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Channel or transporter? The CLC saga continues

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It was recently shown that the putative bacterial Cl^- channel, ClC-ec1, is in reality a Cl^-–H^+ antiporter. Our group has now shown that this is also the case for two human CLCs, ClC-4 and ClC-5. We found that the flux of Cl^- in one direction is stoichiometrically coupled to the movement of protons in the opposite direction, unveiling a behaviour that is typical of a transporter rather than a channel. This discovery will surely stimulate further research to elucidate the molecular elements responsible for the behaviour as a transporter. On the physiological level, the antiport activity of ClC-4/ClC-5 must lead to a review of the role of CLC proteins in intracellular compartments. Small organic molecules have been extremely useful tools for studying ion channels and many commercial drugs target specific ion channel proteins. Several blockers have been found to inhibit the plasma membrane-localized CLC channels ClC-0, ClC-1 and ClC-Ka. These compounds include 9-anthracene-carboxylic acid (9-AC), *p*-chlorophenoxy-propionic acid (CPP) and its derivatives, and 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS). Two different binding sites have been identified, one extracellular and one intracellular. However, high-affinity ligands for most CLC proteins are still missing. Apart from being useful biophysical tools, such drugs may provide a way to modulate protein function *in vivo*. With these tasks to be accomplished, it is definitely an exciting time in the chloride transport field.

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The CLC gene family is present in species ranging from bacteria to humans and comprises Cl^- ion channels and Cl^-–H^+ antiporters. It consists of nine members in mammals, and can be divided into three subbranches on the basis of sequence homology. The first branch comprises ClC-1, ClC-2, ClC-Ka and ClC-Kb channels. These are close homologues of the prototype *Torpedo* channel ClC-0 and localize to the plasma membrane. They are involved in the stabilization of the membrane potential and transepithelial transport. Members of the other two groups, ClC-3, ClC-4 and ClC-5, and ClC-6 and ClC-7, respectively, reside in intracellular membranes and they have been implicated in the acidification of intracellular compartments (endosomes/lysosomes). Mutations in the genes coding for ClC-1, ClC-Kb, ClC-5 and ClC-7 cause human genetic diseases: myotonia, Bartter syndrome, Dent's disease and osteopetrosis, respectively (for review see Jentsch *et al.* 2005).

ClC-0 was identified in the 1980s by Miller as a 'double-barrelled' channel. Two gating mechanisms rule the double-barrelled channel: one acts on single pores and the other simultaneously on both pores as a common gate (Miller & White, 1984). This unconventional architecture was confirmed when the X-ray structure of the bacterial CLC homologue, ClC-ec1 (Dutzler *et al.* 2002), was solved.

Proton–chloride antiporter activity of ClC-4 and ClC-5

When the crystal structure of the bacterial ClC-ec1 protein was first published (Dutzler *et al.* 2002), it was thought to represent the bacterial homologue of the eukaryotic CLC channels for which no high-resolution crystal structure is yet available. This implied that, despite differences in amino acid sequence (identity between ClC-ec1 and ClC-0 is around 15–20%), the general features of the bacterial 'channel' could have been used to extrapolate information about its human homologues. This optimistic view was shaken when a paper from Accardi & Miller (2004) showed that ClC-ec1 is a Cl^-–H^+ antiporter in which the transport of Cl^- in one direction is stoichiometrically coupled to the transport of H^+ in the opposite direction. But if ClC-ec1 is a transporter and not a channel, could it still provide a guideline for the mechanistic interpretation of

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the function of the 'real' CLC channels? And moreover, could it be that the antiporter activity displayed by ClC-ec1 also characterizes other members of the CLC family?

This second question has been addressed by our group. We could show that ClC-4 and ClC-5, which have been assumed to be Cl^- channels (for review see Jentsch *et al.* 2005), but for which no clear single-channel activity has yet been reported, also show Cl^-–H^+ antiport activity like ClC-ec1, and should therefore be considered as transporters (Picollo & Pusch, 2005).

Using pH-sensitive microelectrodes placed close to the membrane of *xenopus* oocytes, we could detect a robust acidification of the extracellular solution in correspondence with the activation of the Cl^- conductance mediated by ClC-5. The pH change correlated with the voltage dependence of ClC-5 activation. In the absence of extracellular Cl^- no pH changes could be detected, showing that H^+ transport through ClC-5 depends on Cl^- and is not mediated by a passive, independent pathway.

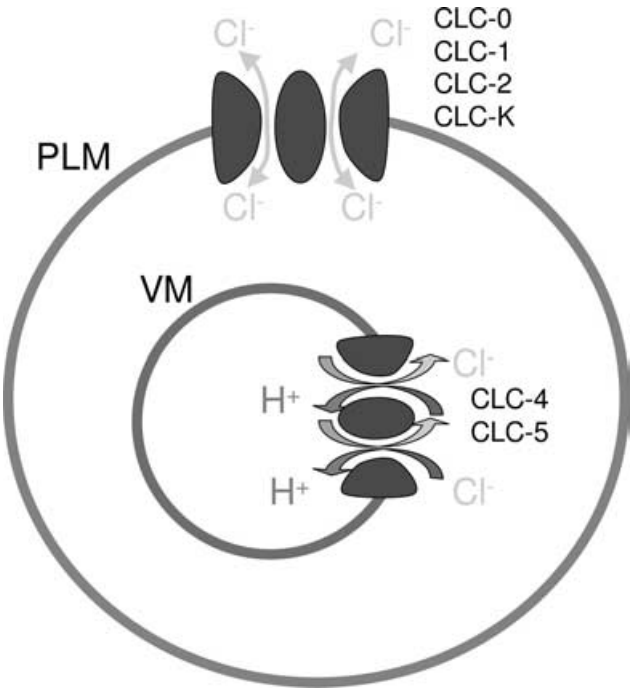

Figure 1. Schematic illustration of channel and transporter activity of CLC proteins
Among the mammalian CLC proteins, the plasma membrane-localized proteins ClC-1, ClC-2, ClC-Ka and ClC-Kb appear to be Cl^- ion channels, with two independent protopores, as indicated by the double-headed arrows (PLM, plasma membrane). The vesicular (VM, vesicular membrane) CLC proteins ClC-4 and ClC-5 (and probably also ClC-3) seem to be Cl^-–H^+ antiporters, like the bacterial ClC-ec1. However, the relationship of the double-barrelled structure and the transport stoichiometry is unknown. Thus, the independence of the two 'proto-transporters', as suggested in the figure, is speculative. The classification of the vesicular CLC proteins ClC-6 and ClC-7 as transporter or channel is uncertain.

Moreover, it was shown that even imposing an inwardly directed H^+ gradient, activation of ClC-5 led to a decrease of the extracellular pH, demonstrating that the energy stored in the downhill gradient of Cl^- was used to actively extrude protons. ClC-4 exhibited a very similar behaviour. ClC-0, ClC-2 and ClC-Ka did not show any proton transport activity. Interestingly, a small but reproducible proton transport activity was detected for ClC-1, the major skeletal muscle Cl^- channel.

The relative contribution of proton transport to ClC-5 activity could not be assessed by measuring the reversal potential because the current–voltage relationship of ClC-5 and ClC-4 is strongly outwardly rectifying and no inward currents can be measured (Friedrich *et al.* 1999; Steinmeyer *et al.* 1995). Therefore, the change in extracellular pH was used to estimate the amount of charge associated with proton transport, while the total charge transported was calculated from the integral of the current. Using this method, we could estimate a transport stoichiometry of about 1:1 for H^+ *versus* Cl^-. Taking into account the large error associated with the pH measurements, this value has to be considered only a rough estimate and therefore is not conflicting with the value of 0.5 found by Accardi & Miller (2004) with a different and more precise method.

The same conclusion was drawn by Scheel *et al.* (2005) using a somewhat complementary approach; measuring the fluorescence emission of a pH sensitive dye, they showed that activation of ClC-4 and ClC-5 led to intracellular alkalinization.

The pathway opened by this discovery has implications not only for the biophysics of Cl^- transport, but it will possibly also provide some general indications about the elements that distinguish channels from transporters that may turn out to be of interest also for other classes of membrane proteins like, for example, the Na^+,K^+-ATPase (Gadsby, 2004). Moreover, the transport mechanism elucidated for ClC-4 and ClC-5 represents a new challenge for the physiologists because the function of Cl^- permeation in intracellular compartments, assumed to be a simple electrical shunt to allow for acidification of the vesicles by proton pumps, remains to be precisely determined (see Fig. 1).

Pharmacology of CLC channels – mechanism of action of *p*-chlorophenoxy-propionic acid (CPP) and 9-anthracene-carboxylic acid (9-AC)

Relatively few specific and high-affinity ligands are known for Cl^- channels. The skeletal muscle background Cl^- conductance, g_{Cl}, which accounts for about 80% of the total resting conductance, has been studied pharmacologically for quite a long time (Bretag, 1987). We now know that g_{Cl} is carried in large part by the CLC

channel, ClC-1 (Steinmeyer *et al.* 1991*a*,*b*) and this fact opened the way for systematically studying inhibitors of CLC channels. CPP and several of its derivatives inhibit the skeletal muscle Cl^- conductance with an apparent affinity of $\sim$20 μM acting from the intracellular side. In particular, CPP inhibits ClC-0 and ClC-1, being much less effective on ClC-2 (Conte-Camerino *et al.* 1988; Pusch *et al.* 2000; Liantonio *et al.* 2002). The efficacy of CPP depends on the internal Cl^- concentration in a manner that is consistent with a competition between Cl^- and CPP for the occupation of the same or closely located sites (Pusch *et al.* 2001). The action of these blockers is voltage dependent, reducing currents at negative voltages but leaving them almost unaltered at positive voltages (Aromataris *et al.* 1999). The voltage dependence arises from a state-dependent binding, with closed channels having higher affinity for the blocker compared to open channels (Pusch *et al.* 2001). Based on the effect of pore mutations on *p*-chlorophenoxy-acetic acid (CPA) block, it was shown that CPA exerts its blocking effect by binding to the channel pore (Accardi & Pusch, 2003).

Estévez *et al.* (2003) identified the binding site of 9-AC and CPA in ClC-1 and ClC-0 by taking advantage of the difference in inhibitor sensitivity between ClC-1 and ClC-2. The binding pocket for the inhibitors is accessible from the cytoplasm and is adjacent to and possibly overlapping with the Cl^- binding site (Estévez *et al.* 2003). This study also proved that the ClC-ec1 structure could provide a good model of the structure of other CLC channels.

An extracellular binding site in CLC-K channels

ClC-K channels are inhibited by derivatives of CPP but only from the extracellular side (Liantonio *et al.* 2002). In particular, for ClC-Ka and ClC-K1, we found that the block was quickly reversible and competitive with extracellular Cl^- (Liantonio *et al.* 2004), suggesting that the binding site is exposed to the extracellular side and is located close to the ion conducting pore (Picollo *et al.* 2004). Among several compounds tested, the most potent inhibitor was the derivative 3-phenyl-CPP. ClC-Ka and ClC-K1 are also inhibited by DIDS with a similar affinity (Picollo *et al.* 2004). By comparison with the sequence of ClC-Kb, which shows 80% identity with ClC-Ka but is much less sensitive to the drug, two residues near the outer mouth of the pore were found to be critical for inhibition of these channels by DIDS and 3-phenyl-CPP.

A DIDS binding site in ClC-ec1

Matulef & Maduke (2005) found that the *E. coli* Cl^-–H^+ antiporter ClC-ec1 is inhibited by DIDS from the intracellular side in a reversible manner. Interestingly, ClC-0 displays an irreversible inhibition by DIDS (Miller & White, 1980). It was therefore suggested that the binding sites for DIDS in ClC-ec1 and ClC-0 differ (Matulef & Maduke, 2005). Identification of the binding site for DIDS on the ClC-ec1, for which the 3D structure is known, may provide guidelines for the design of other more efficient inhibitors and, alternatively, may be useful in understanding at the structural level the differences between ClC-ec1 and the other members of the CLC family.

Putative blockers of ClC-7

Kornak *et al.* (2001) showed that ClC-7 is a lysosomal Cl^- channel. Mutations of ClC-7 induce osteopetrosis, retinal and general neurodegeneration (Kornak *et al.* 2001; Kasper *et al.* 2005). ClC-7 has so far escaped any attempt at biophysical characterization because it does not localize in the plasma membrane in heterologous systems. The compound NS3736 belongs to the group of acidic diphenylureas that has been shown to block a Cl^- conductance in human erythrocytes (Bennekou *et al.* 2001). NS3736 and derivatives (Schaller *et al.* 2004; Karsdal *et al.* 2005) block acidification in resorption compartments and inhibit osteoclastic resorption *in vitro*. While these results suggest that inhibitors of Cl^- channels could be useful in the treatment of osteoporosis, it remains to be shown whether these compounds act directly on ClC-7.

Conclusions

The consequences of the recent discovery that some CLC proteins are Cl^-–H^+ antiporters and not Cl^- channels have still to be explored in the biophysical/mechanistic as well as the physiological dimension. Exciting developments can be expected. Even though recent work has revealed important interaction of several plasma membrane CLC channels with various small organic ligands, the pharmacological tools that are available for CLC proteins in general are still very poor. In particular, practically no modulatory compounds are available for ClC-2, ClC-3, ClC-4 and ClC-5. Identifying such inhibitors is an important challenge of future CLC channel and transporter research.

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