Review

Proton-coupled gating in chloride channels

Jiří Lísal[†] and Merritt Maduke*

*Department of Molecular and Cellular Physiology, Stanford University, 279 Campus Drive,
Stanford, CA 94305, USA*

The physiologically indispensable chloride channel (CLC) family is split into two classes of membrane proteins: chloride channels and chloride/proton antiporters. In this article we focus on the relationship between these two groups and specifically review the role of protons in chloride-channel gating. Moreover, we discuss the evidence for proton transport through the chloride channels and explore the possible pathways that the protons could take through the chloride channels. We present results of a mutagenesis study, suggesting the feasibility of one of the pathways, which is closely related to the proton pathway proposed previously for the chloride/proton antiporters. We conclude that the two groups of CLC proteins, although in principle very different, employ similar mechanisms and pathways for ion transport.

Keywords: proton transport; ion channel; secondary active transporter; chloride; gating

1. INTRODUCTION

Ion channels and secondary active transporters traditionally have been considered as two distinct classes of membrane proteins. This is reasonable. Ion channels form passive pores: they allow ions to move down (and only down) their electrochemical gradient. Secondary active transporters, on the other hand, use the energy derived from the movement of one ion down its electrochemical gradient to catalyse uphill movement of another ion. This cannot be accomplished by a passive pore, but requires active coupling of protein conformational changes to ion binding and transport.

Therefore, it was shocking to discover that more than half of the CLC ‘Chloride Channel’ family members are not channels but rather are secondary active transporters (antiporters) (Accardi & Miller 2004; Picollo & Pusch 2005; Scheel *et al.* 2005; DeAngeli *et al.* 2006; Graves *et al.* 2008). Instead of facilitating Nernstian movement of chloride, these antiporter homologues catalyse the stoichiometric exchange of two chloride ions for one proton (Accardi & Miller 2004; DeAngeli *et al.* 2006; Graves *et al.* 2008).

The CLC channels and antiporters share a number of basic features: (1) structural architecture, (2) anion selectivity, (3) the existence of two ‘gates’, (4) strong modulation by both chloride and protons (Pusch 2004; Chen 2005; Dutzler 2006; Miller 2006; Matulef & Maduke 2007; Zifarelli & Pusch 2007; Jentsch 2008). These similarities strongly suggest that the fundamental difference between the CLC channels and antiporters does not arise from differences in basic design. Indeed,

we hypothesize that the conformational changes involved in channel gating are similar to those involved in antiporter catalysis, and that the key difference between the channels and antiporters lies in the energetics and coupling of the conformational changes.

Conformational changes in both the CLC channels and antiporters are strongly modulated by chloride and protons. For the antiporters, such modulation is expected: the energy of ion binding and transport is used to drive the conformational changes involved in coupling. For the channels, such modulation is a profound indicator of their antiporter ancestry. The focus of this paper is to elucidate the relationship between CLC channels and antiporters by comparing the role of protons in these proteins.

2. PROTON TRANSPORT IN CLC-ec1

The high-resolution structure of the CLC-ec1 chloride–proton antiporter provides a foundation for envisioning proton movement in the CLCs (Dutzler *et al.* 2003). Ideally, such a structure would reveal an unbroken chain of titratable residues and/or water molecules leading entirely through the protein. Unfortunately, the spacing of titratable residues is discontinuous, and water molecules are not sufficiently resolved. Nevertheless, the structure has yielded some very important clues. The most obvious is E148, a glutamate residue positioned directly along the chloride-permeation pathway, at the extracellular face of the protein (figure 1). Mutation of this glutamate (referred to as ‘Glu_{ex}’, (Miller 2006)) abolishes both proton transport and proton activation in CLC-ec1 (Accardi *et al.* 2005). Together with the structure, this result strongly suggests that the chloride and proton permeation pathways converge at this extracellular position in the antiporter. Importantly, this glutamate residue is conserved in most of the CLC channels.

* Author for correspondence (maduke@stanford.edu).

[†] Present address: Institute of Microbiology, Videnska 1083, 14220 Prague 4, Czech Republic.

One contribution of 16 to a Discussion Meeting Issue ‘Membrane transport in flux: the ambiguous interface between channels and pumps’.

Figure 1. The chloride and proton pathways in CLC-ec1. Bound chloride ions (green spheres) and key residues for proton transport (E148 and E203, also known as Glu_{ex} and Glu_{in}) are depicted in a structural model of the CLC-ec1 dimer (pdb 1OTS). Although E203 is not conserved in the channel homologues, it is possible that the adjacent E202 could create a proton pathway. The figure was created using UCSF CHIMERA software.

There are numerous (more than 50) other candidate titratable residues in the membrane domain of CLC-ec1. Although effects of mutation at all of these residues have not yet been functionally examined, a screen of all of the intra-cellularly positioned glutamate and aspartate residues revealed a second key glutamate residue, E203, mutation of which abolishes chloride-coupled proton transport (Accardi *et al.* 2005). In contrast to Glu_{ex}, this glutamate (aka 'Glu_{in}') is positioned away from the Cl[−] permeation pathway, and is not conserved in the CLC channels (figure 1).

3. CLC CHANNEL GATING

At first glance, CLC channel gating appears to be completely idiosyncratic: CLC-0 and CLC-1 (the skeletal-muscle CLCs) are depolarization activated; CLC-2 (expressed ubiquitously) is hyperpolarization activated; and the CLC-K homologues (specific to the kidney and inner ear) are essentially voltage independent. While the differences are not yet completely understood, the extracellular 'glutamate gate' residue is central. This residue, equivalent to Glu_{ex} in CLC-ec1, is thought to act as a physical 'gate' blocking Cl[−] permeation to and from the extracellular side. In CLC-0, 1 and 2, mutation of this residue abolishes voltage-dependent gating of the pores (Dutzler *et al.* 2003; Estevez *et al.* 2003; Niemeyer *et al.* 2003; Traverso *et al.* 2003; deSantiago *et al.* 2005); in CLC-Ka and -Kb, this residue is a valine (Kieferle *et al.* 1994), consistent with its voltage-independent gating.

Our understanding of CLC channel gating has been most advanced by studies on the *Torpedo* CLC-0, because it is the only family member currently amenable to detailed single-channel analysis. The remainder of this review will focus on this homologue together with its closest mammalian relative, CLC-1. In CLC-0 and CLC-1, two modes of gating have been described: in addition to the Glu_{ex}-mediated gating of the channel pore (referred to as 'fast gating'), there is a second mechanism that acts on both the pores simultaneously and probably involves a large

conformational change (Miller & White 1984; Pusch *et al.* 1997). This 'slow gating' mechanism is most readily appreciated for CLC-0, where it is observed at the single-channel level (figure 2). The 'slow' and 'fast' gating mechanisms also operate in CLC-1, although in this case the kinetics of the two processes are similar. Therefore fast and slow gating are also referred to as 'porepore' and 'common' gating.

4. VOLTAGE AND CHLORIDE DEPENDENCE OF FAST GATING

CLC-0 fast gating is weakly voltage dependent, with an apparent gating charge of approximately 1. In addition, the position of the voltage-activation curve is strongly modulated by changes in the extracellular chloride concentration (Pusch *et al.* 1995). Moreover, this gating effect is strongly linked to permeation: the anomalous mole fraction effect on channel conductance (observed with mixtures of the permeant anions Cl[−] & NO₃[−]) is paralleled by an analogous effect on the midpoint of the voltage-dependent activation curve (Pusch *et al.* 1995). This striking result reveals that gating and permeation in this channel are inextricably linked, and has been taken to mean—reasonably so—that the gating charge arises not from the movement of charged residues on the channel but from movement of the chloride ion itself through the transmembrane electric field. Similar conclusions have been made regarding the voltage- and chloride dependence of CLC-1 gating (Rychkov *et al.* 1998).

5. PROTON DEPENDENCE OF FAST GATING

The CLC-0 fast gate is also activated by protons. Lowering the intracellular pH shifts the voltage-activation curves leftward (Hanke & Miller 1983). Thus, activation by intracellular protons is voltage dependent. Lowering the extracellular pH also activates, but does so through a different mechanism, increasing the minimal open probability at negative voltages (Chen & Chen 2001). This process has very little voltage dependence. Glu_{ex} is the obvious candidate as the site of protonation in fast-gate activation by the external protons. Until recently, however, the protonation site for activation by intracellular protons has been a total mystery.

Chloride permeation through the human muscle channel CLC-1 is also enhanced by lowering the intracellular or extracellular pH (Rychkov *et al.* 1996), though in this early study effects on fast gating were not distinguished from those on slow gating. (Recall: in CLC-1, the fast and slow gating processes have similar kinetics, and thus are more difficult to separate.) More recent studies examining the effect of intracellular pH have shown that both the gating processes are affected (Bennetts *et al.* 2007; Tseng *et al.* 2007), but in these studies the focus was on the common gating mechanism, and the effects on fast gating were not explicitly presented. In our analysis of CLC-1, we find that the intracellular pH dependence of fast gating is similar to that observed for CLC-0 (figure 3a–c). Although the extracellular pH dependence of fast gating in CLC-1 has not yet been studied, the overall similarity of the pH effects for CLC-1 and

Figure 2. Two modes of gating in ClC-0. A representative single-channel recording demonstrates the characteristic features of ClC-0 gating. Slow-gate closures are labelled with asterisks. Bursts of fast-gating events, which involve independent opening and closing of the two pores, occur between the slow-gate closures: conductance level 0, both pores closed; conductance level 1, one pore closed, one pore open; conductance level 2, both pores open. The lower trace depicts part of the data from the upper trace with expanded time scale. The recording was made at symmetrical 120 mM Cl^- , pH_{int} 7.5, pH_{ext} 8.5 and -70 mV.

Figure 3. Effect of internal pH on gating in wild-type and mutant ClC-1 channels. (a–c) WT ClC-1; (d–f), the E291D mutant. External pH was held at 7.5 while the internal pH was varied (5.5, black circles; 6.5, red triangles; 7.5, green squares; 8.5, yellow diamonds). (a,d) The combined open probability, (b,d) the slow-gate open probability and (c,e) the fast-gate open probability were determined as described by Bennetts *et al.* 2007. The pipette and bath solutions were composed of (concentrations in mM) 110 NMDG, 110 HCl, 5 MgCl_2 , 1 EGTA, 10 HEPES, pH 7.50, adjusted by NaOH. The buffer compound was substituted by TRIS for pH 8.50 or MES for pH 6.50 and pH 5.50.

ClC-0 suggests that the mechanism of the protopore gate activation in ClC-1 is the same as the mechanism of the fast-gate activation in ClC-0 and involves protonation of Glu_{ex}.

6. PROTON TRANSPORT BY CLC CHANNELS?

Recently, two groups have independently proposed a new mechanism to account for the voltage- and proton dependence of fast gating (Miller 2006; Traverso *et al.* 2006). In his insightful review, Miller (2006) has detailed how all of the previous studies can be grandly unified under the umbrella of a 'degraded transporter' model, in which ClC-0 retains the proton permeation pathway found in the antiporters. Movement of protons through this pathway, towards Glu_{ex} from either side of the membrane, is coupled to fast gating. Thus, it has been proposed that the movement of protons, but not chloride, provides the gating charge.

Traverso *et al.* (2006) has taken things a step further and provided experimental support for such a model. The key results involve a careful analysis of a mutant channel in which the Glu_{ex} residue was mutated to an aspartate. This conservative mutation causes dramatically non-conservative changes in fast gating: specifically, the opening rate is greatly decreased, the closing rate increased and the open probability at physiological pH and voltages is correspondingly extremely low (<0.01). Despite these changes, however, the channel retains its characteristic response to intracellular pH, the main difference between mutant and wild type being a shift in the position of the voltage-activation curves as a function of pH.

Traverso *et al.* has taken advantage of this difference to then re-evaluate the model for pH-dependent gating that has stood for over 20 years. In the original model, although the overall proton activation is voltage dependent, the protonation step itself is voltage independent, and the voltage dependence arises from a channel-opening step subsequent to protonation (Hanke & Miller 1983). Importantly, this model requires that a proton-sensitive residue changes its pK from 6 to 9 upon channel opening. Traverso *et al.* found that application of the same model to the E166D mutant requires the residue to change its pK from 5 to 12—a change of seven orders of magnitude, which seems unlikely. This prompted them to propose an alternative model, in which protonation of the channel from the intracellular side is voltage dependent. They showed that the data for both wild-type and mutant channels fit well to this new model.

Overall, these two proposals have the same basic conclusion: Glu_{ex} can be protonated from the intracellular side, and it is this protonation step that gives rise to the channel's voltage dependence. Since Glu_{ex} can also be protonated from the extracellular side (Dutzler *et al.* 2003), this leads to the striking prediction that protons might be transported via the Glu_{ex} all the way across the membrane. The rate of the proton transport would be on the same time scale as ClC-0 fast gating (approx. 100 H⁺ s⁻¹), measurable in theory, but has not been detected by direct measurements thus far (Picollo & Pusch 2005).

7. CHLORIDE AND PROTON ACTIVATION OF SLOW GATING

As with fast-gating, ClC-0 slow gating too is modulated by voltage, chloride and protons (Hanke & Miller 1983; Chen & Miller 1996; Pusch *et al.* 1997). Although the mechanistic details of these effects have not yet been studied in depth, the very presence of such modulation strongly suggests that the conformational change involved in slow gating is related to the conformational changes involved in H⁺/Cl⁻ exchange by ClC-0's antiporter cousins.

A major insight into antiporter-like behaviour is found, ironically, in single-channel recordings. In such recordings, ClC-0 displays an unusual non-equilibrium gating, seen in the combined behaviour of the fast and slow gates. These gating events are not at equilibrium but rather 'cycle' through conformational states in a fixed direction (Richard & Miller 1990). Specifically, ClC-0 enters the slow-gate closed state predominantly from a state in which only one of the two fast gates is open (conductance level 1, figure 2) and returns from the slow-gate closed state to a state in which both of the fast gates are open (conductance level 2, figure 2). Since maintenance of such a non-equilibrium gating asymmetry requires a source of energy, and since there were scant obvious sources in the minimal recording system, it was proposed that the energy originates from the transport of chloride down its electrochemical gradient (Richard & Miller 1990). This coupling of permeant-ion movement to conformational changes is a hallmark of antiporter behaviour.

In light of the recent suggestions of proton transport in ClC-0 (see above), it is worth re-evaluating the assumption that the chloride ion is the culprit in this ClC-0's non-equilibrium slow gating. Indeed, if protons are transported by ClC-0, they could provide the source of energy that keeps ClC-0 gating away from equilibrium. Strikingly, the original study hints that this might be the case. Richard and Miller found that while varying the transmembrane voltage caused predictable changes in the gating asymmetry, varying the chloride gradient had no effect. This suggests that the effect of the voltage may be due to the proton and not the chloride electrochemical gradient. To test this hypothesis, we measured gating asymmetry at various proton gradients. Our data are quantitatively consistent with a model in which protons but not chloride ions are transported to power the non-equilibrium gating (Lísal & Maduke 2008). These results provide direct confirmation that ClC-0 gating involves transmembrane proton movement.

8. POTENTIAL PROTON PATHWAYS IN CLC CHANNELS

The finding that CLC chloride channels transport protons raises the question: where is the proton pathway?

One straightforward hypothesis is that there is a proton translocation pathway in ClC-0 which has a trajectory similar to that proposed for the Cl⁻/H⁺ antiporter ClC-ec1. As discussed above, two glutamate residues—E148 (Glu_{ex}) at the extracellular side and E203 (Glu_{in}) at the intracellular side—define the proton pathway in ClC-ec1 (Accardi *et al.* 2005;

Figure 4. Neutralization of charge at 'Glu_{in}' in CLC-1 (a) Apparent open probability as a function of voltage and pH for the CLC-1 E291Q mutant. The symbols and colours are identical to those in figure 3. The open probability plots were normalized using open-probability values obtained by noise analysis. (b) Variance of 100–200 sweeps was plotted against the mean current for the E291Q mutant and (c) as a control for the WT CLC-1. The records were obtained at pH_{ext} 7.5 and pH_{int} 6.5 by stepping the membrane voltage repeatedly from +90 mV to –110 mV. The data were fitted by the equation $\langle \sigma^2 \rangle = i \langle I \rangle - \langle I \rangle^2 / N$, where i is the single-channel current and N represents the number of channels in the patch. The open probability at –110 mV was calculated as $P_o = I / iN$, where I is the steady-state current at –110 mV, and yielded values 0.41 ± 0.03 for the WT CLC-1 and 0.08 ± 0.02 for the E291Q mutant.

figure 1). These two residues are also important for proton permeation in the mammalian CLC antiporters (Zdebik *et al.* 2007). Supporting this hypothesis is the evidence, already discussed, pointing towards the role of Glu_{ex} in CLC channel gating and proton permeation. Arguing against this hypothesis is the fact that the Glu_{in} residue is not conserved in the channels but is

a hydrophobic valine that cannot participate in proton transport. However, there is another glutamate adjacent to Glu_{in} (E202 in figure 1) that is conserved in the mammalian CLCs and which could possibly substitute as a titratable residue along the proton permeation pathway. Indeed, malleability of proton pathways has been observed with other proton-transport proteins including cytochrome c oxidase (Namslaue *et al.* 2007) and bacteriorhodopsin (Sonar *et al.* 1994).

We examined the effect of mutations at the position adjacent to Glu_{in} in the CLC channels. Because we did not detect any current upon mutation at this residue in CLC-0, we performed these experiments with CLC-1 (at position E291). Wild-type (WT) CLC-1 exhibits strong dependence of open probability on the intracellular pH (figure 3a–c). When the glutamate was replaced by aspartate (a residue with similar pK_a), the strong pH dependence was preserved; however, the open-probability plots were shifted to higher voltages by approximately 40 mV (figure 3d–f). When the glutamate was replaced by the non-protonatable residue glutamine, the channel open probability was greatly reduced at all voltages and pHs (figure 4). These results are consistent with the E291 position being on the proton pathway. In E291D protons must overcome a higher energy barrier in order to be transported from the aspartate residue to another protonatable residue further up the pathway, and therefore higher voltage is required to achieve the same open probability. For the charge-neutralized E291Q mutant, the protons must 'squeeze' around the glutamine (presumably via water molecules), and thus the proton transport and the interrelated open probability are greatly reduced.

Although mutagenesis experiments cannot definitively identify a proton pathway, our results are exactly as expected for a residue that participates in proton transport: mutation to a protonatable residue shifts proton- and voltage-dependent gating, while mutation to a non-protonatable residue essentially abolishes this gating (making it exceedingly unfavourable). Over the past two decades, CLC-1 and CLC-0 have been extensively mutated—nearly all of the predicted protonatable residues in the transmembrane region have been mutated—yet no other residue exhibits this predicted phenotype (Pusch *et al.* 1995; Ludewig *et al.* 1997; Kurz *et al.* 1999; Fahlke *et al.* 2001; Chen & Chen 2003; Engh & Maduke 2005; Zhang *et al.* 2006). Therefore, our results are in agreement with the hypothesis that this conserved glutamate is a part of the proton pathway in the CLC channels.

An alternative hypothesis is that proton translocation to Glu_{ex} requires no additional titratable residue. Indeed, a continuous chain of water is all that is required to form a proton pathway (Decoursey 2003). Such a pathway could possibly exist anywhere in the protein. One obvious potential pathway is coincident with the chloride permeation pathway. There is clearly room for water to access this pathway; indeed, even large thiol-reactive compounds can reach up to position Y512 in CLC-0, the residue that provides coordination of the central chloride ion (Lin & Chen 2003; Engh & Maduke 2005). Moreover, proton movement appears to require chloride in the permeation pathway. At very

low extracellular chloride, ClC-0-channel opening no longer occurs through the normal voltage-dependent process, but rather the channel opens via a second, nearly voltage-independent process (Chen & Miller 1996). Assuming (as discussed above) that the protons provide the gating charge, this indicates that the presence of chloride in the pore (or bound to an extracellular site) is required for maintenance of the proton permeation pathway. This interdependency implies that the proton and chloride translocation pathways may indeed be coincident.

9. SUMMARY AND CONCLUSIONS

The CLC family is composed of both ion channels and secondary active transporters. At the level of bulk transport, the ion channels display only passive diffusion of chloride. However, in the process of gating, the channels also move protons across the membrane. In an average channel opening event, one proton is translocated, followed by roughly 10^5 chloride ions (considering a 30 ms open time for an 8 pS channel at -100 mV). Therefore, the channels can be considered to be 'broken' chloride-proton antiporters; they still couple the movement of chloride with that of protons, but their (no longer fixed) $H^+ : Cl^-$ stoichiometry falls far short of the obligate 1 : 2 stoichiometry seen in the antiporters. This 'broken transporter' model for the channels helps to explain how these two ostensibly different types of membrane proteins arose from a structurally similar foundation and implies an underlying mechanistic unity in the CLC family, despite the apparent incompatibilities between channel and transporter mechanisms.

We thank Prof. T. Jentsch for the gift of ClC-1 cDNA and Bill Kobertz, Joe Mindell and Rich Reimer for comments on the manuscript. J.L. is supported by HFSP. M.M. is grateful to the NIH (RO1-GM070773) and the Mathers Foundation for support.

REFERENCES

- Accardi, A. & Miller, C. 2004 Secondary active transport mediated by a prokaryotic homologue of ClC Cl^- channels. *Nature* **427**, 803–807. (doi:10.1038/nature02314)
- Accardi, A., Walden, M., Nguitragool, W., Jayaram, H., Williams, C. & Miller, C. 2005 Separate ion pathways in a Cl^-/H^+ exchanger. *J. Gen. Physiol.* **126**, 563–570. (doi:10.1085/jgp.200509417)
- Bennetts, B., Parker, M. W. & Cromer, B. A. 2007 Inhibition of skeletal muscle ClC-1 chloride channels by low intracellular pH and ATP. *J. Biol. Chem.* **282**, 32 780–32 791. (doi:10.1074/jbc.M703259200)
- Chen, M. F. & Chen, T. Y. 2001 Different fast-gate regulation by external $Cl(-)$ and $H(+)$ of the muscle-type ClC chloride channels. *J. Gen. Physiol.* **118**, 23–32. (doi:10.1085/jgp.118.1.23)
- Chen, M. F. & Chen, T. Y. 2003 Side-chain charge effects and conductance determinants in the pore of ClC-0 chloride channels. *J. Gen. Physiol.* **122**, 133–145. (doi:10.1085/jgp.200308844)
- Chen, T. Y. 2005 Structure and function of CLC channels. *Annu. Rev. Physiol.* **67**, 809–839. (doi:10.1146/annurev.physiol.67.032003.153012)
- Chen, T. Y. & Miller, C. 1996 Nonequilibrium gating and voltage dependence of the ClC-0 Cl^- channel. *J. Gen. Physiol.* **108**, 237–250. (doi:10.1085/jgp.108.4.237)
- DeAngeli, A., Monachello, D., Ephritikhine, G., Frachisse, J. M., Thomine, S., Gambale, F. & Barbier-Brygoo, H. 2006 The nitrate/proton antiporter AtCLCa mediates nitrate accumulation in plant vacuoles. *Nature* **442**, 939–942. (doi:10.1038/nature05013)
- Decoursey, T. E. 2003 Voltage-gated proton channels and other proton transfer pathways. *Physiol. Rev.* **83**, 475–579.
- deSantiago, J. A., Nehrke, K. & Arreola, J. 2005 Quantitative analysis of the voltage-dependent gating of mouse parotid ClC-2 chloride channel. *J. Gen. Physiol.* **126**, 591–603. (doi:10.1085/jgp.200509310)
- Dutzler, R. 2006 The ClC family of chloride channels and transporters. *Curr. Opin. Struct. Biol.* **16**, 439–446. (doi:10.1016/j.sbi.2006.06.002)
- Dutzler, R., Campbell, E. B. & MacKinnon, R. 2003 Gating the selectivity filter in ClC chloride channels. *Science* **300**, 108–112. (doi:10.1126/science.1082708)
- Engh, A. M. & Maduke, M. 2005 Cysteine accessibility in ClC-0 supports conservation of the ClC intracellular vestibule. *J. Gen. Physiol.* **125**, 601–617. (doi:10.1085/jgp.200509258)
- Estevez, R., Schroeder, B. C., Accardi, A., Jentsch, T. J. & Pusch, M. 2003 Conservation of chloride channel structure revealed by an inhibitor binding site in ClC-1. *Neuron* **38**, 47–59. (doi:10.1016/S0896-6273(03)0168-5)
- Fahlke, C., Desai, R. R., Gillani, N. & George Jr, A. L. 2001 Residues lining the inner pore vestibule of human muscle chloride channels. *J. Biol. Chem.* **276**, 1759–1765.
- Graves, A. R., Curran, P. K., Smith, C. L. & Mindell, J. A. 2008 The $Cl(-)/H(+)$ antiporter ClC-7 is the primary chloride permeation pathway in lysosomes. *Nature* **453**, 788–792. (doi:10.1038/nature06907)
- Hanke, W. & Miller, C. 1983 Single chloride channels from Torpedo electroplax. Activation by protons. *J. Gen. Physiol.* **82**, 25–45. (doi:10.1085/jgp.82.1.25)
- Jentsch, T. J. 2008 CLC chloride channels and transporters: from genes to protein structure, pathology and physiology. *Crit. Rev. Biochem. Mol. Biol.* **43**, 3–36. (doi:10.1080/10409230701829110)
- Kieferle, S., Fong, P., Bens, M., Vandewalle, A. & Jentsch, T. J. 1994 Two highly homologous members of the ClC chloride channel family in both rat and human kidney. *Proc. Natl Acad. Sci. USA* **91**, 6943–6947. (doi:10.1073/pnas.91.15.6943)
- Kurz, L. L., Klink, H., Jakob, I., Kuchenbecker, M., Benz, S., Lehmann-Horn, F. & Rudel, R. 1999 Identification of three cysteines as targets for the Zn^{2+} blockade of the human skeletal muscle chloride channel. *J. Biol. Chem.* **274**, 11 687–11 692. (doi:10.1074/jbc.274.17.11687)
- Lin, C. W. & Chen, T. Y. 2003 Probing the pore of ClC-0 by substituted cysteine accessibility method using methane thiosulfonate reagents. *J. Gen. Physiol.* **122**, 147–159. (doi:10.1085/jgp.200308845)
- Lísál, J. & Maduke, M. 2008 The ClC-0 chloride channel is a "broken" Cl^-/H^+ antiporter. *Nat. Struct. Mol. Biol.* **15**, 805–810. (doi:10.1038/nsmb.1466)
- Ludewig, U., Jentsch, T. J. & Pusch, M. 1997 Inward rectification in ClC-0 chloride channels caused by mutations in several protein regions. *J. Gen. Physiol.* **110**, 165–171. (doi:10.1085/jgp.110.2.165)
- Matulef, K. & Maduke, M. 2007 The CLC 'chloride channel' family: revelations from prokaryotes (review). *Mol. Membr. Biol.* **24**, 342–350. (doi:10.1080/09687680701413874)

- Miller, C. 2006 ClC chloride channels viewed through a transporter lens. *Nature* **440**, 484–489. (doi:10.1038/nature04713)
- Miller, C. & White, M. M. 1984 Dimeric structure of single chloride channels from Torpedo electroplax. *Proc. Natl Acad. Sci. USA* **81**, 2772–2775. (doi:10.1073/pnas.81.9.2772)
- Namslauer, A., Lepp, H., Branden, M., Jasaitis, A., Verkhovsky, M. I. & Brzezinski, P. 2007 Plasticity of proton pathway structure and water coordination in cytochrome c oxidase. *J. Biol. Chem.* **282**, 15 148–15 158. (doi:10.1074/jbc.M700348200)
- Niemeyer, M. I., Cid, L. P., Zuniga, L., Catalan, M. & Sepulveda, F. V. 2003 A conserved pore-lining glutamate as a voltage- and chloride-dependent gate in the ClC-2 chloride channel. *J. Physiol.* **553**, 873–879. (doi:10.1113/jphysiol.2003.055988)
- Piccolo, A. & Pusch, M. 2005 Chloride/proton antiporter activity of mammalian CLC proteins ClC-4 and ClC-5. *Nature* **436**, 420–423. (doi:10.1038/nature03720)
- Pusch, M. 2004 Structural insights into chloride and proton-mediated gating of CLC chloride channels. *Biochemistry* **43**, 1135–1144. (doi:10.1021/bi0359776)
- Pusch, M., Ludewig, U., Rehfeldt, A. & Jentsch, T. J. 1995 Gating of the voltage-dependent chloride channel ClC-0 by the permeant anion. *Nature* **373**, 527–531. (doi:10.1038/373527a0)
- Pusch, M., Ludewig, U. & Jentsch, T. J. 1997 Temperature dependence of fast and slow gating relaxations of ClC-0 chloride channels. *J. Gen. Physiol.* **109**, 105–116. (doi:10.1085/jgp.109.1.105)
- Richard, E. A. & Miller, C. 1990 Steady-state coupling of ion-channel conformations to a transmembrane ion gradient. *Science* **247**, 1208–1210. (doi:10.1126/science.2156338)
- Rychkov, G. Y., Pusch, M., Astill, D. S., Roberts, M. L., Jentsch, T. J. & Bretag, A. H. 1996 Concentration and pH dependence of skeletal muscle chloride channel ClC-1. *J. Physiol.* **497**(Pt 2), 423–435.
- Rychkov, G. Y., Pusch, M., Roberts, M. L., Jentsch, T. J. & Bretag, A. H. 1998 Permeation and block of the skeletal muscle chloride channel, ClC-1, by foreign anions. *J. Gen. Physiol.* **111**, 653–665. (doi:10.1085/jgp.111.5.653)
- Scheel, O., Zdebik, A. A., Lourdel, S. & Jentsch, T. J. 2005 Voltage-dependent electrogenic chloride/proton exchange by endosomal CLC proteins. *Nature* **436**, 424–427. (doi:10.1038/nature03860)
- Sonar, S., Marti, T., Rath, P., Fischer, W., Coleman, M., Nilsson, A., Khorana, H. G. & Rothschild, K. J. 1994 A redirected proton pathway in the bacteriorhodopsin mutant Tyr-57->Asp. Evidence for proton translocation without Schiff base deprotonation. *J. Biol. Chem.* **269**, 28 851–28 858.
- Traverso, S., Elia, L. & Pusch, M. 2003 Gating competence of constitutively open CLC-0 mutants revealed by the interaction with a small organic inhibitor. *J. Gen. Physiol.* **122**, 295–306. (doi:10.1085/jgp.200308784)
- Traverso, S., Zifarelli, G., Aiello, R. & Pusch, M. 2006 Proton sensing of CLC-0 mutant E166D. *J. Gen. Physiol.* **127**, 51–65. (doi:10.1085/jgp.200509340)
- Tseng, P. Y., Bennetts, B. & Chen, T. Y. 2007 Cytoplasmic ATP inhibition of CLC-1 is enhanced by low pH. *J. Gen. Physiol.* **130**, 217–221. (doi:10.1085/jgp.200709817)
- Zdebik, A. A., Zifarelli, G., Bergsdorf, E. Y., Soliani, P., Scheel, O., Jentsch, T. J. & Pusch, M. 2007 Determinants of anion-proton coupling in mammalian endosomal CLC proteins. *J. Biol. Chem.* **283**, 4219–4227. (doi:10.1074/jbc.M708368200)
- Zhang, X. D., Li, Y., Yu, W. P. & Chen, T. Y. 2006 Roles of K149, G352, and H401 in the channel functions of ClC-0: testing the predictions from theoretical calculations. *J. Gen. Physiol.* **127**, 435–447. (doi:10.1085/jgp.200509460)
- Zifarelli, G. & Pusch, M. 2007 CLC chloride channels and transporters: a biophysical and physiological perspective. *Rev. Physiol. Biochem. Pharmacol.* **158**, 23–76. (doi:10.1007/112_2006_0605)