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PHYSIOLOGICAL FUNCTIONS OF CLC Cl^- CHANNELS GLEANED FROM HUMAN GENETIC DISEASE AND MOUSE MODELS

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■ **Abstract** The CLC gene family encodes nine different Cl^- channels in mammals. These channels perform their functions in the plasma membrane or in intracellular organelles such as vesicles of the endosomal/lysosomal pathway or in synaptic vesicles. The elucidation of their cellular roles and their importance for the organism were greatly facilitated by mouse models and by human diseases caused by mutations in their respective genes. Human mutations in CLC channels are known to cause diseases as diverse as myotonia (muscle stiffness), Bartter syndrome (renal salt loss) with or without deafness, Dent's disease (proteinuria and kidney stones), osteopetrosis and neurodegeneration, and possibly epilepsy. Mouse models revealed blindness and infertility as further consequences of CLC gene disruptions. These phenotypes firmly established the roles CLC channels play in stabilizing the plasma membrane voltage in muscle and possibly in neurons, in the transport of salt and fluid across epithelia, in the acidification of endosomes and synaptic vesicles, and in the degradation of bone by osteoclasts.

INTRODUCTION

The molecular diversity of anion channels may not rival that of cation channels, but these channels belong to several structurally unrelated classes (1). The known Cl^- channels can be grouped into (*a*) CLC chloride channels, which are often gated by voltage; (*b*) ligand-gated $GABA_A$ and glycine receptors, which are related to ligand-gated cation channels such as the nicotinic acetylcholine receptor; (*c*) the cystic fibrosis transmembrane conductance regulator (CFTR), the only member of the ABC transporter family known to function as a chloride channel; and, probably,

(*d*) bestrophins, which may function as Ca^{2+}-activated Cl^- channels (2). The case for bestrophins directly mediating anion currents has been significantly strengthened by structure-function studies (3). This list is most likely incomplete, as some Cl^- channels (e.g., a class of swelling-activated channels) have not yet been identified at the molecular level. For a recent in-depth review on Cl^- channels, see (1).

Chloride channels are present in the plasma membrane and in membranes of intracellular organelles. They are involved in a broad range of functions, including the stabilization of membrane potential, synaptic inhibition, cell volume regulation, transepithelial transport, extracellular and vesicular acidification, and endocytotic trafficking. Many of these functions were discovered through the phenotypes resulting from their inactivation in human inherited disease or in mouse models. In this review, we focus on the surprisingly diverse functions of mammalian CLC chloride channels that were unraveled since the discovery of the CLC gene family in 1990 (4). The role of other classes of chloride channels in health and disease is discussed in recent reviews, such as on the involvement of glycine receptors in myoclonus and startle syndromes (5) and GABA receptor mutations in certain forms of epilepsy (6). Bestrophin (7) is mutated in Best macular dystrophy and is not discussed here.

GENERAL PROPERTIES OF CLC CHANNELS

To understand the pathogenic effects of mutations in CLC genes, it is useful to recall some general properties of these channels. For example, they function as dimers in which each monomer has its own pore (double-barreled channels). The two-pore architecture was first postulated based on an analysis of single channels reconstituted from *Torpedo* electric organ (8). After cloning (4) of this channel (later named ClC-0 (9)), protein purification (10), site-directed mutagenesis, and concatemers (11–13) strongly supported this notion and additionally suggested that each pore is completely contained within one subunit (11, 13). For bacterial CLC proteins, this has now been confirmed by crystallography (14), suggesting that all CLC proteins display the same basic architecture. Interestingly, bacterial CLC proteins may function as cotransporters rather than channels (15), but a mutational analysis of ClC-1 showed that the structure of the bacterial exchanger is highly conserved in the mammalian channel (16).

Each pore of the dimer retains its individual properties such as ion selectivity and single-channel conductance even when forced together in an artificial heterodimer, as shown, e.g., for ClC-0 and ClC-1 or ClC-2 (13). At least in ClC-0, ClC-1 (17), and ClC-2 (13), each pore (protopore) can be opened and closed by an individual gate. In ClC-0 (the best studied channel in this respect), protopore gating is independent of the state of the other gate (8, 18). In addition to the protopore gate (also called the fast gate in ClC-0), there is also (at least in ClC-0 and ClC-1) a common gate that closes both pores in parallel. In ClC-0, its kinetics led to the name slow gate,

but it is much faster in ClC-1 (17). A glutamate side chain that obstructs the pore may play a role in protopore gating (14, 19). The structural basis for the common gate is still obscure.

This architecture has important consequences for the impact of CLC mutations. In contrast to tetrameric K^+ channels, mutations that reduce single-channel conductance or protopore gating will generally not have dominant-negative effects on coexpressed wild-type subunits. Dominant effects can be obtained by those mutations that affect the common gate, which closes both subunits, or by mutations resulting in proteins that, while retaining their ability to associate with wild-type subunits, cause the missorting or degradation of the resulting abnormal dimer. No dimerization signals have been identified in CLC channels. However, genetic data and in vitro studies indicate that most, if not all, truncations within the transmembrane part lack dominant-negative effects, suggesting that these proteins are unable to associate to dimers. Crystal structures of bacterial CLC proteins (14) revealed a broad interface between the subunits, which involves helices H, I, P, and Q.

These considerations suggest that dominant-negative mutations occur less frequently, for example, with CLC channels than with K^+ channels. The dimeric channel structure implies that even the strongest dominant-negative mutations are unlikely to decrease currents to less than 25% in the heterozygous state. This compares with the strong reduction (down to 6%) with dominant mutations in tetrameric K^+ channels. The moderate dominant-negative effects possible with CLC channels explain that dominant myotonia congenita (mutations in ClC-1) (20–22) and osteopetrosis (ClC-7) (23, 24) are generally less severe than the recessive variants, in which both alleles are mutated and thus may be associated with a total loss of function.

Whereas CLC channels can function as homodimers (and this may be the most common situation in vivo), in vitro studies have shown that heterodimerization is possible within the branch of plasma membrane channels (ClC-0, -1, -2) (13, 25). Co-immunoprecipitation experiments suggested that ClC-4 and ClC-5 might interact (26). Whether this occurs to a sizeable degree in vivo is still unknown.

So far, the highly related channels ClC-Ka and ClC-Kb (ClC-K1 and ClC-K2 in rodents) are the only CLC channels known to require a β-subunit (barttin) for functional expression (27). The auxiliary subunit barttin (28) is crucial for an efficient transport of ClC-K proteins to the plasma membrane (27).

The currents through CLC channels can be modulated by voltage, extra- and intracellular anions (29–32), pH (27, 31, 33), extracellular Ca^{2+} (27, 34), cell swelling (33, 35), and phosphorylation (36). CLC channels lack a charged voltage sensor of the type seen in voltage-dependent cation channels (S4-segment). The voltage-dependence of protopore gating is thought to result from the movement of the permeant anion within the pore (30, 37), with the anion acting as gating charge (30). This simple model renders gating dependent on both Cl^- concentration and voltage. Crystal structures of bacterial CLC proteins revealed a glutamate whose side chain obstructs the pore a short distance to the extracellular side of the central Cl^--binding site (14, 19). Indeed, mutations at the equivalent position in

ClC-0 (19), ClC-1 (38), ClC-2 (32), ClC-K1 (39), ClC-4 and -5 (40), and ClC-3 (41) strongly influenced or abolished gating. The pH-dependence of gating was proposed to be due to a protonation of the gating glutamate, and the pH-dependence of gating was indeed abolished when this glutamate was mutated in ClC-0 (19). However, other parts of the protein may also contribute to pH-sensitivity, as ClC-K channels are modulated by pH but lack a glutamate at this position (27, 34, 42).

CLC channels have functions in the plasma membrane (ClC-1, -2, -Ka, -Kb) or in intracellular membranes of the endocytotic-lysosomal pathway (ClC-3 through ClC-7) (Figure 1). The roles of plasma membrane CLC channels include the stabilization of membrane potential, transepithelial transport, and cell volume regulation, whereas endosomal/lysosomal CLC channels are thought to provide an electric shunt for the efficient pumping of the H^+-ATPase (1). Some vesicular channels may also be inserted into specialized domains of the plasma membrane. For instance, the late endosomal/lysosomal ClC-7 has an important role in the ruffled border of osteoclasts (23).

CLC-1 AND MYOTONIA

ClC-1 (9) is the closest homologue of the *Torpedo* electric organ Cl^- channel, ClC-0 (4). ClC-1 is nearly exclusively expressed in skeletal muscle. In rodents, ClC-1 transcripts are upregulated after birth (9). ClC-1 expression depends on muscle electrical activity (43). Immunocytochemistry located ClC-1 primarily to the outer, sarcolemmal membrane of skeletal muscle (44, 45), although physiological investigations revealed that muscle Cl^- conductance is mainly found in t-tubules (46).

ClC-1 has a small single-channel conductance of about 1–1.5 pS (13, 17, 47) and is blocked by 9-anthracene-carboxylic acid and 4-chloro-phenoxy-acetic acid derivates: Their binding sites have been mapped by mutagenesis and molecular modeling (16). As is true for other CLC channels, ClC-1 is partially blocked by I^-. Both the protopore gate and the common gate, which is much faster than that of ClC-0, are activated by depolarization (9, 17).

In an exceptional situation, the resting conductance of skeletal muscle is dominated by Cl^- rather than K^+. This equilibrates the electrochemical potential of Cl^- with the resting potential, which is ultimately generated by the K^+ gradient as in other cells. The large Cl^- conductance stabilizes the resting potential and helps to repolarize action potentials. In skeletal muscle, t-tubules propagate the electrical excitation deep into the muscle fibers, where the voltage-dependent activation of L-type Ca^{2+} channels eventually leads to intracellular Ca^{2+} release and muscle contraction. If the repolarization of t-tubular membranes occurred primarily through K^+ channels, $[K^+]$ would increase in the small space inside these tubules during prolonged muscle activity, thereby leading to a long-lasting moderate depolarization. When Cl^- channels are used for repolarization, the same absolute change in t-tubular $[Cl^-]$ (which is in the 100 mM range as is $[Cl^-]_o$ in general) will lead to a much smaller relative change in $[Cl^-]$, which will not appreciably

change the t-tubular voltage. For this reason, evolution has chosen Cl^- channels to electrically stabilize and repolarize skeletal muscle membranes.

Bryant and colleagues (48, 49) demonstrated a severely reduced Cl^- conductance in the skeletal muscle of human patients with myotonia congenita and of a myotonic goat strain. Myotonia, a symptom found in several genetic diseases, is an impairment of skeletal muscle relaxation after voluntary contraction. It results from an increase in muscle excitability that can be detected in electromyograms in the form of myotonic runs, i.e., long trains of action potentials. In humans, there are two forms of pure nonsyndromic myotonia: autosomal recessive Becker-type myotonia congenita, and autosomal-dominant myotonia or Thomsen disease.

Soon after ClC-1was cloned (9), it was shown (50) that the open reading frame of ClC-1 was destroyed by the insertion of a transposon in the myotonic mouse strain *adr*. This demonstrated that ClC-1 is the major skeletal muscle Cl^- channel essential for maintaining normal muscle excitability. Soon afterward, it was found that ClC-1 also accounts for human myotonia congenita (51). Mutations were identified in dominant myotonia (Thomsen's disease) (20, 52), including mutations in family members of Dr. Thomsen (20), who was also affected. The Thomsen mutation (P480L) exerted a strong dominant-negative effect on wild-type channels coexpressed in *Xenopus* oocytes (20).

More than 80 different ClC-1 mutations have been identified in human myotonia (for a recent review, see 53). Most mutations result in recessive myotonia. This includes all truncations in the membrane portion of the channel. Although these mutations could lead to nonsense-mediated decay of RNA or to protein instability, a likely reason for a lack of a dominant-negative effect is the inability of truncated proteins to associate with wild-type subunits. As discussed above, the broad interface between the two subunits of the dimer also suggests that truncations before helix Q may lead to an inability to associate to dimers. Some recessive mutations, e.g., M485V, drastically reduce single-channel conductance (54). Satisfyingly, the crystal structure of the bacterial CLC protein (14) revealed that the highly conserved phenylalanine directly neighboring this methionine participates in coordinating a Cl^- ion in the narrowest part of the permeation pathway. As the pores of CLC channels are entirely contained within each subunit of the dimer (13, 14), pore mutations are unlikely to affect the conductance of the second subunit in wild-type/mutant heteromeric channels and therefore will generally lack dominant-negative effects. Several recessive mutations, including M485V, also strongly changed the voltage-dependence of ClC-1 gating (54–56).

With the exception of truncations very close to the carboxy terminus of ClC-1, all dominant mutations are missense mutations. Almost all these mutations exert dominant-negative effects by shifting the voltage-dependence of gating of the dimeric channel towards positive voltages where the channel has no impact on membrane repolarization (22). The shift is because of an effect on the common gate that acts on both pores in parallel (17). Indeed, many but not all mutations in dominant myotonia change residues close to the subunit interface, in particular in helices H and I. Site-directed mutagenesis of residues in helices I, G, H, P, and Q affect the common gate of ClC-1 (57–59).

Myotonic mutations in ClC-1 that change the voltage-dependence of gating of homomeric mutant channels have different effects on the voltage-dependence of wild-type/mutant heterodimers, which partly explains the variable penetrance of some of these mutations (60). This variability might be caused by differential effects on the common versus the protopore gate, or by differences in subunit affinities that may lead to preferential assembly of homodimers. Although myotonia has traditionally been classified into recessive (Becker) and dominant (Thomsen) forms, it is now clear that the "border" between these inheritance patterns is blurred. There are indeed mutations that are associated with recessive myotonia in some, and with dominant myotonia in other families (21, 60, 61). On the basis of studies in a small number of patients, it was recently proposed that differences in allelic expression may determine the penetrance of some mutations, thereby influencing the apparent pattern of inheritance (62).

As both alleles are mutated in patients with recessive myotonia, a total loss of ClC-1 channel function may ensue. In contrast, at least 25% of wild-type currents will remain in heterozygous patients carrying dominant-negative mutations, as expected from the dimeric channel architecture. Accordingly, recessive myotonia is clinically more severe than the dominant Thomsen form. As discussed below, even more dramatic differences in disease severity are observed with mutations in ClC-7 that underlie recessive and dominant osteopetrosis (23, 24).

Myotonia is a symptom of myotonic dystrophy (DM), a more severe disease that also affects several other tissues, e.g., the heart and the eye. Skeletal muscle displays the hyperexcitability that is typical for myotonia. In contrast to myotonia congenita, DM is associated with muscle dystrophy that develops with age. Electrophysiological studies on muscle biopsies from patients displayed several abnormalities, including a variable decrease in Cl^- conductance (63). Myotonic dystrophy is caused by CTG or CCTG (i.e., DNA base) expansions in the 3′ end of the DM protein kinase (*DMPK*) gene (in DM1) or in an intron of the zinc finger 9 (*ZNF9*) gene (in DM2), respectively. The aberrant RNAs accumulate in the nuclei. Recent work has shown that this results in mis-splicing of the ClC-1 RNA (45, 64), possibly by recruiting the CUG-binding muscleblind protein, whose knockout in mice also leads to mis-splicing of ClC-1 and myotonia (65). The overall levels of ClC-1 RNA may then decrease by nonsense-mediated decay. However, the repeat-containing DMPK RNA may also sequester transcription factors, which results in an additional reduction of ClC-1 transcription (66). Although several aspects, including the relative specificity for ClC-1, are not yet fully understood, these studies highlight again the importance of ClC-1 in muscle physiology.

CLC-2: A UBIQUITOUSLY EXPRESSED CHANNEL WITH MANY CANDIDATE FUNCTIONS

ClC-2, an almost ubiquitously expressed Cl^- channel (67), is activated by hyperpolarization, cell swelling, and weakly acidic extracellular pH (33, 35, 67). At unphysiologically strong acidic pH values (<pH6), however, its open probability

decreases (68). Similar to ClC-0 and ClC-1, ClC-2 probably has a common gate and protopore gates (69), although the common gate was not detected in single-channel recordings (13). ClC-2 has a single-channel conductance of about 2–3 pS (13). Single-channel currents with similar properties were observed in astrocytes (70), which express ClC-2 (71), as confirmed by the absence of these currents in astrocytes isolated from ClC-2 knockout mice (72). Similar to gating in other CLC channels, the gating of ClC-2 is influenced by anions. However, in contrast to ClC-0 (30), ClC-2 is mainly affected by intracellular Cl^-, with extracellular anions having minor effects (32, 73, 74). In addition, ClC-2 has a Cl > I selectivity (similar to other CLC channels) that was reported to be modulated by cyclin-dependent protein kinases (36, 75) and can be phosphorylated by protein kinase A. However, this does not change its channel activity (76, 77). The knockout of ClC-2 unexpectedly led to testicular and retinal degeneration (78).

The ubiquitous expression of ClC-2 and the various possibilities to modulate its channel activity have invited many speculations on its function. The activation by cell swelling (35) suggests a role in cell volume regulation. However, the swelling-activated Cl^- channel (called VRAC or VSOAC) that is thought to dominate cell volume regulation has quite different properties, most prominently an outward rectification and an I > Cl selectivity (1, 79). When heterologously expressed in *Xenopus* ooyctes (77) or Sf 9 cells (80), ClC-2 accelerated their regulatory volume decrease (RVD) after hypotonic swelling. However, parotid acinar cells from ClC-2 knockout mice recovered their volume as fast as did wild-type cells (81). It is an open question whether RVD of other cells depends on ClC-2. This issue may be best studied using *Clcn2*$^{-/-}$ mice.

The expression of ClC-2 in neurons that display an inhibitory response to GABA suggested that it may play a role in establishing a low chloride concentration in neurons (82). Because $GABA_A$- and glycine receptors are ligand-gated Cl^--channels, their opening results in de- or hyperpolarization when $[Cl^-]_i$ is above or below its electrochemical equilibrium, respectively. Early in development, GABA and glycine are excitatory in most neurons. The excitation later gives way to inhibition as $[Cl^-]_i$ decreases (83). It is now known that the main process lowering $[Cl^-]_i$ is the K-Cl cotransporter, KCC2 (84, 85). Other transport proteins, such as KCC3 (86) or ClC-2, may play additional roles. Indeed, when ClC-2 was transfected into dorsal root ganglion cells, their normally excitatory response to GABA was converted to inhibition (87). This is expected from an equilibration of the electrochemical Cl^- potential with the membrane potential. Opening of $GABA_A$ receptors will then yield neither hyperpolarization nor depolarization, but will stabilize the voltage close to its resting value. In discussing roles of ClC-2 in the central nervous system (CNS), one should be aware that ClC-2 is expressed not only in neurons, but also, prominently, in glia (71, 72, 88) where it may serve in the homeostasis of extracellular ion composition.

According to the proposed role of ClC-2 in lowering neuronal $[Cl^-]_i$, one might expect that its lack gives rise to epilepsy. This, however, was not observed in *Clcn2*$^{-/-}$ mice (78). On the other hand, a locus for multigenic idiopathic generalized epilepsy was mapped to 3q26 close to the ClC-2 locus (89). Screening a large

cohort of patients revealed three sequence abnormalities that cosegregated in an apparently autosomal-dominant fashion with epileptic symptoms in three pedigrees (90). One mutation truncates ClC-2 in helix F, directly predicting a loss of function. The mutant reportedly also exerted a dominant-negative effect on wild-type ClC-2 (90). However, our laboratory and others (91) did not observe such a dominant effect. Indeed, similar truncations in other CLC channels lack dominant-negative effects, as discussed above for ClC-1. The second mutation deletes 11bp of an intron (90). This was reported to alter splicing, thereby increasing the abundance of a protein that lacks 44 amino acids in the intramembrane portion. Expression of a corresponding cRNA was reported to exert a strong dominant-negative effect that exceeded the 75% reduction that is possible with a dimer. Using minigenes, another study (91) failed to detect an effect of the deletion on splicing. Finally, Haug et al. (90) identified a missense mutation (G715E) in a family with three affected siblings. G715 lies between CBS1 and CBS2 in the cytoplasmic tail. G715E was reported to shift the voltage-dependence in a $[Cl^-]_i$-dependent manner to more positive voltages. This is equivalent to a gain of function, contrasting with the loss of function of the truncated channel (90). However, no such gain of function was observed in our laboratory (T.J. Jentsch, unpublished results), nor by others (91). CBS domains were recently shown to bind ATP and other nucleotides (92). Although G715 is located between CBS domains, the G715E mutation decreased the affinity of the ClC-2 carboxy terminus for AMP in vitro (92). In an electrophysiological study (91), the G715E mutant was indistinguishable from wild-type ClC-2 in the presence of 1 mM cytoplasmic ATP, but showed different gating kinetics when ATP was replaced by 2 mM AMP. Whether these conditions occur during development of epileptic seizures is unclear.

Hence, the strongest case for a causative role of ClC-2 in epilepsy is a single family whose affected members are heterozygous for a truncating mutation. In the likely absence of dominant-negative effects, the mutation may act via haploinsufficiency. This contrasts with the lack of epilepsy in mice totally lacking ClC-2 (78). In order to firmly establish a causative role of ClC-2 in epilepsy, it seems desirable to find further epilepsy-associated ClC-2 mutations that compromise channel function. In a recent study, no *CLCN2* mutations were found in 55 families with idiopathic generalized epilepsy (93), but clearly screening of more patients may be required to settle this question.

ClC-2 was also thought to be important for gastric acid secretion (94), a proposal which could not be confirmed in the ClC-2 knockout mouse (78). Because the lung needs to secrete salt and water during development, and because—in contrast to CFTR—ClC-2 is expressed in the lung early on, ClC-2 was proposed to be important for lung development (95–97). The expansion of fetal lung cysts in vitro could be inhibited with an antisense-oligonucleotide directed against ClC-2 (98). This study, however, is inconclusive as it employed the same antisense oligonucleotide that was used previously to knock down an inwardly rectifying Cl^- channel in choroid plexus cells (99). The knocked-down channel differed in several biophysical properties (most notably ion selectivity) from ClC-2, and experiments on *Clcn2*$^{-/-}$ mice revealed that it does not correspond to ClC-2 (100).

Obviously, this oligonucleotide (98, 99) lacks specificity. Convincing evidence against an essential role of ClC-2 in lung development comes from the ClC-2 knockout mouse (78). Lung morphology appeared normal even when both ClC-2 and CFTR were disrupted (101).

The fact that ClC-2 is also found in epithelia fueled speculations that it might modulate the phenotype of cystic fibrosis (CF), a severe disease that is caused by mutations of the cAMP-activated Cl^- channel CFTR. The latter apical channel mediates Cl^- secretion in many epithelia. An optimistic hypothesis holds that pharmacological activation of ClC-2 might create an alternative pathway for apical Cl^- secretion that could be of benefit for CF patients (102, 103). Of course, this requires an apical localization of ClC-2. Depending on the antibody used, however, divergent results were obtained. In lung epithelia, ClC-2 was described in apical membranes (97). It was also reported to localize to apical junctional complexes in an intestinal cell line and to contribute to their anion secretion (103). However, using two different antibodies, other groups described ClC-2 as being in basolateral membranes of intestinal epithelia (74, 104, 105).

If ClC-2 provides a pathway for Cl^- secretion in parallel to CFTR, it would be expected that a disruption of both channels would yield a more severe CF phenotype than the knockout of only CFTR. In particular, there might be symptoms in tissues affected in humans but spared in mice (as lung and pancreas), and the intestinal phenotype of CFTR mouse models could get worse. However, this was not the case (101). Surprisingly, colon from *Clcn2*$^{-/-}$ mice displayed larger cAMP-stimulated Cl^- secretion than wild-type colon. This would be compatible with a basolateral rather than apical localization of ClC-2. Equally surprising, *Cftr*$^{\Delta F508/\Delta F508}$/*Clcn2*$^{-/-}$ mice survived better than *Cftr*$^{\Delta F508/\Delta F508}$ mice (101). A deletion of phenylalanine 508 (ΔF508) is the most common CFTR mutation in Caucasians and leads to a trafficking defect of an otherwise functional channel to the plasma membrane. The better survival of *Cftr*$^{\Delta F508/\Delta F508}$/*Clcn2*$^{-/-}$ mice was hypothesized to be from a slight enhancement of the residual colonic Cl^- secretion by the disruption of basolateral ClC-2 channels (101). The apical exit of chloride may occur through ΔF508 CFTR or another unidentified apical Cl^- channel. The disruption of ClC-2 unexpectedly led to selective male infertility and blindness (78). The observed degeneration of germ cells and photoreceptors may be from a defect in transepithelial transport across Sertoli cells and the retinal pigment epithelium, respectively, which are important to provide an appropriate environment for these cells. Indeed, Ussing chamber experiments revealed a reduction of transepithelial current and resistance across the retinal pigment epithelium (78).

CLC-K/BARTTIN: SALT TRANSPORT IN THE KIDNEY AND THE INNER EAR

Two highly homologous CLC channels are predominantly expressed in the kidney, hence the name ClC-K. The sequences of ClC-Ka and ClC-Kb (ClC-K1 and ClC-K2 in rodents) are about 90% identical (42, 106, 107). Their genes are located

very close to each other on human chromosome 1p36 (108, 109), suggesting a recent duplication. Both channels need the small accessory β-subunit barttin for functional expression (27). The distribution of ClC-K channels has been studied by RT-PCR (27, 42, 106, 107, 110), in situ hybridization (111), immunocytochemistry (27, 34, 112–115) in conjunction with ClC-K1 knockout mice (113, 116), and by the transgenic expression of a reporter gene driven by the ClC-Kb promoter (117). In the kidney, ClC-K1 (ClC-Ka) is expressed in the ascending thin limb of the loop of Henle, where it was found in both apical and basolateral membranes (34). However, in another study, it was found only in basolateral membranes (112). In contrast, ClC-K2 (ClC-Kb) is expressed in basolateral membranes of the thick ascending limb, of the distal convoluted tubule, and of intercalated cells of the collecting duct. In the inner ear, both channels are expressed in basolateral membranes of secretory epithelia, i.e., in marginal cells of the stria vascularis and in dark cells of the vestibular organ (27, 110). Both in the kidney and in the inner ear, ClC-K proteins always colocalize with their β-subunit barttin, which in turn always colocalizes with ClC-K proteins (27). Given the high sequence identity between ClC-Ka and ClC-Kb, these isoforms could not be distinguished by the antibody (27).

Rodent ClC-K1 is the only ClC-K channel known to yield currents by itself (34, 39, 107). In retrospect, the currents published for ClC-K2 (106) were probably endogenous to *Xenopus* oocytes used for expression. Surprisingly, even the human ortholog ClC-Ka did not yield currents when expressed alone (42). The failure to yield currents was puzzling, in particular because immunocytochemistry showed the presence of ClC-K proteins in renal plasma membranes (34, 112), a localization strongly supported by the renal transport defect in Bartter syndrome type III, which is caused by ClC-Kb mutations (109). The solution to this problem came from human genetics: Hildebrandt and colleagues identified a small integral membrane protein, named barttin (see also above), as being mutated in Bartter syndrome type IV (28). Shortly afterward, it became clear that barttin was necessary for the functional expression of ClC-Ka and ClC-Kb and works as a β-subunit for those channels. Barttin drastically increased currents of ClC-K1, both in oocytes and transfected mammalian cells (27). This was due to a large increase in surface expression (27). Currents had an anion selectivity sequence of $Cl \geq Br > NO_3 > I$ for ClC-Ka/barttin and $Cl > Br = NO_3 > I$ for ClC-Kb/barttin. They show only little voltage-dependent gating, consistent with the fact that the gating glutamate that obstructs the pore (14, 19) is replaced by valine in both ClC-Ka and ClC-Kb. Indeed, introducing a glutamate at that position generated voltage-dependent gating (39). Currents through either channel were increased by raising $[Ca^{2+}]_o$ or pH_o (27). Whereas ClC-K1 currents were strongly increased by $[Ca^{2+}]_o$, this effect was blunted upon coexpression with barttin (118). Hence, this β-subunit changes current properties in addition to increasing surface expression. As ClC-K1 channels are normally present in complexes with barttin, this observation also cautions against attributing an important physiological regulatory role to $[Ca^{2+}]_o$.

Barttin, a small integral membrane protein, has two predicted amino-terminal transmembrane domains. Both amino and carboxy termini are inside the cell (27, 28). Barttin does not belong to a larger gene family. ClC-K channels are apparently the only CLC proteins that interact with barttin. Barttin carries a putative PY-motif in its carboxy-terminal tail. Similar motifs have been identified in ClC-5 (119) and in the epithelial Na^+ channel ENaC (120, 121), where they were shown to bind to WW-domain (a protein domain characterized by typical tryptophane residues) containing ubiquitin ligases that down-regulate channel activity by enhancing their endocytosis. Compatible with a similar role in barttin, mutations in its putative PY motif increased currents and surface expression of ClC-K/barttin (27).

As with several other CLC channels, the physiological importance of ClC-K/barttin channels is highlighted by human inherited disease (28, 109) and a mouse model (116). ClC-Kb is mutated in Bartter syndrome type III (109). This syndrome is associated with severe renal salt loss, predominantly through a loss of NaCl reabsorption in the thick ascending limb of Henle's loop. In this nephron segment, NaCl is taken up in a secondary active process by the apical Na-K-2Cl cotransporter (NKCC2). K^+ ions are recycled over the apical membrane by the ROMK (Kir1.1) K^+ channel, whereas Cl^- leaves the cell basolaterally through ClC-Kb/barttin Cl^- channels. This transport model is now strongly supported by genetic evidence: Mutations in NKCC2 lead to Bartter syndrome type I (122), those in ROMK to Bartter syndrome II (123), those in ClC-Kb to Bartter syndrome III (109), and finally, those in barttin to Bartter syndrome IV (28).

ClC-K1 (the rodent ortholog of ClC-Ka) has been disrupted in mice (116), leading to renal water loss reminiscent of diabetes insipidus. A high Cl^- permeability in the ascending thin limb, the site of ClC-K1 expression, is essential for establishing the high osmolarity of the renal medulla in a countercurrent system. Accordingly, the solute accumulation in the inner medulla of ClC-K1 knockout mice was severely impaired (124). No corresponding human disease is known so far, but mutations in both ClC-Ka and ClC-Kb were found in one family (125). As expected, the patients presented with symptoms resembling Bartter syndrome IV, in which the loss of barttin eliminates the function of both Cl^- channels as well.

The renal symptoms in Bartter syndrome III are slightly different (e.g., concerning Ca^{2+} handling) from Bartter syndromes type I and II (126), as might be expected from the additional presence of ClC-Kb in the distal convoluted and collecting tubules. Likewise, renal symptoms in Bartter syndrome IV are more severe than in the other forms as the lack of functional barttin disrupts the function of both ClC-K isoforms. Interestingly, a common polymorphism in the human *CLCNKB* gene led to dramatically increased ClC-Kb/barttin currents in heterologous expression (127). No pathological consequences have been reported as yet.

Barttin mutations in Bartter syndrome type IV additionally lead to congenital deafness (28). This may be explained by a secretory defect of the stria vascularis. This epithelium secretes K^+ into the scala media and generates a positive voltage of this unique extracellular compartment. Its high K^+ concentration ($\sim$150 mM)

and potential (+90 mV) are necessary to drive K^+ through apical mechanosensitive cation channels into sensory hair cells. Marginal cells of the stria, which face the lumen of the scala media, take up K^+ through basolateral Na/K-ATPase pumps and NKCC1 Na-K-2Cl cotransporters. K^+ ions leave the cells apically through KCNQ1/KCNE1 K^+ channels. Similar to ROMK, which recycles K^+ for the NKCC2 cotransporter across apical membranes of the thick ascending limb, ClC-K/barttin channels are needed to recycle Cl^- for the NKCC1 cotransporter over the basolateral membrane of marginal cells (27). RT-PCR indicated the presence of both ClC-K1 and ClC-K2 in the inner ear (27), readily explaining that the disruption of ClC-Kb in Bartter syndrome III does not lead to deafness. The lack of barttin, however, which compromises both channels, reduces Cl^- recycling to such a degree that deafness ensues. This model (27) has been confirmed recently by the finding that the lack of both ClC-Ka and ClC-Kb also results in deafness (125).

CLC-3, AN ENDOSOMAL Cl^- CHANNEL THAT IS ALSO EXPRESSED ON SYNAPTIC VESICLES

ClC-3, -4, and -5 form their own branch of the CLC gene family. They share about 80% sequence identity. These channels are located mainly in membranes of intracellular vesicles, mostly in the endocytotic pathway. Whereas the role of ClC-5 in endocytosis is well established, less is known about the physiological functions of ClC-3 and in particular of ClC-4.

Cell fractionation and transfection studies localized ClC-3 to an endosomal compartment, where it partially colocalized with rab4 and lamp-1 and, additionally, to synaptic vesicles (128). The localization in endosomal/lysosomal compartments and in synaptic vesicles has now been confirmed in several studies (41, 129–131). AP3-deficient mice and cell lines were used to show that the AP3 adaptor complex plays a role in targeting ClC-3 to synaptic vesicles (131). The overexpression in mammalian cells often results in artificial, large intracellular vesicles that are acidic and stain for ClC-3 and lysosomal markers (41; T. Breiderhoff & T.J. Jentsch, unpublished data). However, some ClC-3 protein was also detected at the plasma membrane of ClC-3-overexpressing Chinese hamster ovary (CHO) cells (129).

ClC-3 knockout mice show a severe degeneration of the retina and the brain (128). After a few months, the hippocampus had totally degenerated and was replaced by fluid-filled spaces (128). The neurodegeneration, a consistent feature of three independent mouse models (128, 130, 132), was not restricted to the hippocampus, but was also seen in other brain regions. It was associated with a moderate storage of the subunit c of mitochondrial ATPase in lysosomes (130), a feature considered typical for human neuronal lipofuscinosis (133). The neurodegeneration was associated with an activation of microglia and astrogliosis (128, 132). In spite of the severe neurodegeneration, mice survived more than a year

(128), although there was increased lethality (132). ClC-3 knockout mice show several behavioral abnormalities, including hyperactivity (128, 132). The mechanism by which a loss of ClC-3 leads to neurodegeneration remains unclear and may be related to cellular trafficking defects (128).

Vesicular Cl^- channels are thought to provide an electric shunt for the electrogenic H^+-ATPase, thereby facilitating the acidification of synaptic vesicles and compartments in the endosomal/lysosomal pathway. Indeed, the disruption of ClC-3 partially inhibited the acidification of synaptic vesicles in vitro (128), and the luminal pH of a vesicle fraction mainly representing endosomes was elevated (130). ClC-3 was also reported to be expressed on insulin-containing granules of pancreatic β-cells, but this result hinges on the quality of the antibody (134). It was also proposed to participate in the acidification of insulin-containing granules of pancreatic β-cells and to play an important role in insulin secretion (134). However, ClC-3 knockout mice did not exhibit hyperglycemia, neither under resting conditions, nor following a glucose load (132; A.A. Zdebik & T.J. Jentsch, unpublished data).

As expected from a mainly intracellular localization, many laboratories, including our own, have been unable to obtain plasma membrane currents of ClC-3 upon heterologous expression (129, 135). There are numerous contradictory reports on putative ClC-3 currents. ClC-3 was variably reported to yield protein kinase C-inhibitable, moderately outwardly rectifying Cl^- currents in *Xenopus* oocytes (136), strongly rectifying, $[Ca^{2+}]_i$-inhibitable currents in CHO cells (137), a moderately outwardly rectifying current dramatically activated by CaM-kinase II in tsA cells (138, 139), and moderately activated outward currents that were further activated by cell swelling (140, 141). In spite of these conflicting properties, all these currents (136–141) share an $I^- > Cl^-$ selectivity. This contrasts with the preference of Cl^- over I^- of ClC-0, ClC-1, ClC-2, ClC-K/barttin, ClC-4, and ClC-5 (40, 142, 143) (the latter two being structurally highly related to ClC-3), and even of a bacterial CLC protein (144). On the other hand, Weinman and coworkers reported currents that share the consensus $Cl^- > I^-$ selectivity of CLC channels in CHO cells overexpressing ClC-3 (41, 145). The extremely strong outward rectification of these currents closely resembled that of the highly homologous ClC-4 and ClC-5 channels (40, 142). Furthermore, a neutralizing mutation of the gating glutamate drastically changed the I/V-curve to an inwardly rectifying behavior (41), closely resembling the effect of similar mutations in ClC-4 and ClC-5 (40). Thus these currents (41, 145) almost certainly represent the real ClC-3 currents, whereas the other currents might be endogenous to the expression systems. It seems highly unlikely that the ion selectivity of CLC channels might be significantly changed by accessory proteins potentially present in other expression systems, as their pores are contained within a single protein subunit (13, 14).

The notion that ClC-3 might represent a swelling-activated Cl^- channel ($I_{cl,swell}$) (140) was also invalidated by ClC-3 knockout mouse models (128, 146, 147). Swelling-activated Cl^- currents were not changed in pancreatic and hepatic cells (128), salivary gland cells (146), or in cardiac myocytes (147). The argument

(147) that another swelling-activated Cl^- channel with exactly the same properties is upregulated to compensate for the loss of ClC-3 is not plausible, as the only reasonable candidates are ClC-4 and ClC-5. Their transcription, however, was unchanged in *Clcn3*$^{-/-}$ mice (128, 147), and their channel properties differ drastically from $I_{cl,swell}$ (as, almost certainly, do ClC-3 currents) (41, 145). The different regulation of $I_{cl,swell}$ reported for ClC-3 knockout cells (147) may be from indirect effects, as similar findings were previously observed with multidrug resistance P-glycoprotein (mdr) or $I_{cl,n}$, proteins that are no longer regarded as Cl^- channels (1, 148). Ca^{2+}-activated Cl^- currents were also found unchanged in two independent ClC-3 knockout mouse models (1, 128, 146). However, CamKII-activated Cl^- currents were reported to be strongly reduced in *Clcn3*$^{-/-}$ mice (139), possibly suggesting an effect of ClC-3 on regulatory pathways.

CLC-5, A VESICULAR CHANNEL IMPORTANT FOR RENAL ENDOCYTOSIS

ClC-4 and ClC-5 are about 80% identical to ClC-3. Both reside in endosomal membranes but can reach the plasma membrane to some degree in heterologous expression. Both channels are extremely outwardly rectifying and mediate measurable currents at voltages only more positive than $\sim$+20 mV. These have a selectivity sequence of $NO_3 > Cl > Br > I$ and are inhibited by extracellular acidic pH (40, 142, 143). The physiological significance of the extremely strong outward rectification of these channels is unclear, because voltages more positive than +20 mV are unlikely to be reached under physiological conditions in the plasma membrane or intracellular vesicles. Mutagenesis, particularly a mutation that neutralized the gating glutamate, confirmed that the observed currents are directly mediated by these channels (40). The single-channel properties of ClC-5 and ClC-4 are not yet established. Two groups reported very different values for the single-channel conductance of ClC-4 (143, 149). Overall, the biophysical properties of ClC-4 and ClC-5 are very similar to those reported later for ClC-3 (41, 145). ClC-5, but not ClC-4 or ClC-3, carries a PY motif between the two CBS domains in its carboxy-terminal cytoplasmic tail (119). Destroying this motif by mutagenesis increased surface expression and currents about twofold. This motif probably interacts with the WW-domains of the HECT-ubiquitin ligase WWPII, which is present in the kidney (119, 150). Although ClC-4 and ClC-5 are expressed in most tissues to some degree, ClC-4 is more abundant in brain, muscle, and liver (151, 152), whereas ClC-5 is predominantly found in kidney and intestine (142, 153).

There is still little known about the functional roles of ClC-4. Overexpression of ClC-4 in cell lines led to an increase of copper incorporation into ceruloplasmin (154), which resembles the function of the yeast homologue *Gef1p* in iron metabolism (155–157). However, it is currently unknown whether ClC-4 is necessary for this process, a question that could be answered by analyzing ClC-4 knockout mice.

In contrast, we have a fairly good understanding of the roles of ClC-5. This channel is expressed predominantly in endosomes, where it colocalized with rab5 and endocytosed markers (158). In the proximal tubule of the kidney, it is mainly expressed in apical endosomes below the brush border, where it colocalizes with the H^+-ATPase (158–160). It is also highly expressed in acid-transporting intercalated cells of the collecting duct, where it colocalized with the proton pump in acid-secreting α-, but not acid-reabsorbing β-cells (158). In the intestine, ClC-5 is expressed in apical endosomes as well (153). Although we do not yet understand the role of ClC-5 in intercalated cells, there is now ample evidence that it is essential for apical endocytosis in the proximal tubule. A similar function is likely for ClC-5 in the intestine.

The physiological importance of ClC-5 is illustrated by Dent's disease (161), the pathophysiological mechanism of which has been elucidated by knockout mouse models (162–164). Dent's disease is an X-linked disorder associated with low-molecular weight proteinuria, hyperphosphaturia, and hypercalciuria, which ultimately leads to kidney stones in the majority of patients (165). In addition, there is a variable presence of other symptoms of proximal tubular dysfunction such as glucosuria and aminoaciduria. By now, many different ClC-5 mutations have been identified in patients with Dent's disease. These include early truncations, but also missense mutations. For reasons that are still unclear, the latter mutations cluster at the interface between the two subunits (166). Many missense mutations have been studied in the *Xenopus* oocyte expression system and were shown to abolish or drastically reduce ClC-5 currents (167, 168).

The knockout of ClC-5 led to low-molecular weight proteinuria (162, 164), and, depending on the mouse model, also to hyperphosphaturia (162) or hypercalciuria (164). The proteinuria is the result of a cell-autonomous defect in endocytosis, which extends to fluid-phase endocytosis, receptor-mediated endocytosis, and the endocytosis of integral plasma membrane proteins such as the Na-PO_4 cotransporter NaPi-IIa or the Na/H exchanger NHE3 (162). Endocytosis, however, is not totally abolished, but strongly reduced. The amount of the endocytotic receptor megalin, which mediates the uptake of a wide variety of proteins and other substrates, was significantly reduced, and its presence in the brush border appeared to be reduced (162). This observation probably indicates a role of ClC-5 in recycling endosomes. A strong reduction of megalin in the brush border was also revealed by immunoelectron microscopy (169). A reduction of megalin plasma membrane expression is expected to reduce receptor-mediated endocytosis even further. Renal cortical endosomes, which are predominantly derived from proximal tubules, had a lower rate and extent of acidification than wild-type endosomes in vitro (162, 163). This strongly supports the hypothesis that the Cl^- conductance provided by ClC-5 is needed to dissipate the voltage created by the electrogenic H^+-ATPase, thereby enabling efficient acidification in the endosomal pathway.

How does a defect in endosomal acidification, which in turn leads to a defect in endocytosis, eventually cause kidney stones? Several hormones, including parathyroid hormone (PTH) and vitamin D_3, are filtered into the primary urine.

After binding to megalin, these hormones are normally endocytosed by proximal tubular cells. In the absence of ClC-5, the reduced endocytosis of PTH is expected to result in a progressive increase of luminal PTH concentration along the length of the proximal tubule, while serum concentrations of the hormone remain unchanged (162). The increased luminal levels of PTH will stimulate apical PTH receptors, which in turn enhance the endocytosis of the apical Na-PO_4 cotransporter NaPi-IIa. Indeed, immunocytochemistry revealed that the majority of NaPi-IIa had shifted to intracellular vesicles in knockout mice, whereas it resided in the brush border of wild-type proximal tubules (162). This PTH-dependent decrease of NaPi-IIa in the plasma membrane readily explains the hyperphosphaturia that was observed in the knockout (162) and was found in patients with Dent's disease (165).

PTH is also known to stimulate the transcription of the enzyme α-hydroxylase, the enzyme that converts the inactive precursor 25(OH)-VitD_3 to the active hormone 1,25$(OH)_2$-VitD_3 in proximal tubular cells. As expected from the increased luminal concentration of PTH, mRNA levels of α-hydroxylase and its enzymatic activity were increased in ClC-5 knockout mice (162, 163). These findings suggest increased levels of the active hormone 1,25$(OH)_2$-VitD_3. Indeed, many patients with Dent's disease have slightly elevated serum concentrations of active VitD_3 (165, 170). These may lead to an increased intestinal reabsorption of Ca^{2+}, which in turn could cause the hypercalciuria and kidney stones in Dent's disease. However, there is a major complication: A large part of the precursor 25(OH)-VitD_3 is taken up into proximal tubular cells through megalin-dependent apical endocytosis. The amounts of VitD-binding protein and VitD_3 were drastically increased in the urine of *Clcn5*$^-$ mice (162, 163). Therefore, there are two opposing effects (upregulation of the activating enzyme and loss of substrate) that may lead to an increase or decrease of active VitD_3. The outcome will depend on many factors (including genetic and dietary ones), and may explain the clinical variability of Dent's disease. Such a variability was even observed between the two ClC-5 knockout mouse models: Whereas the knockout mouse generated in our laboratory has decreased serum levels of 1,25$(OH)_2$-VitD_3 and no hypercalciuria (162, 163), the model from Guggino's laboratory has slightly elevated levels of the active hormone and displays hypercalciuria (164, 171). Thus the unifying hypothesis described above explains the complex and variable symptoms of Dent's disease through changes in calciotropic hormones that stem from defects in proximal tubular endocytosis, which in turn result from a defective acidification of endosomes (162, 163).

CLC-7, A LATE ENDOSOMAL/LYSOSOMAL Cl^- CHANNEL IMPORTANT FOR NEURONS AND OSTEOCLASTS

ClC-6 and ClC-7 belong to a third branch of the CLC family and share about 45% sequence identity (108). Both channels are nearly ubiquitously expressed. Like ClC-3, -4, and -5, they are localized in membranes of intracellular compartments (23, 172). This subcellular expression readily explains why our (108) laboratory

and others (173) were unable to measure plasma membrane currents from these channels. The currents reported for ClC-7 (174) may be endogenous to *Xenopus* oocytes in particular, because similar currents were observed by one group (175) upon expression of ClC-4 —but these currents differ in many respects from the established properties of ClC-4 (40, 143). Whereas little is known about the functions of ClC-6, a ClC-7 knockout mouse that resulted in osteopetrosis and neurodegeneration has yielded considerable insights into the roles of ClC-7 (23).

ClC-7 resides in late endosomes and lysosomes (23). Whereas ClC-3 to ClC-7 are all present in the endocytotic pathway, ClC-7 is the only one of these channels that localizes to a large degree to lysosomes (23). It is also present in the ruffled border of osteoclasts, a specialized domain of the plasma membrane that faces the resorption lacuna of these bone-degrading cells. The ruffled border is generated by the exocytotic insertion of late endosomal/lysosomal membranes (176). Hence it contains lamp-1, a marker for late endosomes/lysosomes, and significant amounts of a V-type H^+-ATPase in addition to ClC-7. The exocytotic buildup of the ruffled border is paralleled by a secretion of lytic enzymes such as cathepsins into the resorption lacuna. These lysosomal enzymes, which degrade the organic bone matrix, require an acidic pH for optimal activity. The acidification of the resorption lacuna, which is also needed for the chemical dissolution of inorganic bone material, is mediated by the electrogenic H^+-ATPase of the ruffled border. Similar to the acidification of renal endosomes or synaptic vesicles, which relies on ClC-5 (162, 163) or ClC-3 (128), respectively, it is thought that the neutralizing current through ClC-7 is required for an efficient acidification of the resorption lacuna. ClC-7 might also play a role in the exocytotic trafficking of late endosomal/lysosomal vesicles because the ruffled border was less developed in ClC-7 knockout osteoclasts (23).

The disruption of ClC-7 in mice caused blindness and severe osteopetrosis (23), a disorder characterized by dense, fragile bones devoid of bone marrow and hence associated with extramedullary hematopoiesis (177). In the knockout mice, osteoclasts were present in normal numbers, but could not acidify the resorption lacuna and failed to resorb bone (23). These findings quickly led to the identification of human *CLCN7* mutations underlying infantile malignant osteopetrosis (23). Subsequently, more ClC-7 mutations were identified in this condition as well as in autosomal-dominant osteopetrosis type II (ADOII, Albers-Schönberg disease) and in intermediate forms (24, 178–180). The dimeric structure of CLC channels predicts that the reduction of ClC-7 currents is unlikely to exceed 75% with heterozygous dominant mutations. Indeed, ADOII patients are generally not blind and develop osteopetrosis (whose phenotype is less severe than in malignant infantile osteopetrosis) only during adult life.

Mutations in the a3 subunit of the V-type H^+-ATPase also cause osteopetrosis in mice (181, 182) and humans (183, 184), underscoring the need to acidify the resorption lacuna. Mutations in this gene lead to a recessive, severe infantile form of the disease. As in *Clcn7*$^{-/-}$ mice, the ruffled border of osteoclasts from mice lacking the a3 subunit was poorly developed.

In addition to its function in bone resorption, ClC-7 plays other essential roles in the organism. ClC-7 transcripts were detected in all tissues analyzed, including brain, eye, kidney, liver, and testis, and ClC-7 protein was detected in cultured mouse fibroblasts (23, 108). In brain, expression is strongest in neurons (D. Kasper, R. Planells-Cases, J.C. Fuhrmann, T.J. Jentsch, in preparation). Consistent with the lysosomal localization of the channel, ClC-7-deficient mice develop a neuronal pathology with clear symptoms of lysosomal dysfunction (D. Kasper, R. Planells-Cases, J.C. Fuhrmann, T.J. Jentsch, in preparation). Cortical and hippocampal neurons accumulate osmiophilic storage material positive for periodic acid-Schiff stain. In the brain, the levels of various lysosomal enzymes are increased, as is the amount of subunit c of the mitochondrial ATP synthase. The latter increase, a hallmark of certain lysosomal storage diseases (133), is much more pronounced than in *Clcn3*$^{-/-}$ mice (130; D. Kasper, R. Planells-Cases, J.C. Fuhrmann, T.J. Jentsch, in preparation). Taken together, these changes are reminiscent of the symptoms observed in a group of human pathologies classified as lysosomal storage disease or neuronal ceroid lipofuscinosis. The underlying genetic causes are heterogeneous. In most cases there are mutations in genes encoding lysosomal enzymes, or mutations in other proteins that impair the function of lysosomes (133, 185).

In addition to central neurodegeneration, the disruption of ClC-7 led to a massive loss of photoreceptors and retinal neurons, causing blindness before the age of four weeks (23). Interestingly, osteopetrosis caused by mutations in the a3-subunit of the H^+-ATPase, as in osteosclerotic (*oc/oc*) mice (181), is not accompanied by a fast, primary retinal degeneration and lysosomal storage in central neurons (D. Kasper, R. Planells-Cases, J.C. Fuhrmann, T.J. Jentsch, in preparation). This finding is of clinical relevance because it suggests that molecular diagnosis might influence the choice of therapy.

SUMMARY AND OUTLOOK

Almost immediately after the cloning of the first mammalian voltage-gated Cl^- channel, ClC-1 (9), it became clear that its inactivation causes myotonia (50), an inherited muscle stiffness. During the past ten years, many other CLC channelopathies have been discovered. Progress in this area came from human genetics, as with the roles of ClC-Kb (109) and barttin (28) in two different forms of Bartter syndrome (renal salt loss) or with ClC-5 in Dent's disease (kidney stones) (161), or from mouse models, which led to the identification of human disease genes, as with ClC-7 and osteopetrosis (23). The pathologies associated with CLC defects (muscle stiffness, renal salt loss, deafness, blindness, neurodegeneration, male infertility, osteopetrosis, proteinuria, and kidney stones) revealed a hitherto unsuspected range of physiological functions of chloride channels and stressed their obvious medical importance. The greatest surprise was probably the discovery that the majority of CLC Cl^- channels function in intracellular vesicles and

that their disruption leads to specific and highly diverse diseases. The discoveries that ClC-5 has an important role in renal endocytosis (162) or that ClC-7 is crucial for osteoclast function (23) would have been much more difficult or impossible without the clues provided by mouse models and human disease. As the vesicular CLC channels may have overlapping subcellular expression patterns, which in most cases remain to be rigorously defined, one may expect that additional functions will emerge upon the elimination of redundancies by double knockouts. Furthermore, the pathologies observed with the elimination of ubiquitously expressed channels such as ClC-2, ClC-3, or ClC-7 may represent only the tip of an iceberg, with many interesting discoveries still to be made. Increasingly sophisticated mouse models, as well as a broad spectrum of physiological, biophysical, and cell biological techniques, will be needed to address these issues.

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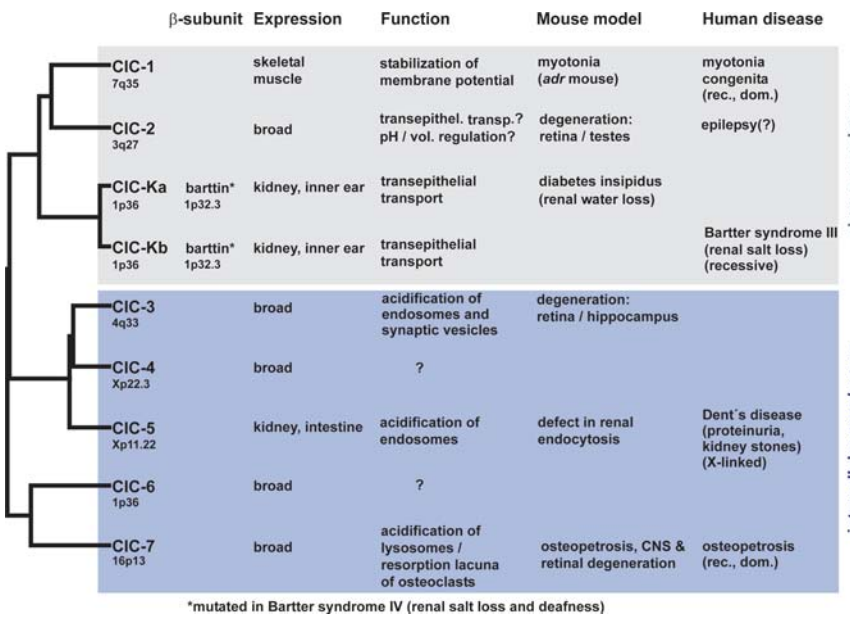

Figure 1 A phylogenetic tree of all mammalian CLC channels, their chromosomal localization in humans, their known subunits, and an overview of diseases associated with loss of these channels in mice and humans. Channels in the gray box mainly function in the plasma membrane, whereas those in the blue box are primarily localized in intracellular membranes. Mouse genes are abbreviated *Clcn*X; the respective genes in humans are written in upper case letters. For proteins, the systematic nomenclature is ClC-X.