From stones to bones: The biology of ClC chloride channels

Alfred L. George, Jr, Laura Bianchi, Elizabeth M. Link and Carlos G. Vanoye

Chloride (Cl^-) is the most abundant extracellular anion in multicellular organisms. Passive movement of Cl^- through membrane ion channels enables several cellular and physiological processes including transepithelial salt transport, electrical excitability, cell volume regulation and acidification of internal and external compartments. One family of proteins mediating Cl^- permeability, the ClC channels, has emerged as important for all of these biological processes. The importance of ClC channels has in part been realized through studies of inherited human diseases and genetically engineered mice that display a wide range of phenotypes from kidney stones to petrified bones. These recent findings have demonstrated many eclectic functions of ClC channels and have placed Cl^- channels in the physiological limelight.

Address: Departments of Medicine and Pharmacology, Division of Genetic Medicine, 451 Preston Research Building, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-6304, USA.

Correspondence: Alfred L. George, Jr
E-mail: al.george@vanderbilt.edu

Current Biology 2001, 11:R620–R628

0960-9822/01/$ – see front matter

Introduction

Ion channels are ubiquitous transmembrane proteins that confer selective ionic permeability to cell surface and intracellular membranes in virtually every cell in every known organism. Research on ion channels during the past 60 years has focused predominantly on proteins that mediate selective permeability to monovalent (Na^+, K^+) and divalent (Ca^{2+}) cations. However, there has recently been intense fascination with ion channels that are selectively permeable to chloride (Cl^-) ions as their importance in human diseases and fundamental cellular events has been elucidated.

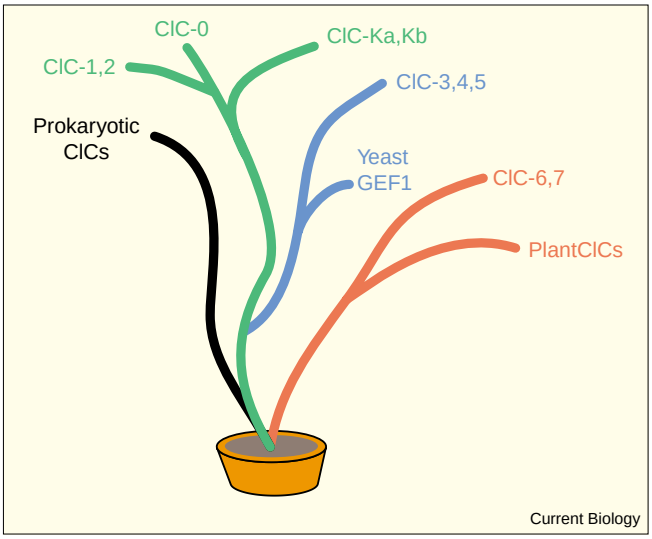
Historically, chloride channels carrying electrical current have been less exciting than their more dynamic cation channel counterparts. This is particularly evident in electrically excitable tissues where the sequential activation of voltage-gated sodium and potassium channels culminates in the generation of the compound action potential. By contrast, chloride channels leave much more subtle physiological footprints. In addition, there are hardly any highly selective pharmacological chloride channel blockers, further lengthening the delay in uncovering the role of these channels in cellular functions.

Despite a lack of attention, the biological importance of chloride channels should not be underestimated. In multicellular organisms Cl^- is the most abundant extracellular anion. Organisms have evolved several diverse molecular mechanisms to enable chloride transport into or out of cells and within the intricate intracellular environment. Chloride channels represent one such mechanism to facilitate passive movement of Cl^- driven by a favorable electrochemical gradient. Increasingly, chloride channels are being recognized as important participants in a wide range of physiological activities. The movement of Cl^- across cell membranes can provide electrical stability in excitable tissues such as skeletal muscle and neurons. Chloride movement is required for the transepithelial movement of salt and water by epithelial tissues such as in the kidney and gut. The movement of chloride also participates importantly in regulating cell volume so that cells may inhabit conditions of changing tonicity. Finally, intracellular chloride channels enable organelles to maintain electrical neutrality during acidification by proton pumping mechanisms.

ClC channels

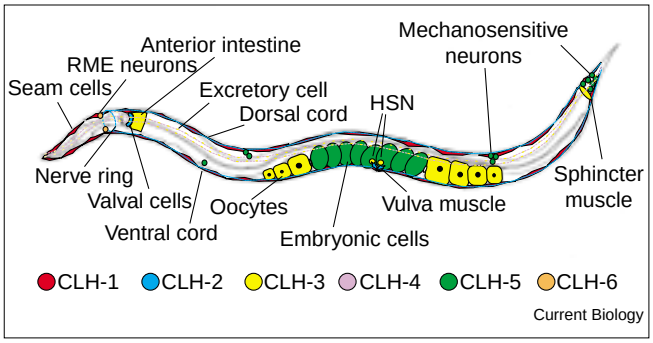
Chloride channels can be formed by diverse molecular structures. During the past ten years a newly recognized class of proteins, the ClC family of chloride-ion selective channels, has been identified through molecular cloning

Figure 1



Phylogeny of ClC channels.

Figure 2



Location of ClC channel homologues in *C. elegans*. Expression patterns of the six worm ClC channels (CLH proteins color coded according to the inset) are illustrated as deduced from reporter gene experiments [3–5].

approaches [1,2]. At least nine mammalian isoforms exist and there is compelling evidence from genome sequencing for multiple ClC channels in a wide range of other species ranging from bacteria to *Drosophila*. Based upon deduced amino acid sequences, ClC channels can be grouped into three evolutionary categories in mammals (Figure 1). Identifiable ClC sequences from *Saccharomyces* (yeast), *Neurospora* (mold), *Arabidopsis* (plant), *Drosophila* (fly) and *Caenorhabditis* (worm) also fit into this phylogenetic tree structure while bacterial ClC channels comprise another more ancient evolutionary branch. Their existence in species separated by billions of years of evolution suggests that ClC channels mediate cellular events that are essential for life.

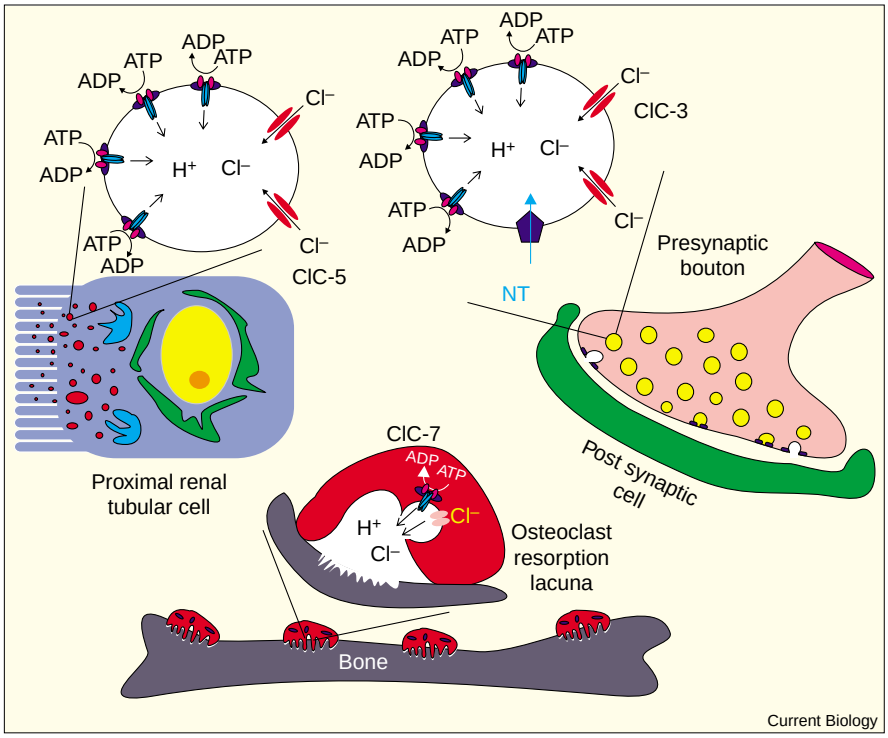
Advances in understanding the importance of ClC channels have emerged from human genetics, and studies of gene inactivation in mice and other model organisms, particularly yeast and *Caenorhabditis elegans*. Specific functions of many mammalian ClC channels may be inferred from the presentation of human diseases caused by natural mutations or from the corresponding null phenotypes of genetically engineered mice (Table 1). Understanding the physiological roles of ClC channels at the cellular level has also moved forward because of recent observations in simple organisms for which entire genome sequence is available, such as *C. elegans* (Figure 2) [3–5]. This synopsis provides an update on many of these recent advances. Readers specifically interested in the role of ClC channels in muscle contraction and renal salt transport are also directed to other recent review articles focused on these issues [6–9].

Table 1

Functions of mammalian ClC chloride channels.

Subtype	Gene (location)[1]	Functional role	Human (murine) phenotype
ClC-1	CLCN1 (7q35)	Sarcolemmal excitability	Myotonia congenita [62,63]
ClC-2	CLCN2 (3q26-qter)	Epithelial Cl^- transport cell volume regulation?	(Retinal degeneration, male infertility) [40]
ClC-3	CLCN3 (4q33)	Synaptic vesicle acidification, cell volume regulation?	(Neurodegeneration, retinal degeneration) [34]
ClC-4	CLCN4 (Xp22.3)	Unknown	
ClC-5	CLCN5 (Xp11.22)	Endosomal acidification	Dent's disease, other X-linked nephrolithiasis syndromes [14]
ClC-6	CLCN6 (1p36)	Unknown	
ClC-7	CLCN7 (16p13)	Bone resorption by osteoclasts	Infantile malignant osteopetrosis [64]
ClC-Ka	CLCNKA (1p36)	Renal Cl^- reabsorption	(Nephrogenic diabetes insipidus) [65]
ClC-Kb	CLCNKB (1p36)	Renal Cl^- reabsorption	Bartter's syndrome [66]

Figure 3



Role of ClC channels in acidification. Illustrations of three cell types in which distinct ClC chloride channels are important for acidification of intracellular or extracellular compartments. See text for further details.

Acidification of intracellular compartments

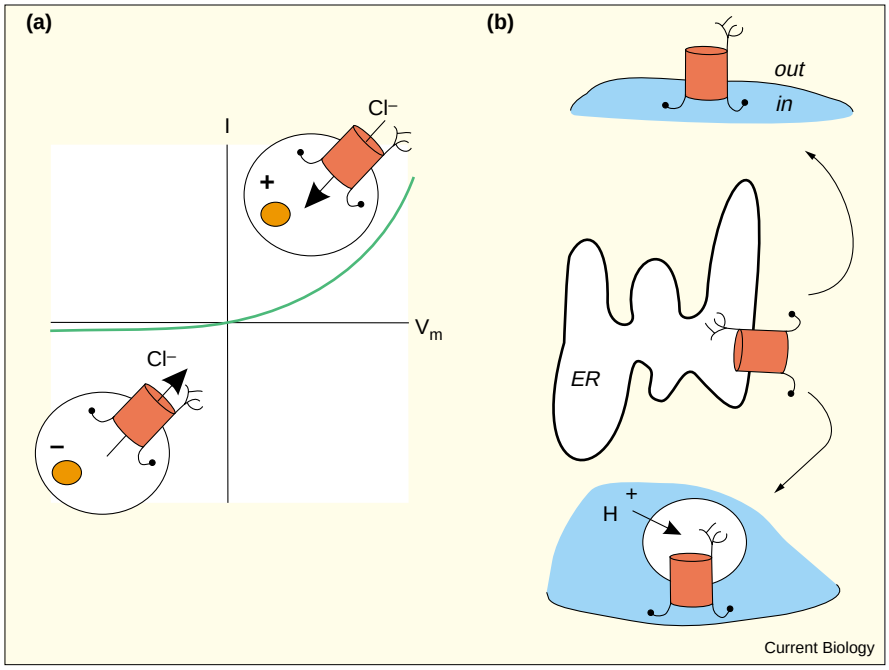
Various intracellular compartments in eukaryotic cells maintain an acidic pH to facilitate vesicular trafficking and related cellular functions [10]. This is accomplished by the active transport of protons (H^+) by a vacuolar type H^+–ATPase [11]. Active H^+ pumping is electrogenic and generates a voltage gradient by creating an asymmetric distribution of charges across the vesicular membrane. This electrical polarization of the intracellular membrane can limit the maximal level of acidification by introducing a positive charge within the vesicle that opposes further movement of protons. This problem is solved by concurrent passive Cl^- movement into vesicular compartments thus restoring electroneutrality and enabling a higher pH gradient to be established (Figure 3). Intracellular organelles possess chloride channels although their molecular identities have not been completely discerned [12]. Recent work now indicates that members of the ClC channel family participate in this process.

Endosomal acidification in renal epithelia

The first evidence for involvement of a ClC channel in the acidification of intracellular vesicles arose from studies of a rare familial disorder, Dent's disease, and related disorders of X-linked hypercalciuric nephrolithiasis caused by mutations in the gene encoding human ClC-5 gene (*CLCN5*, Xp11.22) [13,14]. Individuals with ClC-5 mutations exhibit some or all of the following phenotypic features: elevated urinary excretion of low molecular weight proteins (low molecular weight proteinuria), excessive urinary calcium excretion (hypercalciuria) and intrarenal calcification (nephrocalcinosis) or formation of calcium kidney stones (nephrolithiasis). The appearance of low molecular weight proteins in the urine is abnormal and suggested that ClC-5 mutations might interfere with absorptive endocytosis in the renal proximal tubule where this type of protein is usually reabsorbed [15]. Indeed, ClC-5 has been localized to early endosomes in proximal tubular cells [16–18] and failure to acidify these compartments presumably explains defective endocytosis (Figure 3).

More definitive evidence for the role of ClC-5 in mediating endosomal acidification in the kidney was reported recently from two groups who studied mice with targeted inactivation of the corresponding murine gene, *clcn5* [19,20]. By tracking the excretion of labeled low molecular weight proteins in the mice, these investigators discovered a substantial impairment in proximal tubule protein absorption that correlated with reduced receptor-mediated and fluid-phase endocytosis in proximal tubular epithelial cells. Defective endocytosis may also account for perturbed calcium homeostasis and thus explain other prominent aspects of Dent's disease: hypercalcuria and susceptibility to form kidney stones [19]. Parathyroid hormone (PTH), a

Figure 4



Rectification and transmembrane orientation of ClC-5. (a) Current-voltage relationship for ClC-5 channels exhibiting strong outward rectification. Inset figures indicate the direction of Cl^- flux at positive (inward) or negative (outward) membrane voltage (V_m). (b) Predicted transmembrane orientation of channel proteins in the endoplasmic reticulum (ER), surface membranes (top) or in intracellular vesicles (bottom). A single presumed extracellular glycosylation site is shown as branched lines and the cytoplasmic amino and carboxyl domains are indicated by short lines with terminal knobs.

small peptide hormone, is reabsorbed from glomerular filtrate by the proximal tubule and therefore is predicted to be more concentrated in tubular fluid when ClC-5 is inactive. Stimulation of luminal PTH receptors may increase enzymatic conversion of 25-hydroxy vitamin D3 to the active compound 1,25-dihydroxy vitamin D which in turn promotes intestinal calcium absorption, increases circulating calcium levels, and increases renal calcium excretion (absorptive hypercalciuria). Abnormal membrane recycling of the proximal tubular sodium–phosphate cotransporter and associated urinary phosphate loss is also observed in *clcn5* null mice and this may also contribute to metabolic derangements. Finally, there is speculation that increased intestinal absorption of calcium may also result from impaired membrane recycling of epithelial calcium channels in the duodenum [21], another site of ClC-5 expression [22]. Additional studies are needed to clarify the direct and indirect role of ClC-5 in calcium homeostasis.

In addition to its intracellular location, ClC-5 appears to be expressed on surface membranes of other renal tubular cells such as cortical collecting duct intercalated cells where acid secretion occurs. These same cells exhibit luminal membrane H^+–ATPase activity and subunits of the vacuolar proton pump are expressed here under conditions of acid loading [23]. It is possible that ClC-5 mediates concomitant Cl^- flux during renal acid secretion although the strong outward rectification of the channel may limit its capacity (see below).

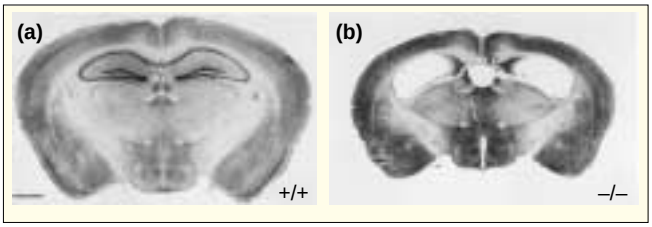
A biophysical paradox

The biophysical properties of ClC-5 are rather dramatic. When expressed in heterologous cell systems such as *Xenopus* oocytes or cultured mammalian cells, recombinant ClC-5 exhibits preferential Cl^- conductance at positive membrane potentials and virtually no permeation at negative membrane potentials (outward rectification, Figure 4) [24,25]. When ClC-5 is present on surface membranes of transfected cells, the channel conducts Cl^- into the cell ('outward' current by convention) during membrane depolarization, but permits only limited Cl^- efflux when the membrane potential is negative. This suggests a biophysical paradox regarding the function of ClC-5 in intracellular vesicles. As pointed out by Sakamoto *et al.* [25], the strong outward rectification of ClC-5 would greatly limit Cl^- movement into the acidifying compartment given certain critical assumptions about the polarity of channel insertion into the vesicular membrane (the intracellular compartment is equivalent to the extracellular space, Figure 4). It is apparent that ClC-5 must behave differently in the intracellular environment or that accessory subunits modulate its activity in order to fulfill its role in endosomal acidification.

Intracellular acidification in yeast

Only one ClC channel sequence has been found within the entire genome of *Saccharomyces cerevisiae* [26,27]. Deletion of this gene (named *GEF1* for glycerol/ethanol Fe-requiring) causes cells to grow slowly on media containing nonfermentable carbon sources in the absence of high

Figure 5



Degeneration of the hippocampus in mice with targeted ClC-3 inactivation. (a) Section of normal mouse brain showing intact hippocampus. (b) Section through the brain of a ClC-3 null mouse illustrating the absence of the hippocampus. Image is from Stobrawa *et al.* [34].

concentrations of iron [27,28]. Cells also exhibit impaired growth on glucose containing media when iron supplies are limited. The protein product of GEF1, Gef1p, has been localized to the medial Golgi apparatus, endoplasmic reticulum and to late and post-Golgi vesicles raising the possibility that this putative chloride channel participates in acidification of intracellular compartments [29]. Its presumed role in acidification has been supported by a similar phenotype resulting from mutation of a vacuolar proton pump subunit, Gef2p. Genetic complementation of the *gef1* phenotype by *Torpedo* ClC-0, murine ClC-6 and certain plant ClC channels has been shown [29–31], further supporting the hypothesis that this yeast gene indeed encodes a related chloride channel.

The relationship between chloride channel function and the observed metabolic phenotype has also been discerned. Poor growth in the presence of low iron concentrations results from a requirement of GEF1 for loading copper ions into the iron oxidase Fet3p, an important component of the high affinity iron uptake system within the yeast secretory apparatus [32]. Chloride ions serve as allosteric effectors of this process which is further augmented at acidic pH. Movement of Cl^- into intracellular compartments maintains electroneutrality during cation accumulation driven by H^+–ATPase and the Cu^{2+} transporting ATPases (Vma1, Ccc2). In addition to growth impairment during iron deficit, mutation of GEF1 confers sensitivity to certain cations including Mn^{2+} [28]. A defect in cation sequestration into intracellular compartments caused by impaired vesicular acidification has been proposed to explain the latter phenomenon.

Acidification of synaptic vesicles

Surprisingly few ClC chloride channels have been identified in the central nervous system. The first ClC channel cloned from brain, ClC-3, is highly expressed in hippocampus [33]. Controversy has surrounded the role of ClC-3 as a volume regulated chloride channel (see below), but recent evidence provides compelling evidence that this channel has a critical role in acidifying synaptic vesicles. Stobrawa *et al.* [34] recently reported the phenotype associated with targeted inactivation of murine *clcn3*. The mice exhibit postnatal growth retardation, blindness secondary to progressive retinal degeneration and behavioral abnormalities associated with profound hippocampal degeneration (Figure 5). Despite these rather dramatic phenotypic features, ClC-3 knockout mice survive and exhibit intact motor development.

Further investigations revealed that ClC-3 is present on synaptic vesicles and participates in their acidification. Stobrawa *et al.* [34] showed that ClC-3 copurifies with several proteins known to reside within synaptic vesicles such as synaptophysin, and vesicular glutamate and GABA transporters. Synaptic vesicles purified from ClC-3 null mice exhibit reduced *in vitro* ATP-dependent acidification rates and diminished glutamate transport that correlate with decreased transporter proteins. At first glance, these findings might suggest a role for excessive extracellular glutamate and excitotoxicity in mediating neurodegeneration in ClC-3 deficient mice but, as the investigators point out, this would not explain retinal degeneration as these cells are insensitive to glutamate receptor agonists. Nonetheless, these experiments provide strong evidence for a biological role of ClC-3 in synaptic function.

Role in bone resorption

Acid secretion by vesicular membranes not only subserves intracellular functions but also participates in selected physiological circumstances requiring extracellular proton accumulation. One such setting is osteoclastic bone resorption [35]. Osteoclasts are multinucleated cells formed by the fusion of mononuclear hematopoietic stem cells belonging to the phagocyte series. When attached tightly to bone, osteoclasts create scalloped spaces, or lacunae, into which are pumped protons and acid hydrolases that digest mineralized bone matrix. This process is critical for bone remodeling during development and healing. Acid secretion is mediated by the fusion of internal vesicles containing vacuolar-type proton pumps into the osteoclast surface membranes adjacent to the bone surface creating the 'ruffled border' where bone resorption occurs. As discussed above for intracellular vesicles, these extracellular acidic lacunae require the counterbalancing movement of Cl^- through chloride channels (Figure 3).

A recent report by Kornak *et al.* [36] defined the role of ClC-7 in osteoclast mediated bone resorption by studying *clcn7* deficient mice. Inactivation of ClC-7 causes a severe phenotype characterized by growth retardation, increased early postnatal mortality, dysmorphic features and short limbs. Underlying the skeletal dysmorphisms were abnormal bone structure characterized by absent marrow cavities in long

bones, fibrosis and a reduced volume of trabecular bone. These findings, collectively consistent with osteopetrosis (bone petrification), could all be explained by impaired osteoclastic bone resorption. Osteoclasts in ClC-7 deficient mice exhibit poorly developed ruffled borders and do not form resorption lacunae. Immunohistochemical staining for ClC-7 in normal osteoclasts demonstrated consistent intracellular staining as well as localization along ruffled border membranes similar to the distribution of the H^+–ATPase. Further functional studies demonstrated a defect in extracellular acidification in osteoclasts from the knock-out animals. These findings indicated a probable role of ClC-7 in osteoclast function. One form of osteopetrosis in humans, infantile malignant osteopetrosis, is also caused by mutations in *CLCN7* [36] thus adding to the repertoire of human chloride channelopathies (Table 1).

Cell volume regulation

Living cells must maintain their internal volume within a narrow range to preserve normal function. Often this requirement provides a significant physiological challenge especially in rapidly changing osmotic conditions. Many cells regulate their internal volume by adjusting the intracellular concentration of osmotically active electrolytes through the activity of a variety of channels and transporters. Virtually all cells respond to swelling or shrinkage caused by osmotic imbalances and the resultant gain or loss of water, respectively, by activating regulatory volume adjustments that either decrease or increase cellular tonicity. An important and ubiquitous volume regulatory pathway involves the activation of chloride currents by cell swelling ($I_{Cl\text{-}swell}$) [37]. In the past few years, several cloned chloride channels have been touted as potential molecular components of $I_{Cl\text{-}swell}$ including two ClC channels, ClC-2 and ClC-3. However, these hypotheses remain largely unproven and controversial.

Role of ClC-2 in cell volume regulation

When initially cloned, ClC-2 was shown to be present in most cell types and to exhibit activation by membrane hyperpolarization and hypotonic cell swelling when heterologously expressed in *Xenopus* oocytes [38,39]. Whether ClC-2 mediates a volume-activated chloride current *in vivo* is still unclear, but based solely upon its biophysical characteristics, it is not likely to represent the major molecular structure responsible for $I_{Cl\text{-}swell}$. Consistent with this notion is the recent observation that inactivation of murine ClC-2 does not produce an overt phenotype such as that expected from impairment of a ubiquitous physiological process such as cell volume regulation [40]. However, this ClC isoform does appear to have a critical role in the development of male germ cells and retinal photoreceptors possibly by enabling the epithelial tissues (Sertoli cells and retinal pigment epithelium, respectively) surrounding these structures to maintain a proper ionic milieu. Incidentally, ClC-2 inactivation does not impair gastric acid secretion nor fluid secretion by developing lung epithelia [40] in contrast to early speculation about the role of ClC-2 in these physiological processes [41,42].

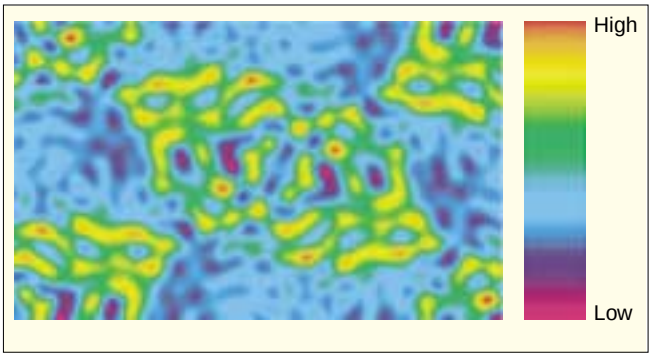
ClC-2 homolog in *C. elegans*

Recent work has demonstrated six ClC channel homologs within the entire genome of *C. elegans* enabling studies using this genetically tractable model organism to elucidate the fundamental biological roles of these chloride channels (Figure 2) [3–5]. A *C. elegans* homolog of mammalian ClC-2 is encoded by the *clh-3* gene (designated for $\underline{Cl}^-$ channel homolog). The functional activity of the corresponding gene product (CLH-3) was characterized in maturing oocytes using patch clamp recording [43]. Similar to ClC-2, CLH-3 is activated strongly by cell swelling and hyperpolarization although this depends greatly upon the developmental stage of the cells. *C. elegans* oocytes progressively enlarge more than 200-fold during development and the requirement for cell swelling to activate CLH-3 progressively diminishes with this growth; for example, the smallest cells require the largest degree of swelling for *clh-3* activation. By the time meiotic maturation occurs, CLH-3 achieves constitutive activation in oocytes. Despite its clear activation by cell swelling, CLH-3 is not required for regulatory volume adjustments in oocytes as demonstrated using double-stranded RNA interference to inactivate this chloride channel. Rather CLH-3 may be involved in signaling to the surrounding myoepithelial sheath cells. Further studies are needed in order to define the physiological role of ClC-2 and its orthologs in diverse species.

Role of ClC-3 in cell volume regulation

Another ClC candidate proposed to mediate $I_{Cl\text{-}swell}$, ClC-3, has stimulated a great deal of debate. Duan *et al.* [44] recloned ClC-3 from guinea pig heart and showed that its heterologous expression in cultured mammalian NIH3T3 cells produced a volume activated chloride current with similarity to $I_{Cl\text{-}swell}$. Further experiments showed that the ion selectivity of the expressed current was changed by mutating residues in ClC-3 that were believed to contribute to permeation indicating that currents recorded from transfected cells were likely generated by ClC-3. More recent studies by the same group of investigators demonstrated the importance of protein kinase C mediated phosphorylation of specific serines in rendering ClC-3 volume responsive, and that polyclonal anti-ClC-3 antibodies blocked native $I_{Cl\text{-}swell}$ in *Xenopus* oocytes and a variety of mammalian muscle cells [45,46]. However, despite these seemingly solid observations, other groups have failed to obtain similar results. Expression of ClC-3 in CHO-K1 cells generates an extremely outward-rectifying Cl^- current — similar to ClC-4 and ClC-5 — that is not activated by cell-swelling [47]. Other groups have reported failed attempts to obtain recordable chloride currents when

Figure 6



Structure of a bacterial ClC channel. The figure represents a projection density map at 6.5 D obtained from cryo-electron microscopic analysis of two-dimensional crystals (color coded for density according to the labeled scale shown on the right). Taken from Mindell *et al.* [57].

ClC-3 is expressed in a variety of heterologous cell systems [48,49]. Furthermore, $I_{Cl\text{-}swell}$ appears intact in selected cell types from the ClC-3 knock-out mouse [34] but cardiac cells or smooth muscle cells, cell types demonstrated by Duan *et al.* [44] to express ClC-3, were not specifically examined [34]. Whether ClC-3 has a limited role in some tissues in mediating $I_{Cl\text{-}swell}$ requires additional studies.

Structural perspectives on ClC channels

Even before the first ClC chloride channel was cloned, there has been robust speculation that this class of ion permeant proteins is uniquely constructed with two independently gated pores, analogous to a double-barreled shotgun [50]. This structural prediction is unprecedented among all other eukaryotic ion channels and was based originally on biophysical arguments. Detailed analyses of single *Torpedo* electroplax Cl^- channels reconstituted into planar lipid bilayers, and of cloned ClC-0 expressed heterologously, have reproducibly demonstrated two equally spaced and independently gated conductance states [51,52] suggesting that the channel consists of two identical ion conduction pathways. More recent single channel recordings of mammalian ClC-1 and ClC-2 support the same conclusion [53,54]. Additional elegant experiments with hybrid ClC channels containing mixtures of wild-type and mutant subunits have provided added evidence for a dual-pore, dimeric channel architecture [55,56].

There is little doubt that active ClC channels are dimers based on a recent low resolution cryo-electron microscopic image of purified bacterial ClC channel protein (Figure 6) [57]. However, this currently blurry view of the channel structure does not provide conclusive evidence for the number of pores and there are functional data seemingly inconsistent with the two pore hypothesis. The strength of the counter argument lies in the identification and characterization of residues that likely line the permeation pathway of the channel [58,59]. One of these residues, a lysine (Lys-231 in human ClC-1), projects its side chain into the path of the permeating chloride ions as demonstrated by site-directed mutagenesis and cysteine accessibility experiments. When converted to cysteine, this residue renders the channel highly susceptible to block by thiol-reactive methanethiosulfates in a manner most consistent with a single pore. Furthermore, this residue can be cross-linked between adjacent subunits, a rather unlikely scenario if both residues were sequestered in separate permeation cavities [60]. However, it is possible that this residue does not reside within the pore itself but rather in a location that facilitates cross-linking preferentially during channel closure which locks the channel in an inactive conformation [61]. Clearly, this issue will be settled shortly in the structural biology arena and a framework for interpreting the biophysical studies can then be constructed.

Summary

Chloride channels belonging to the ClC family are involved in a wide range of cellular and physiological processes, are associated with an eclectic group of inherited human diseases and have fascinating, albeit incompletely understood, biophysical properties. That they were ignored for so long is a shame, but chloride channels have now emerged as important and respectable proteins that will provide enormous opportunities in the future for linking ion channel biology to cell and organ function.

Acknowledgements

This work was supported by grants from the National Institutes of Health (AR44506 and DK58749).

References

1. Jentsch TJ: Molecular biology of voltage-gated chloride channels. *Curr Top Membr* 1994, 42:35-57.
2. Jentsch TJ, Friedrich T, Schriever A, Yamada H: The CLC chloride channel family. *Pflugers Arch* 1999, 437:783-795.
3. Schriever AM, Friedrich T, Pusch M, Jentsch TJ: CLC chloride channels in ***Caenorhabditis elegans***. *J Biol Chem* 1999, 274:34238-34244.
4. Petalcorin MI, Oka T, Koga M, Ogura K, Wada Y, Ohshima Y, Futai M: Disruption of clh-1, a chloride channel gene, results in a wider body of ***Caenorhabditis elegans***. *J Mol Biol* 1999, 294:347-355.
5. Bianchi L, Miller DM, III, George AL, Jr: Expression of a CIC chloride channel in ***Caenorhabditis elegans*** gamma-aminobutyric acid-ergic neurons. *Neurosci Lett* 2001, 299:177-180.
6. Waldegger S, Jentsch TJ: From tonus to tonicity: physiology of CLC chloride channels. *J Am Soc Nephrol* 2000, 11:1331-1339.
7. Uchida S: ***In vivo*** role of CLC chloride channels in the kidney. *Am J Physiol Renal Physiol* 2000, 279:F802-F808.
8. Thakker RV: Molecular pathology of renal chloride channels in Dent's disease and Bartter's syndrome. *Exp Nephrol* 2000, 8:351-360.
9. Fahlke C: Molecular mechanisms of ion conduction in ClC-type chloride channels: lessons from disease-causing mutations. *Kidney Int* 2000, 57:780-786.
10. Futai M, Oka T, Sun-Wada G, Moriyama Y, Kanazawa H, Wada Y: Luminal acidification of diverse organelles by V-ATPase in animal cells. *J Exp Biol* 2000, 203:107-116.
11. Nelson N, Harvey WR: Vacuolar and plasma membrane proton-adenosinetriphosphatases. *Physiol Rev* 1999, 79:361-385.

12. Al-Awqati Q, Barasch J, Landry D: Chloride channels of intracellular organelles and their potential role in cystic fibrosis. *J Exp Biol* 1992, 172:245-266.
13. Fisher SE, Black GCM, Lloyd SE, Hatchwell E, Wrong O, Thakker RV, Craig IW: Isolation and partial characterization of a chloride channel gene which is expressed in kidney and is a candidate for Dent's disease (an X-linked hereditary nephrolithiasis). *Hum Mol Genet* 1994, 3:2053-2059.
14. Lloyd SE, Pearce SHS, Fisher SE, Steinmeyer K, Schwappach B, Scheinman SJ, Harding B, Alessandra B, Devota M, Goodyear P, Rigden SPA, *et al.*: A common molecular basis for three inherited kidney stone diseases. *Nature* 1996, 379:445-449.
15. Hebert SC: Crystal-clear chloride channels. *Nature* 1996, 379:398-399.
16. Günther W, Lüchow A, Cluzeaud F, Vandewalle A, Jentsch TJ: ClC-5, the chloride channel mutated in Dent's disease, colocalizes with the proton pump in endocytotically active kidney cells. *Proc Natl Acad Sci USA* 1998, 95:8075-8080.
17. Devuyst O, Christie PT, Courtoy PJ, Beauwens R, Thakker RV: Intra-renal and subcellular distribution of the human chloride channel, CLC-5, reveals a pathophysiological basis for Dent's disease. *Hum Mol Genet* 1999, 8:247-257.
18. Sakamoto H, Sado Y, Naito I, Kwon TH, Inoue S, Endo K, Kawasaki M, Uchida S, Nielsen S, Sasaki S, Marumo F: Cellular and subcellular immunolocalization of ClC-5 channel in mouse kidney: colocalization with H^+-ATPase. *Am J Physiol* 1999, 277:F957-F965.
19. Piwon N, Günther W, Schwake M, Bösl MR, Jentsch TJ: ClC-5 Cl^--channel disruption impairs endocytosis in a mouse model for Dent's disease. *Nature* 2000, 408:369-373.
20. Wang SS, Devuyst O, Courtoy PJ, Wang XT, Wang H, Wang Y, Thakker RV, Guggino S, Guggino WB: Mice lacking renal chloride channel, CLC-5, are a model for Dent's disease, a nephrolithiasis disorder associated with defective receptor-mediated endocytosis. *Hum Mol Genet* 2000, 9:2937-2945.
21. Yu AS: Role of ClC-5 in the pathogenesis of hypercalciuria: recent insights from transgenic mouse models. *Curr Opin Nephrol Hypertens* 2001, 10:415-420.
22. Vandewalle A, Cluzeaud F, Peng KC, Bens M, Luchow A, Günther W, Jentsch TJ: Tissue distribution and subcellular localization of the ClC-5 chloride channel in rat intestinal cells. *Am J Physiol Cell Physiol* 2001, 280:C373-C381.
23. Bastani B, Purcell H, Hemkin P, Trigg D, Gluck S: Expression and distribution of renal vacuolar proton-translocating adenosine triphosphatase in response to chronic acid and alkali loads in the rat. *J Clin Invest* 1991, 88:126-136.
24. Steinmeyer K, Schwappach B, Bens M, Vandewalle A, Jentsch TJ: Cloning and functional expression of rat CLC-5, a chloride channel related to kidney disease. *J Biol Chem* 1995, 270:31172-31177.
25. Sakamoto H, Kawasaki M, Uchida S, Sasaki S, Marumo F: Identification of a new outwardly rectifying Cl^- channel that belongs to a subfamily of the ClC Cl^- channels. *J Biol Chem* 1996, 271:10210-10216.
26. Huang ME, Chuat JC, Galibert F: A voltage-gated chloride channel in the yeast ***Saccharomyces cerevisiae***. *J Mol Biol* 1994, 242:595-598.
27. Greene JR, Brown NH, DiDomenico BJ, Kaplan J, Eide DJ: The GEF1 gene of ***Saccharomyces cerevisiae*** encodes an integral membrane protein; mutations in which have effects on respiration and iron-limited growth. *Mol Gen Genet* 1993, 241:542-553.
28. Gaxiola RA, Yuan DS, Klausner RD, Fink GR: The yeast CLC chloride channel functions in cation homeostasis. *Proc Natl Acad Sci USA* 1998, 95:4046-4050.
29. Schwappach B, Stobrawa S, Hechenberger M, Steinmeyer K, Jentsch TJ: Golgi localization and functionally important domains in the NH_2 and COOH terminus of the yeast CLC putative chloride channel Gef1p. *J Biol Chem* 1998, 273:15110-15118.
30. Hechenberger M, Schwappach B, Fischer WN, Frommer WB, Jentsch TJ, Steinmeyer K: A family of putative chloride channels from ***Arabidopsis*** and functional complementation of a yeast strain with a CLC gene disruption. *J Biol Chem* 1996, 271:33632-33638.
31. Kida Y, Uchida S, Miyazaki H, Sasaki S, Marumo F: Localization of mouse CLC-6 and CLC-7 mRNA and their functional complementation of yeast CLC gene mutant. *Histochem Cell Biol* 2001, 115:189-194.
32. Davis-Kaplan SR, Askwith CC, Bengtzen AC, Radisky D, Kaplan J: Chloride is an allosteric effector of copper assembly for the yeast multicopper oxidase Fet3p: an unexpected role for intracellular chloride channels. *Proc Natl Acad Sci USA* 1998, 95:13641-13645.
33. Kawasaki M, Uchida S, Monkawa T, Miyawaki A, Mikoshiba K, Maruma F, Sasaki S: Cloning and expression of a protein kinase C-regulated chloride channel abundantly expressed in rat brain neuronal cells. *Neuron* 1994, 12:597-604.
34. Stobrawa SM, Breiderhoff T, Takamori S, Engel D, Schweizer M, Zdebik AA, Bösl MR, Ruether K, Jahn H, Draguhn A, *et al.*: Disruption of ClC-3, a chloride channel expressed on synaptic vesicles, leads to a loss of the hippocampus. *Neuron* 2001, 29:185-196.
35. Teitelbaum SL: Bone resorption by osteoclasts. *Science* 2000, 289:1504-1508.
36. Kornak U, Kasper D, Bösl MR, Kaiser E, Schweizer M, Schulz A, Friedrich W, Delling G, Jentsch TJ: Loss of the ClC-7 chloride channel leads to osteopetrosis in mice and man. *Cell* 2001, 104:205-215.
37. Strange K, Emma F, Jackson PS: Cellular and molecular physiology of volume-sensitive anion channels. *Am J Physiol* 1996, 270:C711-C730.
38. Thiemann A, Gründer S, Pusch M, Jentsch TJ: A chloride channel widely expressed in epithelial and non-epithelial cells. *Nature* 1992, 356:57-60.
39. Gründer S, Thiemann A, Pusch M, Jentsch TJ: Regions involved in the opening of the ClC-2 chloride channel by voltage and cell volume. *Nature* 1992, 360:759-762.
40. Bösl MR, Stein V, Hubner C, Zdebik AA, Jordt SE, Mukhopadhyay AK, Davidoff MS, Holstein AF, Jentsch TJ: Male germ cells and photoreceptors, both dependent on close cell-cell interactions, degenerate upon ClC-2 Cl^- channel disruption. *EMBO J* 2001, 20:1289-1299.
41. Malinowska DH, Kupert EY, Bahinski A, Sherry AM, Cuppoletti J: Cloning, functional expression, and characterization of a PKA-activated gastric Cl^- channel. *Am J Physiol Cell Physiol* 1995, 268:C191-C200.
42. Murray CB, Morales MM, Flotte TR, McGrath-Morrow SA, Guggino WB, Zeitlin PL: ClC-2: a developmentally dependent chloride channel expressed in the fetal lung and downregulated after birth. *Am J Respir Cell Mol Biol* 1995, 12:597-604.
43. Rutledge E, Bianchi L, Christensen M, Boehmer C, Morrison R, Broslat A, Beld AM, George AL, Greenstein D, Strange K: CLH-3, a ClC-2 anion channel ortholog activated during meiotic maturation in ***C. elegans*** oocytes. *Curr Biol* 2001, 11:161-170.
44. Duan D, Winter C, Cowley S, Hume JR, Horowitz B: Molecular identification of a volume-regulated chloride channel. *Nature* 1997, 390:417-421.
45. Duan D, Cowley S, Horowitz B, Hume JR: A serine residue in ClC-3 links phosphorylation-dephosphorylation to chloride channel regulation by cell volume. *J Gen Physiol* 1999, 113:57-70.
46. Duan D, Zhong J, Hermoso M, Satterwhite CM, Rossow CF, Hatton WJ, Yamboliev I, Horowitz B, Hume JR: Functional inhibition of native volume-sensitive outwardly rectifying anion channels in muscle cells and ***Xenopus*** oocytes by anti-ClC-3 antibody. *J Physiol (London)* 2001, 531:437-444.
47. Li X, Shimada K, Showalter LA, Weinman SA: Biophysical properties of ClC-3 differentiate it from swelling-activated chloride channels in Chinese hamster ovary-K1 cells. *J Biol Chem* 2000, 275:35994-35998.
48. Friedrich T, Breiderhoff T, Jentsch TJ: Mutational analysis demonstrates that ClC-4 and ClC-5 directly mediate plasma membrane currents. *J Biol Chem* 1999, 274:896-902.
49. Weylandt KH, Valverde MA, Nobles M, Raguz S, Amey JS, Diaz M, Nastrucci C, Higgins CF, Sardini A: Human ClC-3 is not the swelling-activated chloride channel involved in cell volume regulation. *J Biol Chem* 2001, 276:17461-17467.
50. Miller C, White MM: Dimeric structure of single chloride channels from ***Torpedo*** electroplax. *Proc Natl Acad Sci USA* 1984, 81:2772-2775.
51. Miller C: Open-state substructure of single chloride channels from ***Torpedo*** electroplax. *Philos Trans R Soc Lond B Biol Sci* 1982, 299:401-411.

52. Hanke W, Miller C: Single chloride channels from ***Torpedo*** electroplax. Activation by protons. *J Gen Physiol* 1983, 82:25-45.
53. Saviane C, Conti F, Pusch M: The muscle chloride channel ClC-1 has a double-barreled appearance that is differentially affected in dominant and recessive myotonia. *J Gen Physiol* 1999, 113:457-468.
54. Nobile M, Pusch M, Rapisarda C, Ferroni S: Single-channel analysis of a ClC-2-like chloride conductance in cultured rat cortical astrocytes. *FEBS Lett* 2000, 479:10-14.
55. Middleton RE, Pheasant DJ, Miller C: Homodimeric architecture of a ClC-type chloride ion channel. *Nature* 1996, 383:337-340.
56. Ludewig U, Pusch M, Jentsch TJ: Two physically distinct pores in the dimeric CIC-0 chloride channel. *Nature* 1996, 383:340-343.
57. Mindell JA, Maduke M, Miller C, Grigorieff N: Projection structure of a ClC-type chloride channel at 6.5 Å resolution. *Nature* 2001, 409:219-223.
58. Fahlke C, Yu HT, Beck CL, Rhodes TH, George AL, Jr: Pore-forming segments in voltage-gated chloride channels. *Nature* 1997, 390:529-532.
59. Fahlke C, Desai RR, Gillani N, George AL, Jr: Residues lining the inner pore vestibule of human muscle chloride channels. *J Biol Chem* 2001, 276:1759-1765.
60. Fahlke C, Rhodes TH, Desai RR, George AL, Jr: Pore stoichiometry of a voltage-gated chloride channel. *Nature* 1998, 394:687-690.
61. Maduke M, Miller C, Mindell JA: A decade of CLC chloride channels: structure, mechanism, and many unsettled questions. *Annu Rev Biophys Biomol Struct* 2000, 29:411-438.
62. Koch MC, Steinmeyer K, Lorenz C, Ricker K, Wolf F, Otto M, Zoll B, Lehmann-Horn F, Grzeschik KH, Jentsch TJ: The skeletal muscle chloride channel in dominant and recessive human myotonia. *Science* 1992, 257:797-800.
63. George AL, Crackower MA, Abdalla JA, Hudson AJ, Ebers GC: Molecular basis of Thomsen's disease (autosomal dominant myotonia congenita). *Nat Genet* 1993, 3:305-310.
64. Kornak U, Kasper D, Bösl MR, Kaiser E, Schweizer M, Schulz A, Friedrich W, Delling G, Jentsch TJ: Loss of ClC-7 chloride channel leads to osteropetrosis in mice and man. *Cell* 2001, 104:205-215.
65. Matsumura Y, Uchida S, Kondo Y, Miyazaki H, Ko SB, Hayama A, Morimoto T, Liu W, Arisawa M, Sasaki S, Marumo F: Overt nephrogenic diabetes insipidus in mice lacking the CLC-K1 chloride channel. *Nat Genet* 1999, 21:95-98.
66. Simon DB, Bindra RS, Mansfield TA, Nilson-Williams C, Mendonca E, Stone R, Schurman S, Nayir A, Alpay H, Bakkaloglu A, *et al.*: Mutations in the chloride channel gene, ***CLCNKB***, cause Bartter's syndrome type III. *Nat Genet* 1997, 17:171-178.