are still poorly understood. Recently, patients with mutations in genes coding for both ClC-Ka and ClC-Kb, but not in the gene coding for barttin, have been described (Schlingmann et al., 2004). The disease phenotype was similarly severe as that found for patients with a loss of function of barttin. This result demonstrates that CLC-Ka is of functional relevance in humans and not e.g., a pseudogene. Barttin seems to associate exclusively with CLC-K channels but not other CLC proteins (Estévez et al., 2001). Furthermore, no close homologs of barttin seem to be present in the human genome. Thus, barttin is the only true β-subunit of CLC proteins, even though other cellular proteins have been shown to interact with CLC-proteins (Ahmed et al., 2000; Furukawa et al., 2002; Rutledge et al., 2002; Zheng et al., 2002; Gentzsch et al., 2003; Hryciw et al., 2003; Embark et al., 2004; Hryciw et al., 2004; Hinzpeter et al., 2005). The determination of the precise physiological relevance of these interactions remains an interesting and important future objective of research (Dhani and Bear, 2005).

The physiological roles of intracellular CLC proteins are best understood for ClC-5 and ClC-7. In humans, the mutational loss of ClC-5 leads to Dent's disease, an inherited kidney stone disorder which is also associated with the loss of proteins into the urine (Lloyd et al., 1996). ClC-5 is predominantly expressed in the proximal tubule of the kidney, where it localizes to apical endosomes (Günther et al., 1998). The knock-out of ClC-5 in mice has revealed that the lack of this channel impairs endocytosis of protein and fluid-phase markers (Piwon et al., 2000; Wang et al., 2000). Further, the loss of phosphate and calcium into the urine, which ultimately

leads to kidney stones, may be explained by the decreased renal endocytosis and processing of calciotropic hormones (Piwon et al., 2000). The decrease in endocytosis is associated with an impairment of the luminal acidification of endosomes (Piwon et al., 2000; Günther et al., 2003; Hara-Chikuma et al., 2005). The simplest model to explain a decrease in the acidification rate would be that ClC-5 functions as a $Cl^-$ channel and that this channel activity is important to neutralize the electric current of the $H^+$-ATPase that acidifies these vesicles (Piwon et al., 2000; Jentsch et al., 2005). Without such a neutralization, the voltage over the endosomal membrane would inhibit further $H^+$-pumping, severely limiting endosomal acidification. However, as outlined in more detail below, recently it has been demonstrated that ClC-4 and ClC-5 (and probably also ClC-3) are actually not $Cl^-$ channels but secondary active $Cl^-/H^+$ exchangers (Picollo and Pusch, 2005; Scheel et al., 2005), similar as the bacterial *E. coli* homologue ClC-ec1 (Accardi and Miller, 2004). The precise physiological role of the ClC-5 is thus still relatively unclear.

Vesicular acidification is apparently also the major role of ClC-3, a $Cl^-$ channel or $Cl^-/H^+$ transporter expressed on synaptic vesicles in addition to endosomes (Stobrawa et al., 2001; Li et al., 2002). The genetic knock-out of ClC-3 in mice led to blindness and to a severe degeneration of the hippocampus (Stobrawa et al., 2001). The mechanism of this degeneration, however, is incompletely understood. ClC-3 has also been proposed to act as a "volume regulated" $Cl^-$ channel by several authors (see Jentsch et al., 2002 for review), but these findings could not be reproduced by several laboratories. In fact, ClC-3 is about 80% identical in protein sequence to ClC-4 and ClC-5, and these proteins have very similar biophysical properties (Li et al., 2000; Picollo and Pusch, 2005).

ClC-7 is the only mammalian CLC protein that is prominently expressed on lysosomes in addition to late endosomes (Kornak et al., 2001; Kasper et al., 2005). Surprisingly, its disruption in mice led to a severe osteopetrotic phenotype (Kornak et al., 2001). It was shown that in bone-resorbing osteoclasts, ClC-7 can be inserted together with the $H^+$-ATPase into its ruffled border, a specialized plasma membrane that faces the so-called resorption lacuna. Osteoclasts attach to the bone material, forming a shielded environment, that is also called extracellular lysosome. An acidification of the resorption lacuna is necessary for the degradation of bone. On the one hand, the acid directly dissolves the inorganic bone structure. On the other hand, an acidic pH is needed for specialized proteases that degrade organic material. A steady balance between bone formation mediated by osteoblasts and bone degradation mediated by osteoclasts guarantees the maintenance of bone mass and provides the exquisitely stable structure of bone. Osteoclasts obtained from ClC-7 KO mice fail to acidify the resorption lacuna, readily explaining the osteopetrotic phenotype of the mice (Kornak et al., 2001). Bones in these mice are denser but also more fragile. This phenotype suggested that ClC-7 might also be mutated in human osteopetrosis. Indeed, ClC-7 mutations were found in autosomal recessive malignant infantile osteopetrosis (Kornak et al., 2001) and later also in an autosomal dominant form of the disease (Cleiren et al., 2001). Like with myotonia due to ClC-1 mutations, autosomal dominant osteopetrosis is clinically more benign since 25%

of all ClC-7 dimers are expected to consist entirely of WT subunits, while recessive osteopetrosis is equivalent to a complete loss of ClC-7 function. In addition to osteopetrosis, knock-out of ClC-7 led to blindness and neurodegeneration (Kornak et al., 2001; Kasper et al., 2005). The blindness was caused directly by retinal degeneration and not indirectly by the obstruction of the optic canal (Kornak et al., 2001; Kasper et al., 2005). ClC-7 KO mice survived only for about 6 weeks and it was thus difficult to study in detail the neurodegenerative phenotype of these mice (Kornak et al., 2001). To overcome this problem Kasper et al. (2005) rescued the osteopetrotic phenotype of the ClC-7 mouse by reintroducing the ClC-7 gene under the control of an osteoclast specific promoter. These mice had severe neurodegeneration with the accumulation of electron-dense material in lysosomes, characteristic of a lysosomal storage disease such as neuronal ceroid lipofuscinosis. Surprisingly, however, the pH of the lysosomes was not different in these animal compared to that from WT mice. Thus, the precise role ClC-7 for lysosomal function is not yet fully understood (Kasper et al., 2005).

ClC-4 and ClC-6 remain the only CLC channels for which no disease or mouse knock-out phenotype has been described as yet. The physiological functions of CLC channels, and the pathologies resulting from their disruption, are nonetheless impressive and reveal the previously unsuspected importance of chloride channels. Furthermore, the biophysical effects of CLC mutations found in human disease have often been studied and have greatly increased our understanding of the structure–function relationship of this channel class.

We have learnt that several CLC proteins fulfill their physiological roles in specific intracellular membranes while others are directed to the plasma membrane, either "alone" or in collaboration with the barttin subunit in the case of CLC-K channels. The correct targeting is, of course, of crucial importance. Nevertheless, practically nothing is known about the signals that must be contained in the structure of the various CLC proteins that target them to different membrane compartments within the eukaryotic cell. Several research groups have tried to re-direct intracellular CLCs like ClC-7 to the plasma-membrane using chimeric constructs, without success (e.g., Traverso and Pusch, unpublished results). Thus, the signals that address the intracellular CLC transporters to specific endomembranes are probably not simple "motifs" that are coded by a few amino acids, but represent more complex structural entities that involve several parts of the protein.

## 8.3 Architecture of CLC Proteins

The double-barreled appearance of the single-channel data obtained after reconstitution into lipid bilayers (Miller and White, 1980) strongly suggests by itself at least a homodimeric architecture because it is difficult to imagine how a single polypeptide can form two independent and equal conductance pathways. However, a direct test of this hypothesis is difficult with functional data on native proteins alone. After the cloning of ClC-0 (Jentsch et al., 1990), several important questions regarding

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the double-barreled structure of CLC proteins could be answered using site-directed mutagenesis and functional electrophysiological analysis of mutated channels expressed in "heterologous" systems like frog oocytes, lipid bilayers, and mammalian cell lines. Early on, a multimeric structure was suggested on the basis of dominant negative disease-causing mutations of the muscle channel ClC-1 (Steinmeyer et al., 1994; Pusch et al., 1995b). Such mutants are able to suppress the currents of WT subunits when co-expressed. Using mutations that altered fast gating, slow gating, and/or channel conductance of ClC-0 and combining altered and unaltered channel subunits a homodimeric architecture could be established (Ludewig et al., 1996; Middleton et al., 1996). For example, the cDNA coding for a single subunit can be genetically linked to another copy, that might carry a mutation or not, such that the N-terminus of one subunit is attached to the C-terminus to another subunit (both termini are cytoplasmic). It was then found that when mutations that altered pore properties like single-channel conductance or ion selectivity, or mutations that altered the fast gate, were introduced in one of the two subunits, the resulting single channels behaved like a superposition of an unaltered pore and a mutated pore (Ludewig et al., 1996). In this respect, the two subunits appeared completely independent. However, the presence of a mutation (S123T) in one of the subunits was sufficient to abolish the voltage-dependence of the "slow gate" that closes both pores simultaneously (Ludewig et al., 1996). This shows that the two subunits functionally interact. However, the nature of the slow gate is still one of the biggest secrets of CLC proteins (see below).

It could also be shown that each pore of the double-barreled channel is formed entirely from one subunit (Ludewig et al., 1996; Weinreich and Jentsch, 2001). This architecture is fundamentally different from that found, e.g., in $K^+$ channels that are fourfold symmetric tetramers in which the pore is formed in the central symmetry axis (Doyle et al., 1998). The $K^+$ channel architecture poses restrictions on the topology of the pore: it has to be straight and perpendicular to the membrane. No such restrictions apply to CLC-channels and indeed the crystal structure of bacterial CLC homologues did not reveal a clear straight ion-conducting pore (Dutzler et al., 2002). The crystal structure of two bacterial homologues, a *Salmonella* and an *E. coli* homologue have been determined (Dutzler et al., 2002). The two structures are very similar and thus only the *E. coli* CLC-ec1 structure, for which more recently a higher resolution was obtained (Dutzler et al., 2003), will be discussed here. The overall architecture of CLC proteins revealed by the crystal structure is strikingly consistent with the double-barreled shotgun cartoon: several nearby $Cl^-$ ion binding sites were identified in each subunit, indicating probably the most selective permeation points, but the binding sites of the two subunits are far from each other ($>\sim$40 Å) consistent with the independence of the permeation process in the two pores.

Overall, the crystal structure displays a complex fold with 18 α-helices per subunit. Unexpectedly, the structure revealed an internal pseudo-symmetry within each subunit: the N-terminal half and the C-terminal half have a very similar fold and also some spurious sequence similarity but are oppositely oriented in the membrane. The two halves of each subunit "sandwich" around the central $Cl^-$ ion binding sites

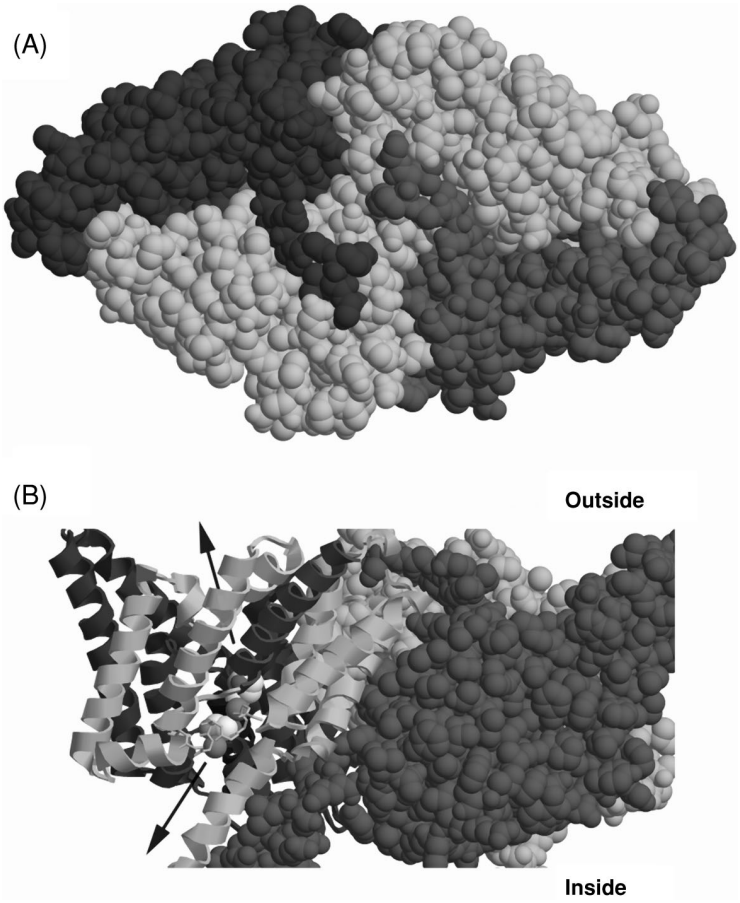


**Fig. 8.3** Overall architecture of ClC-ec1. In (A) the protein is viewed in spacefill representation from the extracellular side. The pseudo-twofold internal symmetry for each subunit is highlighted with the different gray shades for each half-subunit. In (B) the protein is rotated such that the view is more lateral. One subunit is shown in cartoon with a small stretch of helix J shown as backbone-trace for clarity. Chloride ions are shown in light gray, and the amino acids S107 (between the two $Cl^-$ ions) and E148 (above the top $Cl^-$ ion) are shown in spacefill. Arrows indicate the probable extracellular and intracellular ion entry/exit pathways. This figure was prepared with the RasTop program that is based on Rasmol (Sayle and Milner-White, 1995) using the pdb-entry 1OTS from which water and Fab fragments have been removed for clarity.

with helix-ends pointing into the center of the membrane. Thus, CLC proteins have possibly evolved by gene duplication of an ancient protein with half of the length of the "modern" version and assembled as a homotetramer in a dimer-of-dimers arrangement (Fig. 8.3).

The dimeric architecture of CLC proteins could enable, in principle, the formation of heterodimeric complexes with two different subunits. In vitro, the formation of heterodimers could indeed be observed between the plasma membrane channels

ClC-1 and ClC-2 (Lorenz et al., 1996) and ClC-0 and ClC-2 (Weinreich and Jentsch, 2001). Such dimers could be obtained either by co-injecting the respective subunits into *Xenopus* oocytes or by linking two subunits in a single polypeptide in a head-to-tail fashion. The dimeric interface between the two subunits is extensive (Fig. 8.3) and the formation of heterodimeric CLC proteins is probably only possible if both subunits are sufficiently similar in sequence and structure to mimic a homodimeric configuration. Indeed, no functional dimers could be obtained by linking, for example, ClC-7 or ClC-6 to ClC-0 (Brandt, Pusch, and Jentsch, unpublished observation), even though other reasons, like improper plasma membrane targeting, may underlie these negative results. Physiologically, ClC-1/ClC-2 heterodimers are probably of little relevance as suggested by the nonoverlapping phenotype of the respective knock-out mouse models (Steinmeyer et al., 1991a; Bösl et al., 2001). The members of the ClC-3–5 branch are highly homologous and, in particular, ClC-4 and CLC-5 show considerable overlap in their expression profiles. In fact, a physiologically relevant hetero-dimerization of ClC-4 and ClC-5 was suggested by co-immunoprecipitation experiments (Mohammad-Panah et al., 2003). Before describing in more detail the atomic structure of CLC-ec1, basic functional properties of mammalian CLC-channels will be discussed.

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## 8.4 Gating of CLC-0 and Mammalian CLC Channels

As described above, functionally and structurally, animal CLC proteins can be divided into three classes. The first class consists of plasma-membrane channels (ClC-1, ClC-2, ClC-Ka, and ClC-Kb) and also the *Torpedo* channel ClC-0 belongs to this class. These proteins are clearly $Cl^-$ ion channels in contrast to some bacterial homologues and ClC-4 and ClC-5, that are instead secondary active $Cl^-/H^+$ antiporters (see below). Features of these plasma membrane proteins that identify them as channels are a directly measurable single-channel conductance ($\geq$1 pS) (Miller, 1982; Bauer et al., 1991; Pusch et al., 1994; Weinreich and Jentsch, 2001) (Alessandra Picollo and M.P., unpublished results); a reversal potential measured in $Cl^-$ gradients that follows the Nernstian prediction of passive diffusion (Ludewig et al., 1997a; Rychkov et al., 1998; Thiemann et al., 1992); and relatively slow macroscopic gating relaxations that depend on voltage, $Cl^-$ concentration and pH. These channels will be discussed in more detail below. The second class comprises the highly homologous proteins ClC-3, ClC-4, and ClC-5. In vivo, these proteins are mostly expressed in intracellular organelles but at least some functional plasma membrane expression can be achieved in heterologous systems (Steinmeyer et al., 1995; Friedrich et al., 1999; Li et al., 2000) allowing an electrophysiological characterization. Currents induced by these proteins are characterized by an extreme outward rectification—corresponding to movement of $Cl^-$ ions from the extracellular side into the cytoplasm or correspondingly to a movement from the lumen of intracellular vesicles into the cytosol. Currents are measurable only for voltages $>\sim$ +20–40 mV, such that a true reversal potential cannot be measured (Steinmeyer et al., 1995;

Friedrich et al., 1999). Current activation at positive voltages has apparently two kinetic components. A minor component shows relatively slow kinetics while the major component of the current is associated with very fast activation and deactivation kinetics that cannot be resolved using standard electrophysiological techniques. These fast kinetics may actually represent an "instantaneous" activation, i.e., a rectification of the open pore. Noise analysis supports this hypothesis (Hebeisen et al., 2003), however, the single-channel properties of ClC-3, -4, or -5 are still unclear (Vanoye and George, 2002; Hebeisen et al., 2003). Also, the relationship of the double-barreled structure and the functional properties is unknown. The functional properties of ClC-3–ClC-5 are unusual for an ion channel. Indeed, it was recently found that these proteins are in fact not ion channels but $Cl^-/H^+$ antiporters (Picollo and Pusch, 2005; Scheel et al., 2005), like the bacterial ClC-ec1 (see below).

The third group of CLC proteins is composed of ClC-6 and ClC-7. Both proteins are mostly found in membranes of intracellular organelles (Buyse et al., 1998; Kornak et al., 2001). No functional electrophysiological data have so far been obtained for these proteins, and it is thus unclear if they are $Cl^-$ channels, $Cl^-/H^+$ antiporters, or even other transport proteins (Fig. 8.2).

The plasma membrane localized channels ClC-1 and ClC-2, and in particular the "prototype" ClC-0 channel, have been studied most extensively from a functional point of view. A fundamental difference between classical voltage-gated cation channels and the CLC $Cl^-$ channels regards their voltage-dependence. Both type of gates (i.e., the single protopore gate and the common gate) of the *Torpedo* channel ClC-0 are voltage-dependent. The apparent gating valence of the protopore gate is around one and that of the common gate around two, while they exhibit an opposite voltage-dependence (Hanke and Miller, 1983; Pusch et al., 1995a, 1997). However, no clear "voltage-sensor" such as the S4-segment of $K^+$ and $Na^+$ channels is evident from the primary or from the 3D-structure. In fact, the voltage-dependence of the fast gate seems to arise, at least partly, indirectly from the coupling of a transmembrane movement of the permeant anion to channel opening (Pusch et al., 1995a; Chen and Miller, 1996). Lowering the extracellular $Cl^-$ concentration "shifts" the voltage-dependence of the open-probability to more positive voltages, i.e., renders opening more difficult (Pusch et al., 1995a). Also, intracellular $Cl^-$ affects the open-probability of the protopore gate mainly by altering the closing rate constant (Chen and Miller, 1996; Ludewig et al., 1997a). This strong coupling of gating and "down-hill" permeation renders the gating an intrinsically irreversible process. Furthermore, it implies that altering the permeation process (e.g., by mutation) also affects channel gating and vice versa. In fact, many results from mutagenesis studies, and attempts to establish a transmembrane topology have been difficult to interpret in the absence of a crystal structure (see Jentsch et al., 2002 for review). An irreversible coupling of the single protopore gate and the common gate of ClC-0 has been observed by Richard and Miller at the single-channel level: bursts of channel activity (see Fig. 8.1) started more often with both protopores gate open and ended more likely with only one of the protopore gates open (Richard and Miller, 1990). This imbalance, that on principal grounds requires an external energy input, was indeed more pronounced

in stronger electrochemical gradients, demonstrating a coupling of permeation and gating and a coupling of the two types of gates (Richard and Miller, 1990). The protopore gate of ClC-0 depends also strongly on intracellular and extracellular pH (Hanke and Miller, 1983; Chen and Chen, 2001). Acidification on either side favors channel opening, however, with different qualitative effects (see Pusch, 2004 for a review). The pH dependence of fast gating is probably related to the $Cl^-/H^+$ antiport activity of other CLC proteins because the same central glutamate (E166 in ClC-0; E148 in ClC-ec1; E211 in ClC-5) is centrally involved in the pH dependence.

Based on a large temperature-dependence of the kinetics of the common gate it has been suggested that it is accompanied by a substantial conformational rearrangement (Pusch et al., 1997). This is consistent with the finding that mutations in many protein regions affect the common gate (Ludewig et al., 1996, 1997a,b; Fong et al., 1998; Maduke et al., 1998; Lin et al., 1999; Saviane et al., 1999; Accardi et al., 2001; Duffield et al., 2003; Traverso et al., 2003; Estévez et al., 2004). However, the molecular mechanism of the common gate is still obscure. Gating of ClC-1 largely resembles that of ClC-0 (Rychkov et al., 1996; Accardi and Pusch, 2000; Accardi et al., 2001), however, its small single-channel conductance (Pusch et al., 1994) renders a detailed analysis difficult. Qualitatively, also ClC-1 shows a double-barreled appearance (Saviane et al., 1999). However, the two gates seem to be more strongly coupled in this channel compared to ClC-0 (Accardi and Pusch, 2000). ClC-2 shows a quite complicated and slow gating-behavior that, in addition, depends on the expression system, and will not be discussed here in detail (Gründer et al., 1992; Thiemann et al., 1992; Jordt and Jentsch, 1997; Pusch et al., 1999; Arreola et al., 2002; Niemeyer et al., 2003; Zúñiga et al., 2004).

A pair of highly homologous kidney and inner ear-specific channels (ClC-K1 and ClC-K2 in rodents; ClC-Ka and ClC-Kb humans) has been cloned more than a decade ago (Uchida et al., 1993; Adachi et al., 1994; Kieferle et al., 1994). However, these channels expressed only little in heterologous systems despite their elevated sequence similarity to ClC-0. The low expression was also surprising because in vivo immunocytochemistry showed a plasma membrane localization (Uchida et al., 1995; Vandewalle et al., 1997). Furthermore, a basolateral plasma membrane localization of ClC-Kb was strongly suggested by the disease phenotype of Bartter's syndrome (Simon et al., 1997). The lack of expression rendered a detailed biophysical analysis impossible. Only recently it became clear that ClC-K channels need the associated small transmembrane protein barttin for efficient plasma membrane expression (Estévez et al., 2001). The barttin gene was identified by positional cloning of the locus leading to a particular type of Bartter's syndrome associated with deafness (Birkenhäger et al., 2001). Gating of ClC-K channels co-expressed with barttin is slightly voltage-dependent (Estévez et al., 2001; Liantonio et al., 2002, 2004; Picollo et al., 2004). ClC-K1 and ClC-Ka are similarly dependent on voltage being activated at negative voltages, while ClC-Kb shows an opposite voltage-dependence. The voltage-dependence of ClC-K channels is somewhat surprising, because they lack a glutamate in a highly conserved stretch of amino acids, having instead a valine residue. Charge neutralizing mutations of the corresponding glutamate in ClC-0 and

ClC-1 completely abolish any voltage-dependent gating relaxations, while in ClC-2 the neutralization of the glutamate almost completely abolishes gating (Dutzler et al., 2003; Estévez et al., 2003; Niemeyer et al., 2003; Traverso et al., 2003). In fact, re-introducing a glutamate into ClC-K1 confers much more pronounced gating into the channel (Estévez et al., 2001). However, the mechanisms of ClC-K gating and the relationship with the double-barreled structure is not understood.

Interestingly, ClC-K activity can be markedly regulated by the extracellular pH and by the extracellular $Ca^{2+}$ concentration in the millimolar concentration range (Uchida et al., 1995; Waldegger and Jentsch, 2000; Estévez et al., 2001). Since the fast protopore-gate is probably mostly open in these channels, the pH and $Ca^{2+}$ regulation could act via the common gate, but the underlying mechanisms remain to be identified.

## 8.5 Permeation of CLC-0 and Mammalian CLC Channels

$Cl^-$ channels do not generally discriminate strongly among halides, except for fluoride, that is quite impermeant in most $Cl^-$ channels probably due to its strong hydration. Based on anomalous mole fraction effects it has been suggested that CLC pores can accommodate more than one ion at a time (Pusch et al., 1995a; Rychkov et al., 1998) in agreement with the presence of multiple $Cl^-$ ions in the crystal of ClC-ec1 (Dutzler et al., 2003). Ion permeation has been most extensively studied in the muscle channel ClC-1 at the macroscopic level. The channel exhibits an almost perfect selectivity of anions over cations (Rychkov et al., 1998). Among various anions tested the permeability sequence obtained from reversal potential measurements was $SCN^- \sim ClO_4^- > Cl^- > Br^- > NO_3^- \sim ClO_3^- > I^- \gg BrO_3^- > HCO_3^- \sim F^- \gg$ methanesulfonate $\sim$ cyclamate $\sim$ glutamate, where the latter three organic anions are practically impermeable (Rychkov et al., 1998). Furthermore, ClC-1 is blocked in a voltage-dependent manner by various organic anions and permeation and block appear to involve strong hydrophobic interactions (Rychkov et al., 1998; Rychkov et al., 2001). Recently, the permeation properties of ClC-0 and ClC-1 have been modeled using Brownian dynamics based on the crystal structure of ClC-ec1 (Corry et al., 2004). Overall these results predict a "knock-off" mechanism of permeation in which two relatively strongly bound $Cl^-$ ions are destabilized by the arrival of a third ion. The predictions of these simulations remain to be tested experimentally.

## 8.6 The X-ray Structure and Its Functional Implication: A Pivot Glutamate Controls the Protopore Gate

The X-ray structure of ClC-ec1 marked a breakthrough for the structure–function analysis of CLC proteins (Dutzler et al., 2002, 2003). As shown in Fig. 8.4, in each subunit two $Cl^-$ ions could be resolved. The central binding site ($S_{cent}$) is completely

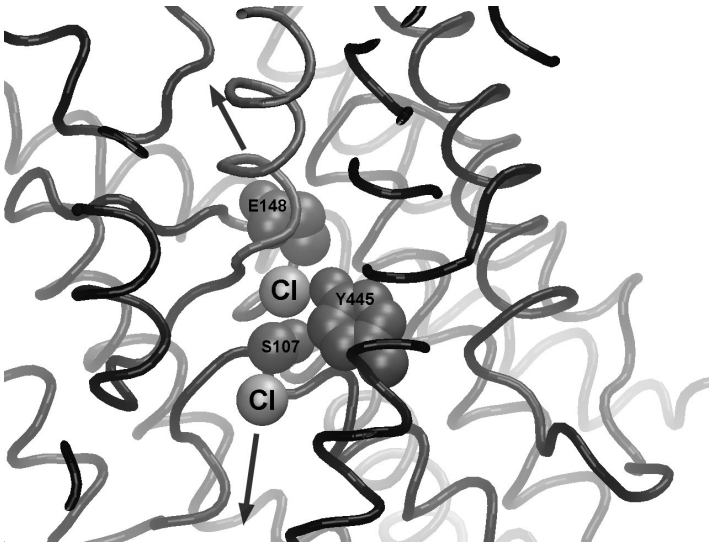


**Fig. 8.4** The $Cl^-$ binding sites in ClC-ec1. Only one subunit is shown with the intracellular side at the bottom. $Cl^-$ ions (light gray) and the amino acids S107 (between the two $Cl^-$ ions), E148 (above the top $Cl^-$ ion) and the coordinating Y445 are shown in spacefill (dark gray). This figure was prepared using the VMD program (Humphrey et al., 1996).

buried in the protein. Major direct protein–ion interactions involve the amide NH groups of some amino acids and the side chains of two residues (Y445 and S107). The directly $Cl^-$ interacting residues are well-conserved among CLC proteins and most of them are located in loops connecting two helices. In fact, several helices have their N-terminal end within the membrane. This topology reflects the pseudo-symmetrical sandwich structure described above (Fig. 8.3). It is interesting to note that none of the directly interacting residues is positively charged. Direct salt-bridging of a $Cl^-$ ion would probably lead to a too strong binding and less efficient conduction. However, simulation studies have shown that several more distantly located positively charged residues make significant contributions to the free energy of $Cl^-$ ion binding (Corry et al., 2004; Miloshevsky and Jordan, 2004; Cohen and Schulten, 2004; Bostick and Berkowitz, 2004). The more intracellularly located binding site, $S_{int}$, is in direct contact with the intracellular solution (Fig. 8.4) indicating the most likely intracellular pore entrance. In agreement with this, and confirming that the ClC-ec1 crystal structure is relevant for eukaryotic CLC channels, mutations in many residues that delineate the putative entrance alter typical pore properties like rectification and single channel conductance in an expected way (Pusch et al., 1995a; Ludewig et al., 1996, 1997a; Middleton et al., 1996; Rychkov et al., 1996; Pusch et al., 1999; Accardi and Pusch, 2003; Chen et al., 2003; Lin and Chen, 2003; Engh and Maduke, 2005).

In contrast, the exit of the central $Cl^-$ ion toward the intracellular side seem to be impeded by the highly conserved serine 107 that is located between $S_{int}$ an $S_{cent}$ (Fig. 8.4) (Corry et al., 2004; Miloshevsky and Jordan, 2004). Probably, some

conformational rearrangement of the intracellular entrance has to occur for an efficient $Cl^-$ conduction. In agreement with the necessity of a conformational change of the inner pore during channel opening strong state-dependent binding of small organic inhibitors from the inside has been described (Pusch et al., 2001; Accardi and Pusch, 2003; Traverso et al., 2003). However, as discussed by Yin et al. (2004), the state-dependent block might be caused by an altered occupancy of $Cl^-$ binding sites in the closed compared to the open state. The exit of the central $Cl^-$ ion toward the extracellular side is clearly impeded by the negatively charged side-chain of a conserved glutamate (E148) (Fig. 8.4) (Dutzler et al., 2002). In order to study the role of E148, Dutzler and colleagues solved the structure of ClC-ec1 in which the glutamate was mutated to alanine (E148A) or glutamine (E148Q). Since at that time direct functional analysis of ClC-ec1 was not yet possible (Maduke et al., 1999), Dutzler et al. used the well-studied *Torpedo* ClC-0 as a model to investigate the functional effect of the mutations (Dutzler et al., 2003). The mutant structures were almost identical to that of WT ClC-ec1 with a single difference: where the negatively charged E148 side-chain in WT CLC-ec1 blocked ion movement was now sitting a third crystallographically identified, third, $Cl^-$ ion, $Cl^-_{ext}$ (Dutzler et al., 2003). This structure appears to be an opened confirmation, at least concerning movement of the central $Cl^-$ toward the outside. Consistent with this simple structural result, both ClC-0 mutants, E166A and E166Q, showed a constitutively open phenotype: they appeared to have lost the voltage- and $Cl^-$-dependence of the open probability (Dutzler et al., 2003; Traverso et al., 2003). Interestingly, WT ClC-0 can also be opened by lowering the extracellular pH, probably by protonation of the corresponding glutamate (Dutzler et al., 2003), mimicking the E166Q mutation. These findings clearly establish E148 as a major player in the gating of CLC proteins. As already discussed above, it is noteworthy in this respect that ClC-K channels are only little voltage-dependent showing almost constitutive activity at all voltages (Estévez et al., 2001; Picollo et al., 2004). The wimpy voltage-dependence is related to the fact that ClC-K channels, and only these, carry a valine residue at the glutamate-position. Also for the strongly outwardly rectifying channels, ClC-3–ClC-5, the conserved glutamate is of prime importance: mutating E211 in ClC-5 (or its equivalents in ClC-3 or ClC-4) to alanine leads to a loss of the strong rectification and to an almost linear current–voltage relationship (Friedrich et al., 1999; Li et al., 2002).

## 8.7 The Function as a $Cl^-/H^+$ Antiporter

Accardi and Miller succeeded recently in obtaining a high-yield and extremely pure protein preparation of ClC-ec1 that allowed them to study macroscopic currents of ClC-ec1 proteins reconstituted in lipid bilayers (Accardi et al., 2004). Currents were voltage-independent but activated by low pH; the apparent single-channel conductance was too low to be reliably estimated even from noise analysis (Accardi et al., 2004). Currents carried by the glutamate mutant E148A were independent of pH (Accardi et al., 2004), in agreement with analogous behavior for ClC-0. A puzzling

finding was however, that the reversal potential measured for WT ClC-ec1 did not follow the Nernstian prediction for a purely $Cl^-$ selective channel but was significantly smaller, indicating the permeation of some cationic species (Accardi et al., 2004; Accardi and Miller, 2004). After detailed analysis Accardi and Miller clearly demonstrated that ClC-ec1 is actually not a diffusive $Cl^-$-selective channel, but instead a secondary active strictly coupled $Cl^-/H^+$ exchanger (Accardi and Miller, 2004). The apparent stoichiometry was found to be an exchange of two $Cl^-$ ions for each transported $H^+$ (Accardi and Miller, 2004). Given the opposite charges of $Cl^-$ and $H^+$, this transporter is highly electrogenic with a net transfer of three elementary charges per transport cycle.

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This result came as a big surprise for the ClC-field as no previous piece of evidence, and in particular not even the atomic resolution structure of the protein, had indicated such a function. The basic mechanism of function of a coupled transporter is a priori quite different from that of a passively diffusive channel and it will be very interesting to decipher the mechanism of this transport in the future. This result does not imply that all CLC proteins are $Cl^-/H^+$ antiporters. Certain well-known ClC proteins such as ClC-0, ClC-1, and ClC-2 are clearly $Cl^-$ ion channels: they have a rather large conductance and they show almost perfect Nernstian $[Cl^-]$-dependence of the reversal potential. Their gating, however, depends on $pH_{int}$ and $pH_{ext}$ (Pusch, 2004) and this dependence is likely related to the transport mechanism of ClC-ec1. In contrast, the function of several intracellular ClC proteins, like ClC-3–ClC-7, has never been clearly related to single $Cl^-$ ion channel activity. For ClC-6 and ClC-7 no functional electrophysiological measurement could be performed so far, leaving open the question if these are channels or transporters. However, for the endosomal proteins ClC-3–ClC-4, it became recently clear that they are indeed not $Cl^-$ channels as assumed previously. In parallel studies, the groups of Jentsch (Scheel et al., 2005) and Pusch (Picollo and Pusch, 2005) demonstrated that the highly homologous proteins ClC-4 and ClC-5 are $Cl^-/H^+$ antiporters, exactly like the bacterial ClC-ec1. Because the electrical currents carried by ClC-3 are very similar to those of ClC-4 and ClC-5, by analogy also ClC-3 is most likely a $Cl^-/H^+$ antiporter (Fig. 8.2). The currents carried by these transporters are so strongly rectifying, however, such that it is impossible to measure a true reversal potential (Steinmeyer et al., 1995; Friedrich et al., 1999). Thus a "simple" verification that these proteins are not channels was not possible and this explains why it had not been discovered previously but only after the example provided by Accardi and Miller for ClC-ec1 (Accardi and Miller, 2004). Scheel et al. (2005) and Picollo and Pusch (2005) showed that ClC-4 and ClC-5 transport protons, coupled to the inward movement of chloride ions. Physiologically, the role of a $Cl^-/H^+$ exchange, that is highly electrogenic, is not at all clear. If it operates as a shunt in acidifying vesicles, the counter-transport of $H^+$ would lead to a waste of pumped protons. It might also be that the chloride concentration itself is an important factor in the physiology of intracellular vesicles, and the regulation of endo- and exocytosis. A major unresolved issue regards the extreme rectification of ClC-3–ClC-5 that is very difficult to reconcile with any physiological role, because the physiological membrane voltages assumed to be

Please change "ClC-4" into "ClC-5".

present in intracellular compartments are opposite to the voltages needed to activate ClC-3–ClC-5 in heterologous expression systems.

Apart from the need to understand in more detail the physiological role of these transporters, one of the most fascinating problems remaining for CLC proteins is to understand how the same basic architecture can produce transport proteins that are either "simple" $Cl^-$ selective channels or stoichiometrically coupled $Cl^-/H^+$ antiporters.

## 8.8 Pharmacology

Small organic molecules and peptides that interact with ion channel proteins have been extremely useful tools for the studying of voltage-gated cation channels and ligand-gated channels (Hille, 2001). In this respect it suffices to recall the potent neurotoxins α-bungarotoxin or tetrodotoxin that act on the nicotinic acetylcholine receptor and the voltage-gated $Na^+$ channel respectively with high affinity (Hille, 2001). Also, many medically useful drugs act on various types of ion channels. Unfortunately, the situation is worse for $Cl^-$ channels in general and for CLC proteins in particular, even though some progress has been made recently. Typical, "classical", $Cl^-$ channel blockers include molecules like 4,4′-diisothiocyanato-2,2′-stilbenedisulfonic acid (DIDS), 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulfonic acid (SITS), 9-anthracenecarboxylic acid (9-AC), niflumic acid (NFA), 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) and others. Most of these drugs block, i.e., reduce the currents, of many $Cl^-$ channels. They are thus quite unspecific. Furthermore, block occurs often with a low affinity ($<100\ \mu M)^{-1}$. There are, however, exceptions. For example, some CLC proteins are particularly resistant to any blocker: no sub 100 μM organic blocker of ClC-2 and no blocker at all of ClC-5 has been identified so far (Pusch et al., 2002). On the other hand, the muscle ClC-1 channel is so far the most "sensitive" CLC protein. It is blocked by 9-AC and by derivatives of p-chloro-phenoxy propionic acid (collectively abbreviated as CPP) at concentrations below 10 μM (Bryant and Morales-Aguilera, 1971; Conte-Camerino et al., 1988; Liantonio et al., 2002). The block of ClC-0 and ClC-1 by these classes of substances (i.e., 9-AC and CPP) has been studied in considerable detail and they have proved to be useful tools to investigate the structure–function relationship of these channels. First, it has been found that both types of drugs can directly access their binding site only from the intracellular side and that they bind to the pore region (Pusch et al., 2000, 2001, 2002; Estévez et al., 2003). Their action is strongly state-dependent in that they bind much more tightly to the closed state than to the open state of the channel leading to a marked apparent voltage-dependence of block (Pusch et al., 2000, 2001, 2002; Accardi and Pusch, 2003). Starting from a critical amino acid identified independently from the crystal structure, Estévez and co-workers then used the bacterial structure as a guide to identify the crucial amino acids involved in blocking the muscle ClC-1 channel by 9-AC and CPP (Estévez et al., 2003). When mapped onto the bacterial structure

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where "-1" must be in superscript. I.e. Interchange ")" and "-1".

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these critical amino acids clustered in a region around the central $Cl^-$ ion binding site (Estévez et al., 2003). On the one hand this rough identification of the binding site opens the way for further analysis of the interaction of these molecules with ClC-0 and ClC-1. On the other hand the results of Estévez et al. demonstrate that the overall structure of ClC-0 and ClC-1 must be very similar to that of the bacterial ClC-ec1, and that, despite the quite different functional properties, the bacterial crystal structure is a good guide for the mammalian homologues.

Intracellularly applied DIDS has been found to irreversibly inhibit the *Torpedo* channel ClC-0, acting on individual protopores of the double-barreled channel (Miller and White, 1984). Irreversible action of DIDS is thought to be mediated by the covalent modification of lysine residues or other free amines. The target of DIDS in ClC-0 has, however, not yet been identified. Interestingly also the bacterial ClC-ec1 is inhibited by intracellular, but not extracellular DIDS (Matulef and Maduke, 2005) in a slowly reversible manner with an apparent $K_D$ of about 30 μM. In an elegant study, Matulef and Maduke showed that the DIDS block could be potentially used to "functionally orient" ClC-ec1 transporters incorporated into lipid bilayers (Matulef and Maduke, 2005). In fact, a general limitation of the reconstitution of purified ClC-ec1 is that the orientation of the transporter in the membrane is practically completely random (Matulef and Maduke, 2005). Inclusion of a saturating DIDS concentration on one side of the bilayers leaves functional only those transporters that face the DIDS solution with their extracellular side. However, the mechanism of DIDS block is still unclear and block was not complete even at concentrations of more than 10-fold larger than the apparent $K_D$ (Matulef and Maduke, 2005). It remains to be seen if there is overlap between the 9-AC/CPA site of ClC-1 and the DIDS site of ClC-ec1.

A different blocker-binding site was identified for certain kidney ClC-K channels. Some derivatives of CPP with two phenyl-groups (abbreviated here as 3-phenyl-CPP) and also DIDS were found to block ClC-K1 and CLC-Ka channels but not ClC-Kb and not other CLC channels (Liantonio et al., 2002; Liantonio et al., 2004; Picollo et al., 2004). ClC-K1, ClC-Ka, and ClC-Kb are about 80% identical to each other, and block by 3-phenyl-CPP and DIDS occurs from the extracellular side in competition with $Cl^-$ ions (Liantonio et al., 2004). Comparing the primary sequence between these channels, and using the ClC-ec1 crystal structure as a guide, two amino acids could be identified that are critically important for the differences in inhibitor sensitivity (Picollo et al., 2004). Both residues (N68/D68 and G72/E72 in CLC-Ka/CLC-Kb, respectively) are located in helix B, and in ClC-ec1 the corresponding side-chains point toward the extracellular channel entrance. The N/D68 residue is the most sensitive residue involved in drug binding and it is highly conserved: practically all CLC proteins, including ClC-ec1, present a negatively charged amino acid at the corresponding position. Mutating this residue in ClC-0 or ClC-1 leads to profound alterations in the gating behavior (Fahlke et al., 1995; Ludewig et al., 1997b). Also, the ClC-Kb D68N mutation drastically alters the gating behavior (Picollo et al., 2004). The precise role of this residue will be interesting to study in the future.

Inhibitors of CLC-K channels might be interesting from a pharmaceutical point of view because they could be used as diuretics (Fong, 2004). Another pharmacologically interesting target is ClC-7 because its blockage might be beneficial to treat osteoporosis (Schaller et al., 2004; Karsdal et al., 2005). Unfortunately, heterologous ClC-7 expression does not induce electrical currents in the plasma membrane of transfected cells such that a direct block cannot be assayed. However, indirect functional assays have been developed that led to the identification of putative ClC-7 blockers (Schaller et al., 2004; Karsdal et al., 2005). It remains to be shown, however, that these substances directly act on ClC-7.

## 8.9 CBS Domains

The crystallized bacterial CLC homologues have short cytoplasmic N- and C-termini. In contrast eukaryotic CLCs have longer N and especially C-termini. These long C-termini are of significant functional importance (Schwappach et al., 1998; Estévez and Jentsch, 2002) and they contain two conserved so-called CBS domains (Ponting, 1997). Probably the two CBS domains of each subunit interact and they seem to play a role in regulating the common gate (Estévez et al., 2004). However, none of the CBS domains is strictly essential for channel function (Hryciw et al., 1998; Estévez et al., 2004; Hebeisen et al., 2004) and their precise role is still unclear (see Babini and Pusch, 2004 for review). A possibly important clue about the role of CBS domains is that (as isolated peptides) they bind intracellular nucleotides like AMP and ATP (Scott et al., 2004). Some disease causing mutations in CBS domains diminish the binding (Scott et al., 2004) suggesting that it plays an important functional role. Among these mutations the amino acid change G715E that is located between CBS1 and CBS2 in ClC-2 reduced the affinity for ATP 10-fold (Scott et al., 2004). This amino acid change was reported to cause epilepsy in humans and to alter the $[Cl^-]_{int}$-sensitivity of the channel (Haug et al., 2003). These gating-effects could, however, not be reproduced (Niemeyer et al., 2004). Nevertheless, Niemeyer et al. found that the G715E-mutation altered a small kinetic effect of intracellular AMP on the gating (Niemeyer et al., 2004). Recently, also the gating of the muscle channel ClC-1 was shown to be dependent on intracellular ATP, AMP, and adenosine (Bennetts et al., 2005). These nucleotides "shifted" the voltage-dependence of the common gating process to more positive voltages compared to the situation in the absence of nucleotides. Modeling a CBS1/CBS2 dimer revealed a putative ATP binding site and mutating amino acids of the site indeed reduced or even abolished nucleotide regulation (Bennetts et al., 2005). These results provide strong evidence for a functional regulation of CLC proteins by intracellular nucleotides. Bennetts et al. speculated that a reduction of ATP levels in skeletal muscle caused by exercise could lead to a significant activation of ClC-1 mediated conductance and a larger threshold for action potential firing, contributing to fatigue (Bennetts et al., 2005). A failure of skeletal muscle action potential generation and/or propagation upon nerve stimulation due to an increased $Cl^-$ conductance remains, however, to be verified

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experimentally. The role of intracellular nucleotides in the regulation of CLC proteins and the involvement of the CBS domains are an exciting new aspect of their physiology.

## 8.10 Conclusion

CLC proteins have been full of surprises right from their first revelation as funny double-barreled, irreversibly gating $Cl^-$ channels. They challenge the classical distinction of ion channel and active transporter. Unexpected physiological roles have been discovered. They are involved in many human genetic diseases. They display an extremely complex three-dimensional structure, with a two-pore architecture of unknown relevance. These fascinating proteins will certainly occupy scientists from disciplines ranging from molecular medicine to molecular dynamics for several years.

## Acknowledgment

The financial support by Telethon Italy (grant GGP04018) is gratefully acknowledged.

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