Ion permeation and selectivity in ClC-type chloride channels

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Fahlke, Christoph. Ion permeation and selectivity in ClC-type chloride channels. Am J Physiol Renal Physiol 280: F748–F757, 2001.—Voltage-gated chloride channels are present in almost every living cell and have many physiological functions. Recently, a novel gene family encoding voltage-gated chloride channels, the ClC family, was identified. The knowledge of primary amino acid sequences has allowed for the study of these anion channels in heterologous expression systems and made possible the combination of site-directed mutagenesis and high-resolution electrophysiological measurements as a means of gaining insights into the molecular basis of channel function. This review focuses on one particular aspect of chloride channel function, the selective transport of anions through biological membranes. I will describe recent experiments using a combination of cellular electrophysiology, molecular genetics, and recombinant DNA technology to study the molecular basis of ion permeation and selection in ClC-type chloride channels. These novel tools have provided new insights into basic mechanisms underlying the function of these biologically important channels.

THE BASIC STRUCTURE OF EVERY biological membrane is a lipid bilayer that is virtually impermeable to charged molecules. Ion transport through membranes is achieved by specialized membrane proteins that permit crossing of ions by different mechanisms of decreasing the Born barrier and are usually classified as channels, carriers, or pumps according to the transport mechanism (47). Certain ion channels have been extensively studied over the last hundred years, and considerable progress has been made in the last few years to provide a detailed understanding of the molecular basis of function of these particular channels (6, 11, 25, 30).

In contrast, the majority of transport proteins remain little studied, and for these the basic function is still insufficiently understood. One of these neglected protein classes is that of the voltage-gated anion or chloride channels, anion-selective channels that display voltage-dependent transitions between open and closed states and do not depend on the binding of a ligand to open (29). These chloride channels do not play major roles in the initiation and spread of excitation in excitable membranes and the neuronal synaptic transmission, but they regulate excitability in nerve and muscle by adjusting length constants and input resistance (1, 29). Anion channels often stabilize the membrane potential of excitable cells by providing an additional conductance, reversing close to the resting potential. They also fulfill important housekeeping functions like volume regulation (46) and pH regulation in organelles (2) and are crucial for transepithelial solute transport (28, 60).

Voltage-gated chloride channels are difficult to study in native preparations. Molecular cloning and subsequent heterologous expression were major breakthroughs in the investigation of this class of ion channels, and this was first accomplished by Jentsch and colleagues in 1990 (34), when they cloned a chloride channel, ClC-0, from the electric organ of Torpedo marmorata. As a large number of homologs were subsequently identified, ClC-0 became the ancestor and the gene precursor of a novel channel family of voltage-gated chloride channels, the ClC family. The ClC fam-
ily presently represents the largest known gene family coding for anion channels (32, 33). At least nine human isoforms (ClC-1 to ClC-7, ClC-Ka, and ClC-Kb) are expressed in various tissues. Some of them are known to play important roles in the function of certain organs, whereas for others the physiological importance has not yet been identified. ClC-1 is almost exclusively expressed in adult skeletal muscle fibers and is responsible for its large chloride conductance at resting potential (57). Mutations in this isoform cause myotonia congenita (24, 39), a muscle disease characterized by stiffness on sudden forceful movement. ClC-2 is ubiquitously expressed and seems to fulfill various tasks in several organs. ClC-2 was originally proposed to be an ubiquitously expressed volume-sensitive chloride channel (26), but this role has been disputed in the last few years (38). In neuronal cells, heterologous expression of ClC-2 decreases internal chloride concentration, suggesting an important role of this isoform in adjusting the chloride equilibrium potential in neurons (56). Furthermore, a role in transepithelial transport in the gastrointestinal tract was recently demonstrated (8, 28). In cardiac myocytes, ClC-2 forms an inwardly rectifying chloride channel with a possible role in the regulation of excitability (13). ClC-3 was also suggested to be a ubiquitous volume-activated chloride channel (12), but the discussion of this suggestion has not yet been settled (38). Moreover, there is uncertainty about the functional properties of this isoform, as some groups have reported successful heterologous expression and others have not. ClC-4 and ClC-5 share high sequence identity and are functionally very similar (22). In humans, ClC-5 is kidney specific, whereas ClC-4 is expressed in muscle, brain, and heart. Mutations in the gene coding for ClC-5, CLCN5, cause Dent's disease, an inherited renal disorder associated with hypercalciuria, nephrolithiasis, and low-molecular-weight proteinuria (40). ClC-5 is thought to form an anion channel in endosomes of proximal tubule cells that is crucial for proper acidification (10, 27, 52). A similar role for ClC-4 appears possible, but this has not been demonstrated as yet. ClC-6 and ClC-7 are ubiquitously expressed, but nothing is known about their functional or physiological properties (5). ClC-K1 and ClC-K2 in rats, or ClC-Ka and ClC-Kb in humans, represent kidney-specific isoforms that are involved in fluid resorption within the nephron (42, 55, 60). Genetic alterations of CLCNKB are responsible for type III Bartter's syndrome, a salt-wasting renal tubular disorder causing hypovolemia, hyponatremia, and hypokalemia (55, 60).

ClC channels are clearly of high physiological importance, and understanding basic properties of ClC channels at the molecular level will provide important information regarding many physiological questions. Studying ClC channels in heterologous expression systems provides the opportunity to combine cellular electrophysiology and recombinant DNA technology to gain molecular insights into how chloride channels work. In the following pages I will review the basic properties of ClC channels and recent progress in our understanding of how anion channels fulfill their primary task, the selective transport of anions through cellular and subcellular membranes.

**PHYSIOLOGICAL ROLE OF CHLORIDE CHANNELS**

The physiological task of ClC channels is quite different from the role of cation channels. Various cations of similar sizes exist in intra- and extracellular solutions, and channels that selectively permit the passage of Na⁺ or K⁺, but not of another ion, are key for many physiological processes. In contrast, there are only two anions, Cl⁻ and HCO₃⁻, at millimolar concentrations in biological media, and perfect discrimination seems not to be crucial, as these anions are similarly distributed over the cell membrane. Similarly, many anion channels are permeable to both chloride and bicarbonate. Moreover, perfect anion-to-cation selectivity of chloride channels appears necessary only in certain tissues. For example, in excitible cells, a small Na⁺ or Ca²⁺ permeability through anion channels would provide a component Na⁺ or Ca²⁺ conductance at resting potential and greatly disturb the normal excitation processes (16). In contrast, in epithelial cells such a leak cation conductance would be much less harmful, as in those cells there are many active cation transport mechanisms.

**FUNCTIONAL PROPERTIES OF CLC-TYPE CHLORIDE CHANNELS**

The various ClC isoforms do not share a high degree of sequence identity, and they display a wide variety of gating and permeation properties. Table 1 gives an overview of functional properties of mammalian ClC isoforms, illustrating the functional divergence within this gene family. Figure 1 demonstrates representative current recordings from four ClC isoforms: ClC-0 in *T. marmorata* electric organ (Fig. 1A), hClC-1, the human muscle isoform (Fig. 1B), rat ClC-2 (Fig. 1C), and human ClC-4 (Fig. 1D). The four isoforms were chosen because there is general agreement about the functional properties of these ClC channels. For each isoform, currents were recorded by whole cell patch-clamp recordings from transiently transfected tsA201 cells. Cells were held at 0 mV between pulses, and voltage steps between −165 and +75 mV in 40-mV intervals are shown. Conduction and gating properties differ among the isoforms. ClC-0 and ClC-1 are open at 0 mV, and currents deactivate on membrane hyperpolarization. ClC-0 displays a linear instantaneous current-voltage relationship; hClC-1 is pronouncedly inwardly rectifying. ClC-2 is closed at positive potentials, and voltage steps between −165 and +75 mV in 40-mV intervals are shown. Conduction and gating properties differ among the isoforms. ClC-0 and ClC-1 are open at 0 mV, and currents deactivate on membrane hyperpolarization; the current-voltage curve of the open channel is linear. ClC-4 outwardly rectifies and activates on membrane depolarization.

Selectivity among different anions, albeit physiologically not important, is of special importance for this review as selectivity has provided important insights into the function of the pores of ClC channels. Many ClC channels display a Cl⁻ > Br⁻ > I⁻ (Table 1)
permeability sequence, in contrast to other anion-selective channels, such as glycine and GABA receptors or volume-sensitive chloride channels (4, 46). Obviously, there are functional differences between the ion pores of members of the CIC family and those of many other anion-selective channels. There are two exceptions, CIC-3 (12) and hClC-4 at an external pH (pH$_o$) of 4.5 (35). The results for CIC-3 are presently a matter of controversy (12, 21, 36, 37), as there are conflicting published data and several groups have failed to reproduce these results because they could not functionally express CIC-3.

Interestingly, for CIC-type chloride channels, the anion permeability sequence is affected by changing the pH$_o$. Native chloride channels in frog muscle exhibit an anion permeability sequence of Cl$^-$ > Br$^-$ > NO$_3^-$ > I$^-$ at physiological pH$_o$, but an inverted one (Cl$^- <$ Br$^- <$ NO$_3^-$ < I$^-$) at pH$_o$ of 5.0 (31). For cloned CIC channels, a similar result was reported for hClC-4 (16). Friedrich et al. (22) observed a Cl$^-$ > I$^-$ permeability sequence at physiological pH, whereas Kawasaki et al. (35), using the same clone in a similar expression system, observed an I$^-$ > Cl$^-$ sequence at pH$_o$ of 4.5. These examples establish that anion selection by this class of channels is under the control of a titratable side chain.

Table 1. Functional properties of several CIC isoforms

<table>
<thead>
<tr>
<th>CIC Isoform</th>
<th>Permeability Sequence</th>
<th>Open-Channel I-V Relationship</th>
<th>Gating</th>
<th>Origin of Tested Channels (Ref. No.)</th>
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<tbody>
<tr>
<td>CIC-0</td>
<td>Cl$^-$ &gt; Br$^-$ &gt; NO$_3^-$ &gt; I$^-$</td>
<td>Linear</td>
<td>Fast gate activating on depolarization, slow gate on hyperpolarization</td>
<td>Native channels, reconstituted in planar lipid bilayers (45)</td>
</tr>
<tr>
<td>CIC-0</td>
<td>Cl$^-$ &gt; Br$^-$ &gt; NO$_3^-$ &gt; I$^-$</td>
<td>Linear</td>
<td>Fast gate activating on depolarization, slow gate on hyperpolarization</td>
<td>Expressed in Xenopus oocytes (34)</td>
</tr>
<tr>
<td>rClC-1</td>
<td>SCN$^-$ &gt; Cl$^-$ &gt; Br$^-$ &gt; NO$_3^-$ &gt; I$^-$</td>
<td>Inwardly rectifying</td>
<td>Activation on depolarization</td>
<td>Expressed in SB9 (51)</td>
</tr>
<tr>
<td>hClC-1</td>
<td>Cl$^-$ &gt; SCN$^-$ &gt; Br$^-$ &gt; NO$_3^-$ &gt; I$^-$</td>
<td>Inwardly rectifying</td>
<td>Activation on depolarization</td>
<td>Expressed in HEK293 (16)</td>
</tr>
<tr>
<td>rClC-2</td>
<td>Cl$^-$ &gt; Br$^-$ &gt; I$^-$</td>
<td>Linear</td>
<td>Activation on hyperpolarization</td>
<td>Expressed in Xenopus oocytes (59)</td>
</tr>
<tr>
<td>hClC-2</td>
<td>Cl$^-$ &gt; I$^-$ &gt; Glu$^-$</td>
<td>Linear</td>
<td>Activation on hyperpolarization</td>
<td>Endogenous channels in human immortalized airway cells (54)</td>
</tr>
<tr>
<td>hClC-2</td>
<td>Cl$^-$ &gt; I$^-$ &gt; Glu$^-$</td>
<td>Linear</td>
<td>Activation on hyperpolarization</td>
<td>Heterologously expressed in human immortalized airway cells (54)</td>
</tr>
<tr>
<td>rbClC-2</td>
<td>Cl$^-$ &gt; Br$^-$ &gt; I$^-$ &gt; F$^-$</td>
<td>Linear</td>
<td>Activation on hyperpolarization</td>
<td>Expressed in Xenopus oocytes (23)</td>
</tr>
<tr>
<td>rClC-3</td>
<td>I$^-$ &gt; Cl$^-$ &gt; Br$^-$</td>
<td>Outwardly rectifying</td>
<td>Inactivation on depolarization</td>
<td>Expressed in Xenopus oocytes (37)</td>
</tr>
<tr>
<td>rClC-3</td>
<td>I$^-$ &gt; Cl$^-$ &gt; Br$^-$</td>
<td>Outwardly rectifying</td>
<td>Activation on depolarization</td>
<td>Expressed in CHOK1 (36)</td>
</tr>
<tr>
<td>gpClC-3</td>
<td>I$^-$ &gt; Cl$^-$</td>
<td>Outwardly rectifying</td>
<td>Inactivation on depolarization</td>
<td>Expressed in NIH3T3 (12)</td>
</tr>
<tr>
<td>hClC-4</td>
<td>NO$_3^-$ &gt; Cl$^-$ &gt; Br$^-$ &gt; I$^-$</td>
<td>Outwardly rectifying</td>
<td>Activation on depolarization</td>
<td>Expressed in CHO-1 (22)</td>
</tr>
<tr>
<td>hClC-4</td>
<td>I$^-$ &gt; Cl$^-$ &gt; F$^-$</td>
<td>Outwardly rectifying</td>
<td>Activation on depolarization</td>
<td>Expressed in CHO-1 (35)</td>
</tr>
<tr>
<td>rClC-5</td>
<td>NO$_3^-$ &gt; Cl$^-$ &gt; Br$^-$ &gt; HCO$_3^-$ &gt; I$^-$</td>
<td>Outwardly rectifying</td>
<td>Activation on depolarization</td>
<td>Expressed in Xenopus oocytes (58)</td>
</tr>
<tr>
<td>rClC-K1</td>
<td>Br$^-$ &gt; Cl$^-$ &gt; I$^-$</td>
<td>Outwardly rectifying</td>
<td>Activation on depolarization</td>
<td>Expressed in Xenopus oocytes (61)</td>
</tr>
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CIC, voltage-gated chloride channel; I-V, current-voltage; Glu, glutamate; r, rat; h, human; rb, rabbit; gp, guinea pig; CHO, Chinese hamster ovary.

Fig. 1. Representative current recordings from several voltage-gated chloride channels (CIC) isoforms: CIC-0 (A), hClC-1 (B), rClC-2 (C), and hClC-4 (D). Currents were recorded by using tight whole-cell recordings from tsA201 cells, transiently transfected with coding regions of various CIC isoforms subcloned into the pRc/CMV vector as previously described (16). Cells were bathed in a standard external solution containing (in mM) 140 NaCl, 4 KCl, 2 CaCl$_2$, 1 MgCl$_2$, and 5 HEPES, pH 7.4, and perfused with a standard internal solution (in mM: 130 NaCl, 2 MgCl$_2$, 5 EGTA, and 10 HEPES, pH 7.4). A holding potential of 0 mV was chosen, and voltage steps between −165 and +75 mV were applied as shown in the pulse diagram (inset).
FUNCTIONAL CHARACTERIZATION OF THE CLC ION CONDUCTION PATHWAY

A number of electrophysiological studies using permeant and impermeant anions have provided insights into the functions of the pore and the selectivity mechanism of CLC channels. These studies, pioneered by Miller and White (45), focus on two closely related CLC isoforms, CLC-0 and CLC-1. For other CLC channels, such an analysis is still missing. Miller and White probed native CLC-0 channels in planar lipid bilayers with various anions. They investigated single-channel and macroscopic current amplitudes at several concentrations of Cl\(^-\) and other anions, as well as reversal potentials under bi-ionic conditions. In their hands, CLC-0 is permeable only to Cl\(^-\) and Br\(^-\) and is blocked by other anions, including I\(^-\) and SCN\(^-\). CLC-0 exhibits a low-affinity binding site for Cl\(^-\) [dissociation constant (K\(_D\)) = 75 mM], and several tests revealed that the channel is only singly occupied (absence of anomalous mole fraction, concentration independence of reversal potentials under bi-ionic conditions, and concentration independence of the parameters describing the blocking action of SCN\(^-\)). A later study of CLC-0 channels heterologously expressed in Xenopus laevis oocytes (48) reported anomalous mole fraction behavior of macroscopic currents and concluded that this CLC isoform exhibits multiple occupancy. The reason for these different experimental results is unclear.

The muscle CLC isoform, CLC-1, is similar to CLC-0 in many respects but differs in others. For CLC-1, the reversal potential determined under bi-ionic conditions with a constant concentration ratio changes with alterations of the absolute concentration (18). This result is clear evidence that CLC-1 is multiply occupied. Various permeant anions, such as I\(^-\), SCN\(^-\), NO\(_3^-\), or Br\(^-\), block Cl\(^-\) current through hCLC-1 by binding to sites within the ion conduction pathway with higher affinity than does Cl\(^-\) (16, 18, 51). These anions remain at this site longer, and this feature reduces their permeability and accounts for the observed block of Cl\(^-\) current (15, 16, 31, 49). Cl\(^-\) current block is accompanied by kinetic effects that depend on the side of the membrane to which the blocking anions are applied. On membrane hyperpolarization, muscle chloride channels deactivate with a biexponential time course (20). Although the time constants are almost voltage independent, the relative amplitude of the fast-deactivating, slow-deactivating, and nondeactivating current components are highly voltage dependent. External application of I\(^-\), SCN\(^-\), NO\(_3^-\), or Br\(^-\) changes the voltage dependence of the fractional current amplitudes but leaves the time constants of deactivation unaffected (18, 51). In contrast, internal application slows the deactivation time constants (18, 51). These results demonstrate that there are at least two distinct binding sites within the hCLC-1 ion conduction pathway (16). The two binding sites differ in their relative anion selectivity; i.e., the extracellular site exhibits an affinity sequence of SCN\(^-\) > I\(^-\) > NO\(_3^-\) > CH\(_3\)SO\(_3^-\) > Br\(^-\) > Cl\(^-\) > F\(^-\) and the intracellular one of I\(^-\) > NO\(_3^-\) > SCN\(^-\) > Cl\(^-\) > F\(^-\) (18). By using the characteristic kinetic changes induced by external and internal I\(^-\), the K\(_D\) of the internal and external binding sites could be independently determined. The K\(_D\) values so obtained are in the millimolar range (18). Because iodide binds more tightly than chloride, this value represents a lower limit for the chloride K\(_D\), indicating that hCLC-1 binds anions with affinities that are more than an order of magnitude lower than the permeant ion affinities of potassium (62) and calcium channels (9). Another important result of this study is that the concentration and voltage dependences of the effect of I\(^-\) on the fast-deactivating component are distinct from those on the slow-deactivating and the nondeactivating components. For all three current components, the K\(_D\) (0 mV) as well as the voltage dependence of the K\(_D\) are different (18). These results suggest that hCLC-1 undergoes conformational changes of the ion conduction pathway during transition between the three kinetic states.

To study the functional basis of the distinct relative anion permeabilities of various anion channels, we studied the interaction of anions within the ion conduction pathway for several CLC channel constructs exhibiting distinct anion permeability sequences. The first CLC channel construct demonstrated as exhibiting a larger permeability for I\(^-\) than for Cl\(^-\) was a mutant ClC-1 channel that carries a disease-causing G230E mutation (16). G230E hClC-1 exhibits an inverted anion permeability sequence of SCN\(^-\) > NO\(_3^-\) > I\(^-\) > Br\(^-\) > Cl\(^-\). Experiments revealed that neither external nor internal I\(^-\) blocks Cl\(^-\) currents, although its ability to change gating properties indicates that I\(^-\) still binds better than Cl\(^-\) (16). The effect of I\(^-\) on various CLC channel constructs that exhibit a larger I\(^-\) than Cl\(^-\) permeability is, however, not uniform. Figure 2 shows representative voltage-clamp current recordings and instantaneous current amplitudes in external Cl\(^-\) and in external I\(^-\) for four CLC channel constructs: WT hClC-1, G230A hClC-1, K231A hClC-1, and the ClC-3/ClC-1 chimera described above. WT hClC-1 exhibits a P\(_I/P_Cl\) of 0.34, where P is permeability, while all other constructs exhibit a higher I\(^-\) than Cl\(^-\) permeability (P\(_I/P_Cl\) ~ 2.5) (21). WT hClC-1 displays a I\(^-\) block over the whole voltage range, accompanied by a shift of the current reversal potential to the right, causing the Cl\(^-\) > I\(^-\) permeability sequence of this CLC isoform. For G230A hClC-1, there is a similar blocking action, and the I\(^-\) conductivity at positive potentials is very small, but the reversal potential shifts in the opposite direction. For cells expressing K231C hClC-1, Cl\(^-\) currents in the negative-voltage range are blocked by I\(^-\), but the I\(^-\) current at positive potentials is larger than the Cl\(^-\) current. The fourth illustrated construct is a rClC-3/hClC-1 chimera that was generated by replacing the D3-D5 segment of hClC-1 with that of rClC-3 (21). Over the whole voltage range, no blocking action of I\(^-\) was observed (15). Moreover, the changes in current amplitudes were similar to the predictions of the Goldman-Hodgkin-Katz equation (29), suggesting that different anion fluxes only minimally interfere with each other.
higher anion flux at positive potentials. At negative potentials, the presence of I\(^-\) markedly reduces channel conductance. This indicates a clear interaction between simultaneously bound anions. For the chimera, the virtual absence of interaction between the two anions suggests that a single site determines ion conductance through the pore.

Other anions that can be also used to probe the ionic pore of ClC channels are F\(^-\) and CH\(_3\)SO\(_3\)\(^-\). Although ClC-0 and ClC-1 are not measurably permeant for F\(^-\) or CH\(_3\)SO\(_3\)\(^-\) (15, 51), ClC-4 exhibits a permeability for these two anions (35). These results allow predictions about the pore narrowing of different isoforms.

A MODEL FOR THE MECHANISM OF SELECTIVITY IN CLC-TYPE CHLORIDE CHANNELS

Selection between anions and cations can be easily explained with electrostatic interactions between ions and the channel protein. Cation channels use negative charges in pore vestibules to attract cations and to repel anions, and it is easy to imagine that anion channels use a similar mechanism, a positive electrostatic potential to prevent the permeation of cations and thus be anion selective. Studying the mechanism by which ClC channels select among anions has provided much more information about basic pore properties than the anion-cation selection process.

Ions in aqueous solution are surrounded by a shell of water molecules. The electrostatic interaction of the water dipole with the ion charge is quite strong, with free energy values in the range of 100 kcal/mol. For the majority of selective ion channels, the first step in ion permeation is a partial dehydration, reducing the size of the permeating complex and therefore allowing the ion without water molecules, or with a few, to pass through the pore (14). The energy necessary for dehydration is provided by the binding of dehydrated ions to the amino acids of the ion conduction pathway. For hClC-1, F\(^-\), the smallest tested anion, is almost impermeant whereas Cl\(^-\) the anion with the next larger diameter, has the highest permeability among all ions tested. This behavior indicates that a dehydration step is occurring during anion permeation through this channel and is critically involved in selection among anions. Binding of anions within ClC channel pores occurs with low affinity, and it thus appears reasonable that the interaction of the channel pore with the permeating anion is dominated by electrostatic forces. These features demonstrate the feasibility of using the Eisenman approach to describe anion binding within ClC channel pores. The Eisenman approach (14, 29) to describing the binding of ions within pores takes only electrostatic interactions and dehydration energies into account, and this simplification allows an easy mathematical treatment of channel ion binding. This theory predicts that, for a given set of ions, only a certain number of permeability sequences exist, the so-called “Eisenman sequences.” Within these sequences, two extreme cases can be distinguished. One is that the binding site provides a weak...
electrostatic field. In this case, the dehydration energy is the dominating factor of binding. A weak binding site will therefore prefer large anions over small ones as the dehydration energy of these anions is smaller. The other case is an electrostatically strong binding site, in which the electrostatic term will dominate. Such a site will prefer small anions over larger ones because they provide smaller electrostatic energy terms. For hClC-1, larger and polyatomic anions bind with higher affinities to sites within the ionic pore. Therefore, according to the Eisenman theory, binding sites within the hClC-1 pore are weak-field-strength sites. They provide little electrostatic binding energy, and binding selectivity is largely determined by the dehydration energy; thus they prefer larger anions over smaller. The better binding of polyatomic anions such as SCN\(^-\), NO\(_3\), and CH\(_2\)SO\(_3\) suggests that hydrophobic components contribute to the binding sites. hClC-1 selects among SCN\(^-\), I\(^-\), NO\(_3\), Br\(^-\), and Cl\(^-\) by selective binding to a low-affinity binding site. The tighter binding of certain anions reduces their turnover, and this feature decreases the permeability as well as the conductivity of these anions (15, 31). Such a selectivity mechanism is not possible for singly occupied pores, as shown by Bezanilla and Armstrong (3), but is feasible for ClC-1 because it is multiply occupied. The proposed selectivity process of hClC-1 has the interesting feature of producing a selectivity sequence that prefers small anions over large ones (and would be called a strong-field interacting site in Eisenman terminology) by means of selective binding to binding sites that are weak-field interacting sites, i.e., that prefer large and polyatomic anions over small anions. The weak interacting sites within the hClC-1 pore are unable to provide the energy necessary to dehydrate F\(^-\), and the hydrated F\(^-\) cannot permeate the narrow part of the pore as it is too bulky. In the context of this reasoning, the increased F\(^-\) permeability of ClC-4 (35) may be explained by suggesting a larger pore narrowing. If hClC-4 exhibits a larger diameter of the pore narrowing than ClC-1, hydrated F\(^-\) could permeate hClC-4 but not hClC-1. Experiments with G230E hClC-1 demonstrated that channels with a I\(^-\) > Cl\(^-\) permeability sequence can also exhibit binding sites preferring large and polyatomic anions (16). A changed permeability selectivity is therefore not due to changed binding affinities. The major difference between ClC channels with Cl\(^-\) > I\(^-\) and those with I\(^-\) > Cl\(^-\) is that the latter do not exhibit a block by permeant anions. The following selectivity mechanism is in agreement with all published experimental results.

ClC-type chloride channels select among ions by selective binding to several anion-selective binding sites. All these sites prefer large or polyatomic anions over small uniatomic ones; they are weak interacting sites in Eisenman terminology (14, 29). By binding to these anionic sites, anions dehydrate and the smaller radius of dehydrated anions permits passage through a narrow pore constriction. Some anions simply do not permeate as they are too large to pass; for all others, the binding to and unbinding from these sites is crucial in determining passage of the anion through the channel. In channels with Cl\(^-\) > I\(^-\) or Cl\(^-\) > NO\(_3\) selectivity, the rate-limiting step in anion permeation is the dissociation of anions from one of these sites. Anions that bind with higher affinity to this site are less permeant and cause block of current flow by anions that bind with lower affinity. In channels with I\(^-\) > Cl\(^-\), or NO\(_3\) Cl\(^-\) selectivity, the association, not the dissociation, of permeant anions to one of these binding sites is the rate-limiting step. For this reason, the permeability sequence in these channels follows the affinity sequence of the binding sites. ClC channels with a I\(^-\) > Cl\(^-\) permeability sequence differ from those with Cl\(^-\) > I\(^-\) in the absolute value of the interaction energy between binding site and anion, not in the selectivity of

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**Fig. 3.** A: graphical illustration of the postulated passage of anions through a ClC channel pore. The hydrated anion dehydrates by binding to a side within the pore, then passes the pore narrowing, binds to a distinct side, and leaves the pore after rehydration. B: primary sequence regions that were identified to determine anion permeation and selectivity in ClC-type chloride channels (17, 21). Transmembrane domains D1/D2 and D6–D12 are shown as blank rectangles, and only the region between D3 and D5 is shown in more detail. Residues, the exchange of which alters pore properties, are shown in white, black, and gray circles. Residues in gray circles denote those accessible to hydrophilic methanethiosulfonate (MTS) reagents from the external solution; those in dark circles are accessible from the internal solution. Residues in open circles represent positions at which substituted cysteines cannot be functionally modified by MTS residues. With D5, nonaccessible residues are not shown. The 3 likely pore-forming regions (P1, P2, and P3) are shown as rectangles with dotted borders. N, NH\(_2\) terminus; C, COOH terminus.
these binding sites. As shown in Fig. 2, a single binding site with such properties is sufficient to endow an anion channel with larger permeability for $I^-$ than for $Cl^-$. This again demonstrates the variability of structural changes that can cause an inversion of the permeability sequence of CIC-type chloride channels.

The selectivity mechanism of anion channels is clearly distinct from that of voltage-gated $K^+$ and $Ca^{2+}$ channels. Compared with the elegance with which these channels perform their extremely challenging task, selection in CIC-type chloride channels appears almost awkward. The reason for such an “imperfection” is clearly that selection among different anions is biologically not important. What could be the physiological importance of anion channels with a $Cl^- > Br^- > I^-$ selectivity sequence compared with others exhibiting the inverse sequence? We do not know as yet, but a possible answer emerges from the above-mentioned selectivity mechanism. As discussed above, the two types of anion channels differ in the absolute value of anion affinity of their ion conduction pathway. Thus it appears possible that channels with $Cl^- > Br^- > I^-$ sequence will be more selective for anions over cations because of the higher electrostatic potentials of their ion conduction pathway. The functional properties of CIC-1 responsible for the large $Cl^-$ conductance in skeletal muscle are in agreement with this hypothesis. CIC-1 has a $Cl^- > I^-$ permeability sequence and exhibits a very small relative cation permeability, as even a small cation conductance at resting potential would drastically alter muscle excitability. Clearly, evaluation of this hypothesis awaits accurate determination of the relative cation permeabilities of various CIC isoforms.

IDENTIFICATION OF MOLECULAR DETERMINANTS OF ION SELECTIVITY IN CIC CHANNELS

Functional studies of the anion selection process of CIC channels provide indirect information about the structural elements involved. We know that CIC channels exhibit weak Eisenman interaction sites that interact with permeating anions with low affinity. The obvious dependence of the anion permeability sequence on $pH_o$ demonstrates that titratable sites are critically involved in anion binding. As all known CIC channels are anion selective, we expect an evolutionarily conserved anion-selective pore that exhibits positively charged side chains protruding into the aqueous ion conduction pathway to create a positive electrostatic potential. CIC channels distinguish among anions by selective binding to low-affinity binding sites, and therefore we expect that the anions do not make intimate contact with structures within the pore wall. AllostERIC and other long-range effects of point mutations can have considerable influence on the interaction between the channel and permeant anions and thus on the selectivity and single-channel current amplitude. These properties pose a particular problem for approaches using a combination of site-directed mutagenesis and cellular electrophysiology to identify pore residues. The effect of a single amino acid exchange on ion conduction properties (16, 41, 48, 64) does not define this region as contributing to the formation of the ion conduction pathway, and special care has to be taken in defining pore-forming regions using such an approach.

There are nevertheless certain regions within the CIC primary sequence that fulfill all the criteria for a selectivity filter and pore. A disease-causing mutation, found in a family with Thomsen’s dominant myotonia (24), resulting in the substitution of a glycine by glutamic acid COOH terminal to the third transmembrane domain (G230E), dramatically alters the ion conduction pattern of hCIC-1 and inverts the anion permeability sequence form $Cl^- > Br^- > I^-$ to $I^- > Br^- > Cl^-$ (16). In the framework of the proposed selectivity mechanism described above, the result suggests that amino acid 230 is close to ion binding sites within the pore. An additional negative charge at this position would weaken the interaction with the permeant anion and increase the off-rate constant. This could explain the higher permeability of larger and polyatomic anions. It would make the electrostatic potential within the pore less positive, thus accounting for the observed increase in cation permeability. G230 is part of a highly conserved eight-amino acid stretch present in every CIC channel identified (GKxGpxxH) (21) (Fig. 3B), and such an evolutionary conservation is expected for a region that represents a core structural element of the pore of CIC channels. We tested this hypothesis by a combination of cellular electrophysiology, site-directed mutagenesis, and chemical modification. Every amino acid substitution made within this region affects the anion selectivity of hCIC-1 (21). In two of the eight positions (231 and 237), there is a clear dependence between substituted charge and anion selectivity (21). Such an association implies a close interaction between the side chain and the permeating anion and suggests that these two amino acid side chains, K231 and H237, protrude into the ionic binding sites (Fig. 3B). Of eight single substituted cysteines in the P1 region, three (K231C, P234C, and H237C) react with methanethiosulfonate (MTS)-reagents MTS-ethyltrimethylammonium (MTSET) and MTS-ethylsulfonate (MTSES), resulting in channel block (21). K231C is only reactive to extracellular MTS reagents, whereas P234C and H237C only react with intracellular reagents. As a short stretch of three amino acids cannot span the entire lipid bilayer, this result is only possible if all three cysteines protrude into an aqueous pore that connects the extra- and intracellular medium. MTS ethylammonium (MTSEA), which has the same charge as MTSET, did not result in channel block but in a change of channel gating and selectivity. Both MTSET (positively charged) and MTSES (negatively charged) have a diameter of 6Å (which is larger than that of MTSEA and the estimated minimum pore diameter of hCIC-1), and they effectively block current flow. These results imply that the P1 region lines the narrowest part of the CIC pore.
The P1 region is responsible for the high anion-to-cation selectivity of hClC-1 (21). Several mutations in the first four residues of the P1 region (GKEG in hClC-1) change the cation permeability. Estimating the electrostatic potentials near substituted cysteines by comparing the reaction rate with MTSES and MTSET (7) reveals that the cavity formed by the P1 region is inherently anion selective. Because two of these cysteine substitutions (K231C and H237C) remove a positive charge, the electrostatic potential in the WT hClC-1 will be even more positive and could be fully responsible for the low cation permeability of CIC channels (21). The GKEG motif within the P1 region appears to be of special importance for ion selectivity in CIC channels. Several results indicate that K231 projects into the ionic pore, and similar approaches suggest that the negatively charged side chain of E232 points away from the ion conduction pathway. Because of the flanking glycine residues, the position of this two-amino acid dipole is flexible and may be affected by interaction with other parts of the CIC sequence. Small positional changes because of alteration of the conformation of interacting protein parts may alter the electrostatic potentials close to the P1 region and account for the observed isoform-specific permeation differences in CIC channels. The two positively charged side chains (K231 and H237) that project into the pore represent likely anion-binding sites. The combination of cationic groups and hydrophobic side-chain and backbone structures in the putative narrow pore can account for the experimentally observed higher binding affinity for large and polyatomic anions (16, 18). Although the P1 region exhibits accessible charged amino acid side chains, it cannot account for the effect of external acidification on anion permeability, as the exposed basic amino acids will not lose their charge at acidic pH. Moreover, it is obvious that this short stretch of amino acids cannot form an ion conduction pathway by itself. Searching for additional pore-forming regions, we focused on highly conserved sequence motifs. Within D5, we have identified another highly conserved amino acid stretch, GVLFSI in CIC-1 (designated as the P2 region) (Fig. 3B). Exchange of single amino acids in P2 affects the anion permeability sequence. Substituted cysteines at certain positions within this motif are accessible to hydrophilic MTS reagents, and that within P2, there is a change of sidedness of MTS modification; i.e., at the NH₂-terminal end, substituted cysteines are accessible from the internal solution, and at the COOH-terminal end, from the external solution. We concluded from these results that this region, together with the P1 region, lines the narrow part of the pore (21) Using similar approaches, we showed that a highly conserved eight-amino acid motif (P3) located in the linker between transmembrane domains D2 and D3 contributes to the formation of a pore vestibule facing the cell interior (17) (Fig. 3B). The role of these motifs has also been demonstrated for other isoforms (22, 63), supporting the notion that they represent evolutionarily conserved pore regions.

To gain insights into the dimensions of the pore, we used cysteine-specific reagents of different sizes (17). The reactivity of MTS-propyltriethylammonium (MTS-PTrEA) and monobromotrimethyl-ammoniobimane (qBBr) with the substituted cysteines at these positions reveals that the pore diameter is wider than 10 Å at the level of K231 (within the P1 region) and G190C (within the P3 region) and that E193 (P3 region) and H237 (P1 region) are located in pore regions that are more restricted. These marked differences in the diameter of pore-forming regions imply that CIC channels exhibit a pore architecture quite similar to that of certain cation channels: a narrow constriction containing major structural determinants of ion selectivity is neighbored by wide vestibules on both sides of the membrane. The result that Cys²³¹ can be modified with large cysteine-specific reagents is astonishing as cysteines at position 231 of both subunits of a functional dimeric hClC-1 channel can form a disulfide bridge (19). These two experimental results suggest that the P1 region is very flexible. Another experimental finding that indicates a flexible pore-forming region is the absence of periodicity in the reactivity of substituted cysteines within P3. Cysteines introduced between positions 189 and 193 are all reactive, and this could be explained by conformational changes of this region (17). Flexible pore regions could explain the experimental results suggesting conformational changes of the hClC-1 ion conduction pathway (18) and could be a structural feature explaining the tight coupling of permeation and gating observed for CIC-0 (48) and CIC-1 (51).

Most probably, there are additional pore regions that remain to be discovered. It is likely that certain regions will remain elusive in mutagenesis experiments as they do not interact with the permeant anions and thus do not contribute to the selectivity process, and for these novel approaches will be necessary.

PORE STOICHIOMETRY

CIC-0 exhibits unique single-channel behavior, with two equally spaced and independently gated conduction states. To account for this finding, Miller (44) postulated that an individual channel exhibits two ion conduction pathways, i.e., a novel double-barreled pore stoichiometry for this channel. In the last few years, several experimental results have been reported that support the idea that a CIC channel consists of two subunits and that each subunit forms an individual ion pore (41, 43, 53). Nevertheless, there are some experimental results that appear to be incompatible with this concept. For hClC-1, three independent experimental lines of evidence support the idea that a major determinant of pore properties, the P1 region functionally interacts with the corresponding region of the other subunit (19) in a way that would be expected if the two P1 regions form a single pore vestibule. This finding was originally interpreted as evidence for a unipore ion conduction pathway in CIC channels (19). One possible explanation that could account for all presently known
experimental results would be two protopores that share a common vestibule, with important structural determinants of ion permeation; another is that the P1 regions simply form adjacent walls of the two protopores. Recently, Ramjesingh and colleagues (50) reported an exciting new finding that could provide a third possibility for resolving this controversy. They showed that ClC-2 channels purified from Sf9 cells form functional channels in a dimeric as well as in a tetrameric subunit stoichiometry. Reconstituted in planar lipid bilayers, tetrameric channels exhibited two conductance levels, and dimeric only a single one.

**SUMMARY AND OUTLOOK**

Work in the last several years has provided the first insights into the function and structural determinants of the ion pore of ClC-type chloride channels. Our understanding of this family of channels is clearly just beginning. Additional experiments, employing functional, molecular, and structural approaches, will be necessary to fully understand the molecular basis of anion permeation. Because of the widespread importance of this channel family in normal and pathological physiology, these results will have a major impact on biology and medicine.

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