Commentary

Knocking on Channel's Door

The Permeating Chloride Ion Acts as the Gating Charge in ClC-0

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The well known chloride channel from *Torpedo* has been around since 1979 (White and Miller, 1979), but it continues to supply us with new surprises. In this issue of *The Journal of General Physiology* Chen and Miller analyze in great detail the voltage- and chloride-dependent activation mechanism of the peculiar "double-barreled" channel (Chen and Miller, 1996), which turns out to be completely different from that of the more familiar voltage-dependent cation channels.

The *Torpedo* chloride channel had been discovered by Chris Miller and colleagues in their attempts to study reconstituted acetylcholine receptors from *Torpedo* electroplax (see Miller and Richard, 1990 for review). Instead of the nicotinic receptor, they consistently observed a voltage-dependent Cl^- selective channel. Miller continued to characterize the chloride channel in lipid bilayers and revealed several important aspects of its function. The channel opens in bursts and within each burst two equidistant conductance levels are seen. The gating of the two conductance levels can be almost perfectly described assuming two separate and independent conduction pathways. Single "protopores" are activated at positive voltages (fast gate) whereas the occurrence of bursts is favored at hyperpolarized voltages (slow gate) (Miller and Richard, 1990). This "double-barreled model" was supported by experiments in which individual protopores could be inhibited by DIDS[1] (Miller and White, 1984), a blocker of ClC-0 when applied intracellularly. An additional feature of ClC-0 is that its gating is evidently coupled to the electrochemical gradient of chloride. This coupling was inferred indirectly from a violation of microscopic reversibility in channel gating, observations greatly facilitated by the double-barreled structure of the channel (Richard and Miller, 1990).

If history had stopped at that point one would have been left with a biophysically interesting but possibly exotic fish channel that was probably unimportant for human physiology. But the story of mammalian voltage-dependent chloride channels started anew with the expression cloning of the *Torpedo* channel by Jentsch et al. (1990). Based on homology, nine different human ClC genes have been identified by now (for a recent review, see Jentsch, 1996). The role of most of these channels (or putative channels) still has to be clarified. Only for the muscular chloride channel, ClC-1, is the function definitely known: it serves to stabilize the membrane potential of the muscle membrane. For several other homologs intriguing possible functions have been proposed that have yet to be put on a firm experimental basis. The importance of ClC channels is underscored by two human genetic diseases caused by defects of ClC genes. Mutations in ClC-1 lead to recessive or dominant myotonia, a disorder that is characterized by a hyperexcitability of the muscle fibers (see Jentsch, 1996). Defects of the recently cloned ClC-5 lead to several forms of hereditary kidney stone diseases (Lloyd et al., 1996), although the pathophysiology of this disease is still obscure.

The original ClC-0 has a relatively large single-channel conductance (in contrast to ClC-1). Given the amount of knowledge that exists for this channel, it gains considerable importance as a "model" channel that could help us to better understand the structure and function of newly cloned homologous chloride channels. This presupposes, however, that basic mechanisms of ClC channel function have been preserved during evolution. Importantly, ClC-0 can be conveniently expressed in heterologous expression systems such as oocytes or mammalian cells, and its sequence and structure can be manipulated using molecular biological methods. Another pleasing feature of ClC-0 is that its gating is, at least phenomenologically, rather well understood. Fast gating appears as an apparent two-state process with monoexponential kinetics. Slow gating can be separated from fast gating both at the single channel level, because the slow gate closes both protochannels simultaneously, and macroscopically due to the much slower kinetics. In contrast, the gating of ClC-1 appears to be more complex, as the presence of several exponential components in the gating kinetics complicate the interpretation of the measurements.

[1]*Abbreviations used in this paper:* α, opening rate; β, closing rate; DIDS, 4-4′-diisothiocyanostilbene-2,2′ disulfonic acid; $[Cl]_{int}$, intracellular chloride concentration; $[Cl]_{ext}$, extracellular chloride concentration; α, opening rate; β, closing rate; p_{open}, steady state open probability; SCN^-, thiocyanate.

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What is known about the gating of ClC-0? In contrast to the voltage-dependent cation channels, which have a distinctive charged structure (S4-segment) that was proposed to be the voltage sensor right after cloning (Noda et al., 1984), no such structure is present in ClC-channels. Also, it was found that fast gating of ClC-0 is strongly dependent on $[Cl]_{ext}$ (Pusch et al., 1995) with a shift of the open probability (p_{open}) curve toward more positive potentials by ~50 mV per 10-fold reduction in $[Cl]_{ext}$. Evidently, chloride can bind to the channel from the extracellular side and thereby favor the open state (in Fig. 1 several possibilities for a coupling of Cl^- ion binding to channel opening are shown). If the chloride binding site is located within the electrical field and if binding of chloride is necessary for opening, then p_{open} automatically becomes voltage dependent just because of this binding (Fig. 1, *Model A*). If all of the voltage dependence is caused by chloride binding, the steepness of the $p_{open}(V)$ curve is essentially determined by the distance of the binding site from the extracellular side. Alternatively, extracellularly bound chloride could modulate conformational changes that are intrinsically voltage dependent (Fig. 1, *Model B*). Pusch et al. (1995) reasoned that if the binding site is located deep in the conducting pore (as in *Model A*), gating properties and open-pore properties should be similarly affected when ionic conditions are varied. This was in fact observed (Pusch et al., 1995); only permeant ions are able to open the channel, the ion selectivity of gating is reflected by the ion selectivity of conduction, and an anomalous mole fraction effect of the conductance was mirrored by a similar anomalous effect of gating parameters (Pusch et al., 1995). The interaction of several ions in the pore, as suggested by the anomalous effects, ruled out simple models with one binding site (*Model A* and *Model B*). Instead, a model with two interacting binding sites was proposed (Fig. 1, *Model C*). In this model, the voltage dependence of channel opening does not arise from voltage-dependent binding of chloride from the outside but rather from the movement of chloride ions in the pore, which is closed towards the intracellular side.

Chen and Miller attacked this same problem with improved methods. First, they used ClC-0 reconstituted in lipid bilayers, thus eliminating endogenous channels and enabling precise control of intracellular and extracellular solutions. Second, they studied the gating at the single-channel level, allowing an unambiguous determination of both opening rate α and closing rate β of the fast gate. This allowed them to concentrate on α and thus avoid difficulties arising from the nonequilibrium situation when the channel is open, where downhill ion flow could create complex relationships. Chen and Miller were thus able to uncover important new properties of the ClC-0 channel. First, external chloride ions increase not only p_{open} of individual protopores, but also stimulate the occurrence of bursts, i.e., stimulate slow gating. The relation of the chloride dependence of fast and slow gating processes remains to

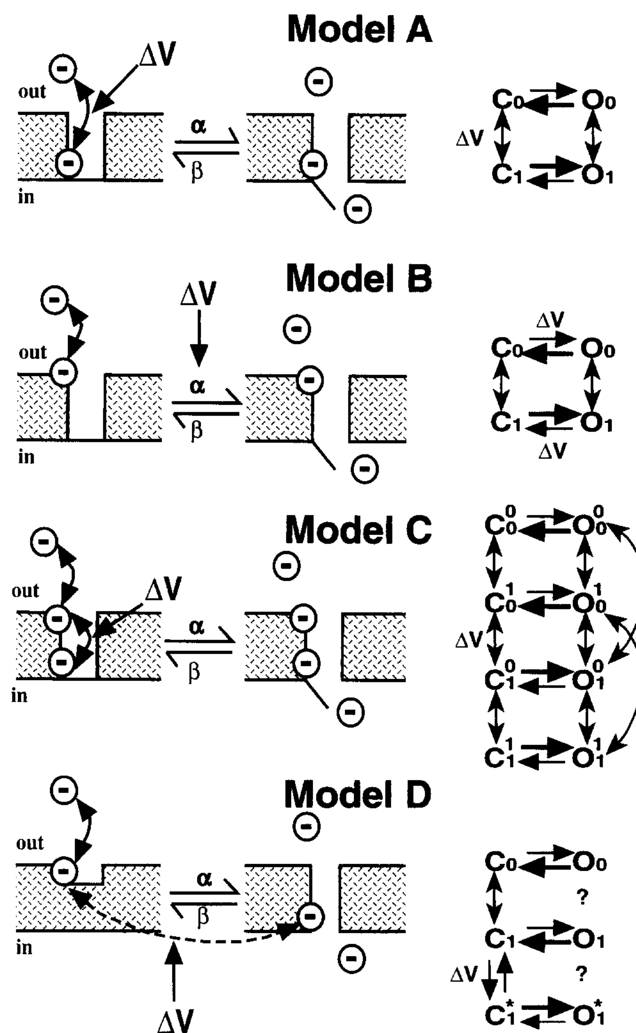

FIGURE 1. Possible schemes to describe the voltage and Cl^- dependence of ClC-0. In the diagrams on the right side, double-headed arrows indicate fast ion binding or ion movement steps, single headed arrows represent conformational changes of the protein. Steps that carry the main voltage dependence for the opening rate are indicated by ΔV. In model A, voltage-dependent binding of Cl^- to a single binding site deep within the pore confers the voltage dependence. In model B, voltage dependence arises from an intrinsic voltage sensor, whereas Cl^- binding is voltage independent. The index 0 or 1 indicates the occupancy of the binding site. Model C is a refined version of model A with two (interacting) anion binding sites. Movement of Cl^- from the outer binding site to the inner site is the major voltage-dependent step. Superior and inferior numbers indicate the occupancy of the outer and inner binding site, respectively. Model D is the model proposed by Chen and Miller. Here, the effective movement of the superficially bound Cl^- within the electric field (which could be a real transport step or a rearrangement of the electric field) is directly accompanied by a conformational change. The voltage dependence of transitions among the various open states are more difficult to model and are not considered in detail.

be identified. Second, Chen and Miller found an unexpected rise of α at negative voltages. The voltage dependence of the opening rate therefore is non-monotonic. The origin of the charge movement underlying this process (if it is, for example, also coupled to chloride) remains an open question. Third, intracellular chloride mainly affects the closing rate, and at high $[Cl]_{int}$ channels deactivate incompletely.

The main finding of the paper, however, is that binding of chloride appears to be voltage independent. Whereas model A predicts that α keeps increasing with increasing $[Cl]_{ext}$, Chen and Miller found that α saturates at higher $[Cl]_{ext}$. The saturation occurs at higher chloride concentrations (>100 mM) than had been investigated previously by Pusch et al. (1995). At a first glance the new results are compatible with model B, i.e., a binding of chloride at a more superficial location.

But things turned out to be more complicated. The agreement of model B with the data was good only in a limited voltage range. When analyzed over the complete voltage range, qualitative discrepancies with the 4-state model B led Chen and Miller to add an additional chloride-liganded state, as shown in model D. The transition of the first chloride-liganded state (C_1) to the new state (C_1*) represents the voltage-dependent step. From this state opening occurs very rapidly. The most intriguing aspect of the new model is that in order to keep the channel voltage dependent, Chen and Miller propose that the voltage dependence of the C_1→C_1* step arises from the transfer of the bound Cl^- across the electric field during the conformational change. This could occur either by a "transport" step and/or by a change of the geometry leading to a different field distribution in the channel protein.

Two different models therefore can explain the coupling of extracellular chloride to the voltage-dependent opening of ClC-0 (*Model C* and *Model D*). The schemes are representatives at opposite ends of a continuum of models. In either model, the movement of Cl^- within the electrical field in the closed channel is the source of voltage dependence. But there is an important difference. Whereas in model D the movement of Cl^- is coupled directly to the conformational change, in model C Cl^- moves "freely" in the fixed structure of the closed pore. Can one distinguish between these possibilities? Chen and Miller measured the binding of Cl^- indirectly (via the opening rate and a specific model), so the conclusion that binding of Cl^- is voltage independent is model dependent. The complexities in ion conduction through multiple-occupied channels, however, provide model C with a very large spectrum of behaviors. Experiments with different anions such as nitrate or SCN^- that have proven the presence of multiple binding sites at least in the open channel (White and Miller, 1981; Pusch et al, 1995) also cannot distinguish between the models.

In addition, one should not forget that still alternative models, as one proposed by Finkelstein and Peskin (1984), may need to be invoked in order to explain the nonequilibrium gating seen for ClC-0.

Gating-current measurements should, in principle, be able to distinguish between model C and model D because different kinds of charge movement are predicted for the opening process. For model C where chloride moves freely in the closed channel, gating currents would be caused by the extremely fast binding of chloride ions in the pore, whereas model D predicts (on-) gating currents with the same kinetics as the mean open probability, i.e., the ionic currents. Unfortunately, no high affinity blockers similar to those that allow gating current measurements for voltage-dependent cation channels are available for chloride channels. If available, such a blocker would also be of little use if it acted from the outside because this would prevent Cl^- binding (and channel opening). A blocker that binds from the inside and impedes ion flow without altering gating could work, but such things seem to exist only in the dreams of ever-optimistic biophysicists.

Even crystal-structure data of closed and open conformations of the channel may not completely resolve the problem. But because such data are unlikely to be available in the near future, further functional experiments and quantitative analysis of model expectations under various conditions seem to be the only way to limit the possible models that are able to describe the gating. For the physiologist, the question is probably of minor importance. The critical point, that fast gating does not use an intrinsic voltage-sensor and that, instead, the permeating anion serves as the gating charge in ClC-0, seems to be established beyond reasonable doubt. From the perspective of channels as enzymes, ClC-0 can be considered as an enzyme that is activated by its own substrate.

Is this mechanism relevant also for the other members of the ClC-family or even for other channels? How does extracellular and/or intracellular Cl^- affect protein function in the various mammalian homologs? Especially in salt-secreting and salt-absorbing epithelia a mechanism similar to that found in ClC-0 could function as a Cl^- sensor. Chloride channels that are sensitive to intracellular Cl^- also could be involved in adjusting the contribution of the Cl^- equilibrium potential to the membrane potential of neuronal cells, which could be important for determining cell excitability.

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