

# A DECADE OF CLC CHLORIDE CHANNELS: Structure, Mechanism, and Many Unsettled Questions

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■ **Abstract** CLC-type chloride channels are ubiquitous throughout the biological world. Expressed in nearly every cell type, these proteins have a host of biological functions. With nine distinct homologues known in eukaryotes, the CLCs represent the only molecularly defined family of chloride channels. CLC channels exhibit features of molecular architecture and gating mechanisms unprecedented in other types of ion channels. They form two-pore homodimers, and their voltage-dependence arises not from charged residues in the protein, but rather via coupling of gating to the movement of chloride ions within the pore. Because the functional characteristics of only a few CLC channels have been studied in detail, we are still learning which properties are general to the whole family. New approaches, including structural analyses, will be crucial to an understanding of CLC architecture and function.

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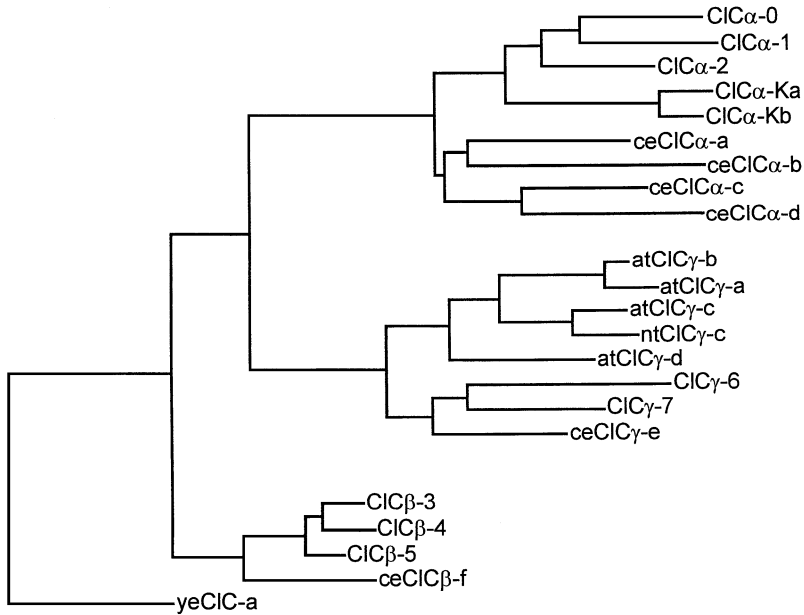
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## INTRODUCTION

Chloride is the most abundant aqueous ion on earth. All living organisms have accordingly evolved membrane transport and ion channel proteins to exploit  $\text{Cl}^-$  toward varied physiological ends. Despite their biological ubiquity,  $\text{Cl}^-$  channels have been relegated to the sidelines in the grand trajectory of ion channel studies of the past fifty years, mainly because their specific biological roles have been, until recently, only vaguely glimpsed. In their seminal discovery, Hodgkin & Huxley (31) described three conductances— $\text{Na}^+$ ,  $\text{K}^+$ , and “leak”—underlying neuronal action potentials. As the research they inspired progressed, complex collections of cellular currents were dissected into components consisting of many  $\text{K}^+$ ,  $\text{Na}^+$ , and  $\text{Ca}^{++}$  channels. These and other cation-conducting ion channels became a central focus for research on the molecular foundations of neurobiology, while the inescapable leak languished as an experimental irritant. It is now appreciated, however, that “leaks” are largely mediated by  $\text{Cl}^-$  channels and that these channels play diverse functional roles, belying their original pejorative label, from regulation of blood pressure, muscle tone, and cell volume to control of synaptic transmission and cellular excitability.

While  $\text{Cl}^-$  currents were recognized electrophysiologically long ago, an understanding of their molecular underpinnings has only recently begun to emerge. The key breakthrough occurred when Jentsch and colleagues (35) cloned a voltage-gated  $\text{Cl}^-$  channel from an electric fish. This result led swiftly to the discovery of an abundant, widespread, and ancient molecular family of  $\text{Cl}^-$  channels (35, 66). These “CIC” genes are found in virtually all organisms, from humans and invertebrates to plants, protists, and prokaryotes. In mammals, nine CIC homologues have been identified, and these fall into three subfamilies (Figure 1). In addition to sequence similarity (30–80% within, ~20% between subfamilies), subfamily members share some general functional features such as anion selectivity, voltage-dependent gating, and macroscopic current rectification.

With some reluctance, we suggest an updated nomenclature for CIC channels from multicellular organisms, which heretofore have been named on an ad hoc basis, mostly by sequential numbering without reference to subfamily. It is awkward and unappealing to refer to the “0/1/2” or “3/4/5” subfamily (Figure 1), and so we propose a supplementary subfamily designation  $\alpha$ ,  $\beta$ ,  $\gamma$ —to be inserted within the current nomenclature; thus, CIC-0 and CIC-6 become  $\text{CIC}\alpha$ -0 and  $\text{CIC}\gamma$ -6, respectively. This naming is timely because it is likely that all CIC subfamilies from higher organisms have now been identified; the three subfamilies encompass



**Figure 1** CIC subfamily organization. A. Phylogenetic tree of selected eukaryotic CIC genes, derived by Clustal analysis of aligned sequences, is shown. The  $\alpha$ ,  $\beta$ ,  $\gamma$  subfamilies are indicated in the nomenclature introduced here. Nonvertebrate genes are annotated with lowercase species prefixes “ce” (*C. elegans*), “ye” (*S. cerevisiae*), “at” (*A. thaliana*), “nt” (*N. tabacum*). B. A table translating old nomenclatures of CIC channels into the naming format used here. The plant channel ntCIC $\gamma$ -c is so classified because it is a close orthologue of atCIC $\gamma$ -c.

all known CIC genes from animals and plants, the latter of which are so far exclusively of the type  $\gamma$  (29, 48). The six CIC channels from the complete *C. elegans* genome fall into all three subfamilies (81). We further suggest that as new orthologues are identified in nonvertebrate organisms, they should in addition be labeled by a species prefix and denoted by letters so as not to be confused with the numeral denotation of the vertebrate CIC genes. CIC channels from unicellular organisms cannot be classified in this way; the single CIC channel in the yeast genome (32) and all prokaryotic CIC genes are too distant in sequence to be shoehorned into the above subfamilies. Table 1 provides a nomenclature translation scheme for all eukaryotic CIC channels reported to date.

The CICs make up the only recognized molecular family of  $\text{Cl}^-$  channels, and several excellent reviews have recently dealt with them from different viewpoints (33, 93). In this review, a recurrent theme will be that CIC channels differ utterly in form and function from “conventional” ion channel proteins such as S4-type voltage-gated channels, neurotransmitter-activated channels, and gap junctions. These familiar channel proteins are all constructed according to a barrel-stave plan in which the ion-conducting pore lies along the axis of symmetry formed by

**TABLE 1** Translation of existing CIC names into proposed nomenclature

	Old name	New name
<i>Vertebrate species</i>		
	C1C-0	C1C $\alpha$ -0
	C1C-1	C1C $\alpha$ -1
	C1C-2	C1C $\alpha$ -2
	C1C-Ka	C1C $\alpha$ -Ka
	C1C-Kb	C1C $\alpha$ -Kb
	C1C-3	C1C $\beta$ -3
	C1C-4	C1C $\beta$ -4
	C1C-5	C1C $\beta$ -5
	C1C-6	C1C $\gamma$ -6
	C1C-7	C1C $\gamma$ -7
<i>Plants</i>		
	atC1C-a	atC1C $\gamma$ -a
	atC1C-b	atC1C $\gamma$ -b
	atC1C-c	atC1C $\gamma$ -c
	atC1C-d	atC1C $\gamma$ -d
	Ntl	ntC1C $\gamma$ -c (orthologue of atC1C-c)
<i>S. cerevisiae</i>		
	GEF-1	yeC1C-a
<i>C. elegans</i>		
	CeC1C-1	ceC1C $\alpha$ -a
	CeC1C-2	ceC1C $\alpha$ -b
	CeC1C-3	ceC1C $\alpha$ -c
	CeC1C-4	ceC1C $\alpha$ -d
	CeC1C-5	ceC1C $\beta$ -f
	CeC1C-6	ceC1C $\gamma$ -g

the conjunction of four, five, or six identical or similar subunits. In contrast, CIC pores are formed from a single subunit, and therefore they must be lined by regions of protein scattered throughout the primary sequence. Also in contrast to conventional channels, the voltage-dependence of gating in CIC channels arises not from movement of charge on the protein but from movement of the permeant ion through the transmembrane electric field. Together, the unique features of the CICs foreshadow an entirely new structure-function paradigm for this family of ion channels, one in which the vast experience gained from mutagenesis and analysis of familiar cation-conducting channels will be of little use in guiding experiments.

## PHYSIOLOGICAL ROLES OF CLC CHANNELS

CIC channels act in many different biological contexts for numerous physiological purposes. Despite their widespread distribution in virtually all cells, or perhaps because of it, the functions of most of these channels remain obscure. In a few

cases, though, we have a good understanding of ClC physiology. Other reviews (33, 93) have discussed this topic in some detail, and our purpose here is only to outline the few examples of known ClC functions.

## Skeletal Muscle Excitability

The most thoroughly studied example of ClC biology is found in mammalian skeletal muscle, where ClC $\alpha$ -1 provides the preponderance of the resting conductance. In contrast to most cells, which are K<sup>+</sup>-selective at rest, vertebrate skeletal muscle is Cl<sup>-</sup> selective, a circumstance that allows it to maintain an unusually high resting potential (−90 mV). The resting conductance to Cl<sup>-</sup> is mediated by ClC $\alpha$ -1, as was originally demonstrated from analyzing the genetic defect in a line of myotonic mice (90). These animals, which along with ClC $\alpha$ -1-defective goats (4, 6) provide models for certain human myotonias (24, 90), have muscle cells that are hyperexcitable because they lack the outward currents (inward Cl<sup>-</sup> flux) carried by ClC $\alpha$ -1. In addition to stabilizing the resting membrane, this channel also provides repolarization current to help terminate the action potential in skeletal muscle; however, this effect is probably small compared to the action of voltage-gated K<sup>+</sup> channels in repolarization, since ClC $\alpha$ -1 is approximately 50% open at the resting potential (18, 74), and consequently Cl<sup>-</sup> conductance can increase only about twofold in response to depolarization.

A similar physiology in a very different cellular context is illustrated by ClC $\alpha$ -0, the first ClC channel to be identified at the molecular level. This is a close homologue of ClC $\alpha$ -1 and is found in the specialized electroplax organ of marine electric rays. The electric organ is laid out as a series of disc-shaped cells piled on top of each other; the cells are polarized, with the negative, innervated side of the disc richly endowed with nicotinic acetylcholine receptors, and the positive side loaded with ClC $\alpha$ -0. As in skeletal muscle, the Cl<sup>-</sup> channel establishes a high-voltage, low-resistance pathway across the resting membrane. When a nerve signal activates the acetylcholine receptors in all the electroplax cells simultaneously, the stacked structure causes the individual cellular voltages (−90 mV for each cell in the stack) generated by ClC $\alpha$ -0 to add in series, generating 50–100 V across the entire organ and producing the fish's lethal jolt. This physiology is actually close to that of skeletal muscle (from which the electroplax is evolutionarily derived); the main difference is the absence of regenerative action potentials in the electroplax. Indeed, skeletal muscles of electric rays express ClC $\alpha$ -0 (34), possibly for the same purpose as ClC $\alpha$ -1 in higher vertebrates.

## Renal and Intravesicular Ion Transport

Investigations of human genetic diseases of blood pressure regulation have led to deeper understanding of two other ClC genes, both involved in renal function. Bartter's syndrome is an inherited disorder of NaCl reabsorption, the salt-concentrating process of the mammalian kidney. Lifton's group (85) has shown that Bartter's is caused by defects in any one of three separate transport proteins involved in salt resorption: the Na-K-Cl cotransporter, a K<sup>+</sup> channel, and a Cl<sup>-</sup>

channel recently identified as  $\text{ClC}\alpha\text{-Kb}$ . A detailed analysis of NaCl transport defects in patients with Bartter's localized the physiological disruption to the thick ascending limb of the loop of Henle. This nephron segment is known to bring about NaCl reuptake (86), with the Na-K-Cl cotransporter and  $\text{K}^+$  channel cohabiting the apical membrane to clear NaCl from the lumen, and the  $\text{Cl}^-$  channel allowing the accumulated cytoplasmic  $\text{Cl}^-$  to flow "downhill" across the basolateral membrane back into the blood. The fact that mutations in any one of these transporter genes indeed cause Bartter's syndrome provides elegant support for this physiological mechanism, even in the absence of direct measurements of  $\text{ClC}$ -mediated  $\text{Cl}^-$  currents in kidney tubules.

The second human renal disease associated with a  $\text{ClC}$  channel is Dent's disease, caused by mutations in the  $\text{ClC}\beta\text{-5}$  gene, which is prominently expressed in kidney (42, 91). Dent's disease is a polymorphic disorder associated with kidney stones, elevated urinary protein, and chronic depletion of phosphate and vitamin C. No unifying physiological principle has been definitively identified to tie these defects together. However, Günther and coworkers (27) noticed that  $\text{ClC}\beta\text{-5}$  is predominantly localized to intracellular vesicles, and they proposed that  $\text{ClC}\beta\text{-5}$  is normally involved in the physiology of endosomal ion transport, in particular intravesicular acidification. This idea makes sense: Many intracellular compartments, including lysosomes and endosomes, are maintained at acidic pH by  $\text{H}^+$ -ATPases, which utilize  $\text{Cl}^-$  conductance to shunt the membrane and thereby allow mass transport of protons (1, 95).

Genetic studies of *Saccharomyces cerevisiae* frequently produce insight into the functions of mammalian genes, and the case of  $\text{ClC}$  channels is no exception. The yeast genome contains a single  $\text{ClC}$  gene ( $\text{yeClC-a}$ ) originally called GEF1, mutants of which are impaired for aerobic growth because of a limitation in Fe transport (25). Gaxiola and coworkers (23) posed the question: Why should a  $\text{Cl}^-$  channel defect lead to a requirement for Fe? They tracked down a rather complex pathway in which defective  $\text{Cl}^-$  conductance inhibits intravesicular acidification as above, and this in turn suppresses the ATP-dependent transport of  $\text{Cu}^{2+}$  into post-golgi vesicles; since Cu is a cofactor for the high-affinity Fe-uptake system, GEF1 mutants require high levels of Fe to grow. Moreover, disruptions of either GEF1, which is localized in golgi membranes, or of the vacuolar  $\text{H}^+$ -ATPase produce similar phenotypes (82). These experiments, along with the rescue of GEF1 mutant phenotypes by  $\text{ClC}$  genes from vertebrates or green plants (23, 29, 56), strongly imply that this yeast  $\text{ClC}$  gene provides a  $\text{Cl}^-$  shunt pathway accompanying proton uptake into intracellular compartments. However, no direct electrophysiological demonstration of this channel's  $\text{Cl}^-$  transport function has been reported.

## Cell Volume Regulation

When exposed to osmotically altered bathing solutions, many cells counteract swelling or shrinkage by dumping KCl or taking up NaCl and thus maintain their

original cell volume.  $\text{Cl}^-$  channels activated or inhibited by hyper- or hypo-osmotic challenge have been observed in numerous cell-types as rate-determining components of this process (59). The molecular identities of these channels have been extremely difficult to deduce, but recent results suggest that at least two CLC channels,  $\text{CLC}\alpha$ -2 and  $\text{CLC}\beta$ -3, may be involved in cell volume regulation.  $\text{CLC}\alpha$ -2 is found ubiquitously in mammalian tissues, and when expressed in *Xenopus* oocytes, this channel induces  $\text{Cl}^-$ -selective currents that increase upon applying hypo-osmotic condition leading to cell swelling (26). The appearance of similar currents in many  $\text{CLC}\alpha$ -2-expressing native tissues suggests by correlation that this is a volume-sensing channel in native tissue. However, the currents observed in physiological preparations differ in detail from those observed with heterologous expression of  $\text{CLC}\alpha$ -2. This raises several possibilities: that the native osmotically sensitive currents are mediated by a different CLC isoform (or an unknown non-CLC channel), that the channels in native systems are functionally modulated by accessory subunits, or that they result from heteromers of different CLC isotypes. The dearth of specific high-affinity blockers for CLC channels has frustrated attempts to convincingly associate native channels with CLC genes, and in fact the very involvement of CLC channels in physiological volume-regulated anion currents remains controversial (60).

$\text{CLC}\beta$ -3 has also been tied to volume regulation. Duan and colleagues (12, 13) observed that guinea pig  $\text{CLC}\beta$ -3 produces an osmosensitive current when expressed in NIH-3T3 cells. As monitored by whole-cell recording,  $\text{CLC}\beta$ -3 induces an outwardly rectifying  $\text{Cl}^-$  current that increases when the cells are exposed to hypotonic solutions and decreases in hypertonic media. Remarkably, phosphorylation of  $\text{CLC}\beta$ -3 by protein kinase C (PKC) is a critical element in the osmotic regulation pathway. Hypotonic exposure leads to inhibition of endogenous PKC and thereby to net dephosphorylation of  $\text{CLC}\beta$ -3, a modification that increases channel activity (13, 37); conversely, hypertonic conditions activate PKC and downregulate the channel. The site of osmoregulatory phosphorylation has been identified: Ser51, located in the cytoplasmic N-terminal domain of  $\text{CLC}\beta$ -3 (12). These results provide a real breakthrough in dissecting the molecular foundations of volume regulation, which has been a persistently murky area of investigation (10). Nevertheless, even in this case there are major differences between volume regulated currents in native tissues and those arising from heterologous expression of  $\text{CLC}\beta$ -3 (93).

## Control of GABA-ergic Neurons

An important and novel neurophysiological function has been proposed for  $\text{CLC}\alpha$ -2 in central neurons: to ensure that  $\text{GABA}_A$  receptors produce the classic inhibitory response. Cytoplasmic  $\text{Cl}^-$  concentrations are maintained differently in different types of neurons. In some,  $\text{Cl}^-$  is actively accumulated in the cytoplasm, thereby establishing an equilibrium potential ( $E_{\text{Cl}}$ ) positive to the cell's resting potential. Other neurons are constitutively more permeable to  $\text{Cl}^-$  and have

passively distributed  $\text{Cl}^-$  and resting potentials closer to  $E_{\text{Cl}}$ . These differences in  $\text{Cl}^-$  handling can lead to dramatic differences in GABA synaptic physiology. In cells where  $E_{\text{Cl}} \sim V_{\text{rest}}$ , GABA<sub>A</sub> receptors ( $\text{Cl}^-$  channels themselves) are inhibitory; their activation stabilizes the resting potential and counteracts action potential generation. In contrast, in cells of low resting  $\text{Cl}^-$  conductance and high  $\text{Cl}^-$  content, GABA<sub>A</sub> receptors are excitatory (62). GABA-inhibitory neurons often contain a hyperpolarization-activated  $\text{Cl}^-$  current, which provides the relevant  $\text{Cl}^-$  conductance near the resting potential (89). Staley's group (87) noticed that this current is similar to heterologously expressed  $\text{ClC}\alpha$ -2. They demonstrated that  $\text{ClC}\alpha$ -2 is expressed in neurons with inhibitory GABA<sub>A</sub> responses, but not in those with excitatory GABA<sub>A</sub> responses; accordingly they proposed that  $\text{ClC}\alpha$ -2 expression provides a key molecular control on the qualitative character of a given neuron's GABA<sub>A</sub> response. Staley and colleagues (88) elegantly tested this hypothesis by genetically introducing  $\text{ClC}\alpha$ -2 into rat dorsal root ganglion neurons, which lack the hyperpolarization-activated  $\text{Cl}^-$  current and normally show an excitatory GABA<sub>A</sub> response. This maneuver resulted in the appearance of the hyperpolarization-activated  $\text{Cl}^-$  current and concomitantly converted the cells into GABA-inhibitory neurons. The combination of in situ and heterologous expression results thus makes a convincing case for this proposed function of  $\text{ClC}\alpha$ -2 in GABA-ergic neurons.

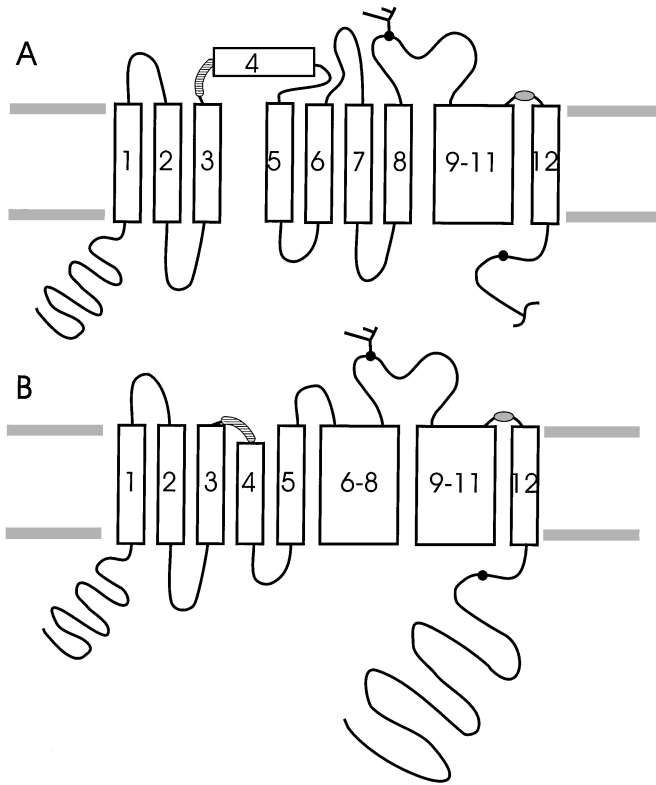
## MOLECULAR ARCHITECTURE OF $\text{ClC}$ CHANNELS

### Transmembrane Topology

The first glimpse of  $\text{ClC}$  primary sequence provided by the cloning of  $\text{ClC}\alpha$ -0 (35) revealed a protein of roughly 800 residues with 12-13 transmembrane helical stretches, D1-D13, a feature present in all eukaryotic orthologues subsequently discovered (Figure 2). The following topological characteristics are definitively known from work on the  $\text{ClC}\alpha$  subfamily. The N- and C-termini are both cytoplasmic (26, 50), as is the residue (K519 in  $\text{ClC}\alpha$ -0) just following D12 (52); the D8-D9 loop carrying the single N-linked glycosylation site is extracellular (51), and D13 is part of the cytoplasmic C-terminal domain, not a transmembrane sequence (26, 36, 50). The extracellular location of the D8-D9 loop presents a conundrum as to the exact number of transmembrane crossings, since it constrains the polypeptide chain to cross the membrane an odd number of times from D1 to D8 and from D9 to D12; in each of these regions, therefore, the number of actual membrane crossings is probably one less than the number of transmembrane sequences assigned by hydrophathy analysis. All eukaryotic  $\text{ClC}$  channels so far examined contain in the C-terminal region two copies of a CBS domain, a ~50-residue sequence motif of known structure but unknown function originally recognized in various globular proteins (63).

By 1997, a consensus (Figure 2) had been reached for  $\text{ClC}$  transmembrane topology on the basis of the above facts, combined with a determined assault on





**Figure 2** Alternative views of CLC transmembrane topology. The figure presents (A) Schmidt-Rose & Jentsch (79) and (B) Fahlke et al (20) models of CLC topology, with extracellular side up. Definitely established positions that are not in contention are explicitly indicated. Residues N365 and K519 (dark circles, CLC $\alpha$ -0 numbering) are singled out as solidly established in membrane sidedness, as is the conserved EXT sequence in the D11-D12 linker (shaded patch). The striped patch near D4 represents the conserved sequence GKEGP, which has been located near the center of twofold symmetry of homodimeric CLC $\alpha$ -1.

the problem by glycosylation-mapping, protease-protection, and cysteine-modification studies (79). However, additional data have recently called this picture into question in the region around D4 and D5. In particular, Fahlke and colleagues (20) showed that a cysteine substitution at the C-terminal end of D5 could be modified by a thiol-reactive reagent only when the reagent was added to the external side of the membrane. This result starkly clashes with Schmidt-Rose & Jentsch's (79) demonstration that an asparagine residue engineered in the D4-D5 loop can be glycosylated in truncated constructs. We do not know how to reconcile the straightforward interpretations of these two results, but they cannot both be correct, at least when extended to wild-type CLC channels. Until this

question is resolved, our view of CIC transmembrane topology will remain seriously blurred.

## Quaternary Structure

In contrast to the prevailing uncertainty over transmembrane topology, CIC quaternary structure is not in contention; all CIC channels examined are dimeric. Indeed, quaternary structure is one of the few properties that has been studied in widely divergent CIC family members. The effects of dominant negative mutations indicated early on that CIC $\alpha$ -1 is multimeric (24, 90), and sedimentation studies on CIC $\alpha$ -0 and CIC $\alpha$ -1 suggested that these channels are homodimers (16, 51). Examination of single CIC $\alpha$ -0 channels formed by co-expression of wild-type and point mutants with altered unitary conductance definitively confirmed this channel's dimeric architecture (46, 52).

Recently, an *E. coli* CIC homologue—the product of the yadQ gene only 15% identical in sequence to any eukaryotic CIC channel—was overexpressed and purified (49). Functional reconstitution has been so far limited to Cl<sup>-</sup> fluxes in liposomes, but even in the absence of direct electrical recordings it is clear that the protein acts as a Cl<sup>-</sup> channel with ion selectivity properties expected from the eukaryotic homologues. The functional channel is readily produced in large quantities (1–5 mg/L culture) and thus is amenable to protein-level biochemical analysis. Three complementary experimental techniques—chemical cross-linking, gel filtration, and analytical ultracentrifugation—confirmed the dimeric structure of this prokaryotic CIC channel. Since this result applies to a CIC channel so distant from the eukaryotic homologues, it suggests that dimeric architecture is a general hallmark of all CIC channels and not merely an idiosyncrasy of CIC $\alpha$ -0 and CIC $\alpha$ -1.

## BASIC FUNCTIONAL PROPERTIES OF CLC CHANNELS

### Anion Permeation

The prominent functional characteristics of the S4 family of Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup> channels can be succinctly stated: strongly voltage-dependent opening upon depolarization, and specific selectivity for the appropriate cation. (Cyclic nucleotide gated channels, however, lack strong voltage dependence although they are formally within the S4 family.) Likewise, the defining properties of classic neurotransmitter-activated channels are easily summarized: activation by binding of multiple ligands, desensitization upon sustained application of neurotransmitters, and ionic selectivity on the basis of electrical valence. In contrast, only a single functional property is common to all CIC channels studied so far: strong selectivity for inorganic anions. All CIC channels studied selectively conduct Cl<sup>-</sup>, Br<sup>-</sup>, and NO<sub>3</sub><sup>-</sup>, and some are also permeable to the low-conductance blockers I<sup>-</sup> and SCN<sup>-</sup>. No universal “signature” ion selectivity sequence is known, possibly

because no single set of ionic conditions has been used for selectivity measurements on all the ClC channels. This situation is unhelpful to the electrophysiologist hoping to identify the molecular identity of a cellular Cl<sup>-</sup> current, especially since many anion currents have been described—some the products of known non-ClC genes, and some molecularly unidentified—and all of them display roughly similar interanionic selectivities.

A careful and extensive examination of ClC selectivity (75) shows that ClC $\alpha$ -1 is not a simple anion sieve. Although interanionic permeability values (measured by macroscopic reversal potential) generally decrease with ion size, there are striking exceptions; ClO<sub>4</sub><sup>-</sup> and SCN<sup>-</sup> are more permeant than Br<sup>-</sup>, NO<sub>3</sub><sup>-</sup>, or ClO<sub>3</sub><sup>-</sup>, and hydrophobic acids like benzoate and hexanoate are more permeant than much smaller anions BrO<sub>3</sub><sup>-</sup>, formate, and bicarbonate. If the “anomalous” permeabilities of the larger hydrophobic anions are ignored, the size-dependence of permeability shows a cutoff at about 5 Å ionic diameter, similar to the values for the anion-selective GABA<sub>A</sub> and glycine receptor channels (5). In other ion channels, such size cutoffs have been taken as empirical measures of the narrowest constriction along the conduction pathway (30), and this has now been directly verified in the case of a bacterial K<sup>+</sup> channel (11).

Very little is understood about the actual mechanism of anion permeation and selectivity of ClC channels. Our ignorance of this most basic process is mainly the consequence of a technical problem: the difficulty of making direct measurements of anion permeation through ClC channels. Since ion permeation is so tightly coupled to gating (see below), interpretation of the effects of ion substitution on macroscopic currents are nearly always ambiguous. In principle, this ambiguity can be resolved by direct single-channel studies of permeation, but because of the small conductances of ClC channels, very few such studies have been made. Early work (94) on the variation of single-channel conductance with Cl<sup>-</sup> concentration showed that ClC $\alpha$ -0 saturates in a simple Michaelis-Menten fashion, as expected for a channel that can accommodate only one Cl<sup>-</sup> ion at a time. More recently, experiments showing complex dependence of macroscopic currents in ionic mixtures led to proposals that ClC pores allow two ions simultaneous occupancy (15, 69).

## Voltage-Dependent Activation

The most thoroughly studied ClC channels—ClC $\alpha$ -0 and ClC $\alpha$ -1—are voltage dependent. They display fast activation gating upon depolarization, with weak voltage-sensitivity (e-fold increase in opening for ~25 mV, five- to tenfold weaker than in S4-type channels). The position of the voltage-activation curve, i.e., the set-point along the voltage axis, is strongly modulated by cytoplasmic pH and extracellular Cl<sup>-</sup> (9, 28, 69, 74), for physiological reasons that remain completely unknown. ClC $\alpha$ -2 gating is also voltage-dependent, but with polarity opposite of that of ClC $\alpha$ -0 and ClC $\alpha$ -1 (92). Remarkably, the voltage dependence of ClC $\alpha$ -0 and ClC $\alpha$ -1 is “plastic,” with many mutations of these channels producing reversed-polarity gating (19, 44, 45, 50). All three mammalian ClC $\beta$  channels

show outward rectification of macroscopic currents (13, 22), but it is not known how much of this reflects voltage-dependent gating and how much open-channel permeation. No mammalian members of the  $\text{ClC}\gamma$  subfamily have been functionally expressed, so their electrophysiological properties are unknown. However, strong inward rectification was observed in  $\text{ntClC}\gamma\text{-c}$ , a  $\gamma$ -subfamily channel from a green plant, tobacco (48).

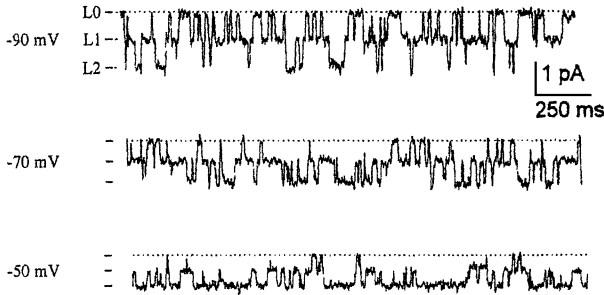
## Inactivation Gating

Upon maintained depolarization,  $\text{ClC}\alpha\text{-0}$  inactivates. Qualitatively, this gating process is similar to inactivation observed in many voltage-dependent channels, but there are differences in detail that warrant discussion. Inactivation of  $\text{ClC}\alpha\text{-0}$  is extremely slow (10–100 sec timescale) and astonishingly temperature-dependent, with a 10-degree temperature increase leading to a 40-fold rate enhancement (8, 21, 53, 68). The biological purpose of inactivation—if there is one—is unknown. Like fast activation, inactivation is favored by depolarization, but the set-point of the equilibrium inactivation curve is about 60 mV displaced in the positive direction along the voltage axis from the activation curve. For this reason, there is a wide voltage window in which the channel is often open at equilibrium, in contrast to S4-type channels, where inactivation curves typically lie negative to the activation curve, and the open state is in consequence only transient. At this time, it is not known whether inactivation is intrinsically voltage-dependent, or whether its voltage dependence arises from coupling to activation gating, as in some S4 channels. Like activation, inactivation is also modulated by cytoplasmic pH and extracellular  $\text{Cl}^-$  (9, 54, 67).

The mechanism of inactivation is unknown, and there is no reason to suppose that a  $\text{K}^+$  channel-like ball-and-chain mechanism (97) is involved. Extensive chimera studies aiming to localize determinants of inactivation in the  $\text{ClC}\alpha\text{-0}$  sequence showed that the process can be affected by manipulations of numerous regions (21), much like C-type inactivation in  $\text{K}^+$  channels. Recently Chen (8), following up on the known “block” of  $\text{ClC}$  channels by transition metal cations (39), showed that extracellular  $\text{Zn}^{2+}$  or  $\text{Cd}^{2+}$  inhibits  $\text{ClC}\alpha\text{-0}$  by promoting inactivation, not by pore plugging, most likely by coordination with a cysteine residue. Chen’s group went on to locate a cysteine in D5, C212, whose replacement by serine eliminates all traces of inactivation without affecting activation gating (41). The mechanism by which C212 exerts its influence on inactivation is unknown.

## Single-Channel Gating Behavior of $\text{ClC}\alpha\text{-0}$

General mechanisms of  $\text{ClC}$  gating remain obscure for a simple reason: the scarcity of information at the single-channel level. Most  $\text{ClC}$  channels that have been functionally expressed are very low in conductance (<5 pS) and therefore inaccessible to detailed microscopic examination (70, 75). The only exception is  $\text{ClC}\alpha\text{-0}$ , which is large enough (~10 pS) to allow such measurements. For this reason, nearly everything we know about single  $\text{ClC}$  channels comes from  $\text{ClC}\alpha\text{-0}$ , and



**Figure 3** Fast activation gating in  $\text{ClC}\alpha\text{-0}$ . A single inactivation-removed  $\text{ClC}\alpha\text{-0}$  channel is shown in a *Xenopus* oocyte membrane patch. The three substate levels are labeled, and opening is downward. Data taken from Lin et al (41).

it is uncertain whether the conclusions reached for this one channel apply to the entire molecular family. Nevertheless, it is worth reviewing the single-channel behavior of  $\text{ClC}\alpha\text{-0}$ , which has provided unique insight into both gating mechanism and molecular structure.

In Figure 3, records of a single inactivation-removed  $\text{ClC}\alpha\text{-0}$  channel are shown at several voltages. The striking feature of these recordings is their multistate character. Simple inspection shows the channel fluctuating among three levels of current: a nonconducting level (L0) and two conducting levels (L1, L2), of which one appears to be about twice the current of the other. At hyperpolarized voltages, transitions among the substates are frequent; at more depolarized voltages, the channel spends more of its time in L2, and excursions into L0 and L1 become short-lived and less frequent. This is the microscopic manifestation of  $\text{ClC}\alpha\text{-0}$  activation gating: a depolarization-promoted shift from the low to the high substates. The channel's open probability levels off at a nonzero value at very hyperpolarized voltages (9), as is also seen in  $\text{ClC}\alpha\text{-1}$  (18). Not shown in this figure is the single-channel symptom of inactivation at depolarized voltages: long-lived nonconducting intervals that segment the channel record into "bursts" of activity (53). Recently, in a series of heroic experiments (77), single  $\text{ClC}\alpha\text{-1}$  channels were observed directly. Although at 1 pS the channels are much lower in conductance than  $\text{ClC}\alpha\text{-0}$ , the qualitative features of  $\text{ClC}\alpha\text{-0}$  are all present in  $\text{ClC}\alpha\text{-1}$ : three substates involved in voltage-dependent activation gating and sojourns into a longer-lived nonconducting state analogous to the inactivated intervals of  $\text{ClC}\alpha\text{-0}$ .

A great deal of discussion and controversy has been expended on the underlying molecular meaning of the  $\text{ClC}\alpha\text{-0}$  (and now  $\text{ClC}\alpha\text{-1}$ ) substates. On one side (2, 53), the substates are seen as a direct reflection of an unusual molecular architecture: a double-barreled construction in which the functional channel carries two identical  $\text{Cl}^-$  conduction pores that gate independently on the fast timescale of activation, but are coupled together in an obligatory dimeric complex. These two pores inactivate via a slower "common gate" that occludes or exposes both pores simultaneously. On the other side (17), the substates represent multiple conformations of a single

pore. Since this controversy is so fundamental to all molecular understanding of these channels, we review below the experimental evidence on which the two views of CIC proteins are based.

## Experimental Support for Double-Barreled Architecture

To recapitulate the fundamental fact: CIC $\alpha$ -0 shows multistate bursting behavior. When the channel leaves the long-lived inactivated state, it engages in a burst of transitions among three substates, L0, L1, L2, distinguishable by their different conductances (0,  $\sim$ 10, and  $\sim$ 20 pS, respectively, in symmetrical 200 mM Cl $^-$ ). There are three experimental lines of evidence that these substates reflect the independent random opening and closing of two separate, distinct, and identical Cl $^-$  diffusion pores.

1. Equally spaced conductance. An obvious property of CIC $\alpha$ -0 substates is their equal spacing in the single-channel record, a feature observed with native Torpedo membranes, heterologous expression systems, and the purified protein (2, 28, 51, 53). L0 is nonconducting, and L2 is twice the conductance of L1 to a precision better than  $\pm$ 2%. This twofold ratio holds across a 250 mV voltage span and under diverse ionic conditions, where absolute single-channel currents vary nearly tenfold. Moreover, the Cl-Br-selectivity and the blocking potency of SCN $^-$  are identical in L1 and L2. These properties are obviously demanded of a double-barreled channel with independent permeation pores. They have never been observed together in single-pore channels showing substates. In cyclic nucleotide-gated channels, for example, in which different protonation states of a single pore give rise to three substates, the conductances are unequal (58, 72), and the spacing depends strongly on ionic conditions (M Root, R MacKinnon, unpublished results). Likewise, Kv channel substates, which reveal partially open conformations along the voltage-activation pathway, are unequally spaced in conductance and display different ion selectivities (98).
2. Binomial gating. Another remarkable property arguing for two independent, identical structures in substate gating is strict adherence to a binomial distribution (2, 9, 28, 41, 53). As long as the inactivated states are long-lived enough to be unambiguously distinguishable from the brief closed states, the probabilities,  $f_i$ , of substate appearance can be predicted without any adjustable parameters:

$$f_0 = (1 - p_0)^2 \quad f_1 = 2 p_0(1 - p_0) \quad f_2 = p_0^2$$

where  $p_0$  is the fundamental open probability obtained measured directly from the integrated channel record. In addition to this equilibrium behavior, the kinetics of the three substates are tightly constrained by the double-barreled channel assumptions. Specifically, the time constants of

the single-exponential dwell-time distributions of the three substates are obligatorily related to each other:

$$2/\tau_1 = 1/\tau_0 + 1/\tau_2 \quad \tau_2/\tau_0 = p_0/(1 - p_0)$$

The fact that these quantitative relations have all been repeatedly confirmed (2, 9, 28, 41, 47, 51, 53) over a wide set of conditions is a powerful argument for any model invoking independence and equivalence. In the cyclic nucleotide-gated channel, a binomial distribution of substates was observed (72), and this result argued compellingly for the equivalence and independence of the substate-generating mechanism, in that case two protonation reactions in a single pore.

A further experimental feature of substate gating is demanded of this picture: forbidden transitions between L0 and L2. In terms of the binomial model, this is a simple consequence of the fact that the closing of two open pores requires two independent events, which cannot happen at exactly the same time. This predicted feature of ClC $\alpha$ -0 gating has also been confirmed (53). Forbidden transitions like this are consistent with multistate models in single-pore channels, but they are not required of them; for example, in a K<sup>+</sup> channel from sarcoplasmic reticulum, all possible transitions among the three conductance levels were observed (40).

3. Independent behaviors of the substates. Another argument that the substates represent separate pores, each with its own activation gate, is based on experiments in which the substates are independently manipulated. There are now four such examples. First, the effect of DIDS, an irreversible inhibitor of ClC $\alpha$ -0, was examined at the single-channel level (55). The reagent caused disappearance of the channel in a two-hit process, each hit apparently occurring on a separate pore. The first hit converted the three-substate bursts into conventional two-state open-closed channel gating, as in a single-pore channel; the second hit eliminated all channel activity. Moreover, the putative single-barreled channel gating in the interval after the first hit but before the second was quantitatively as predicted from the binomial kinetics observed before inhibitor was added. These facts are easy to understand in terms of two independent gating entities, each inhibited separately by DIDS, but would be difficult to reconcile with a single pore fluctuating among multiple conducting conformations.

Second, point mutations were made at various positions that influence single-channel conductance, and mixed-subunit channels were examined (46, 52). Heterodimers containing one wild-type and one mutant subunit display a striking new property: bursts with four substates instead of the usual three. This result is demanded by the double-barreled picture, since in this case L1 loses the degeneracy of the homodimer: the value of L1 conductance now depends on which of the pores is open—the wild-type or

mutant pore. Most importantly, the values of the single-pore conductances of the heterodimers are identical to those in the “parental” homodimers; again, the individual pores behave independently of one another. This kind of behavior is nearly incomprehensible in terms of a single pore.

Third, a pore-counting experiment was performed using a chemically reactive cysteine residue substituted at a position that electrostatically influences single-channel conductance (52). While recording the single channel, the cysteine was modified with a positively charged thiosulfonate reagent, a maneuver that places a lysine-like moiety at this position. The experiment demonstrated conversion of a low-conductance “double-cysteine” channel to a high-conductance “double lysine-like” channel in exactly two chemical steps, one acting on each substate.

Finally, heterodimeric channels were constructed from parental channels with different activation-gating properties (47). In this case, the substate behavior was no longer binomial; instead, the gating could be quantitatively modeled by two nonequivalent, but independently gating pores, each with gating characteristics observed in the parental homodimers. None of these results rigorously rules out single-pore construction for  $\text{ClC}\alpha\text{-0}$ . Only direct structure determination can do that. However, these independent lines of evidence force upon any single-pore model contortions so extreme as to cast it into great doubt. In contrast, all of the results cited are not only interpretable in terms of a double-pore structure of the channel dimer, they are required of it a priori. It is this feature of prediction before the experiment that makes the case for a double-barreled construction of  $\text{ClC}\alpha\text{-0}$  so compelling.

## Experimental Support for Single-Pore Architecture

The usual explanation of substates in a single-channel record invokes multiple conformations of a single pore. This idea is well supported for numerous cases of substate behavior in channels known to consist of a single pore: voltage-gated and  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels (7, 43, 98),  $\text{Na}^+$  and  $\text{Ca}^{2+}$  channels (38, 65, 78), cyclic nucleotide-gated channels (72, 73), and NMDA receptors (64). Fahlke and colleagues (17) recently argued that  $\text{ClC}\alpha\text{-1}$  is a homodimer containing a single pore. This conclusion is based on a series of experiments employing cysteine replacements in the vicinity of transmembrane sequence D4. The experiments first argued that D4 lines a major part of the anion conduction pathway (20), since MTS reagents applied to channels cysteine-substituted in this area cause inhibition of macroscopic currents, depending on which side of the membrane the reagent is added. With the stage thus set, MTS inhibition at several such positions was carefully compared in tandem homo- and heterodimers. If the cysteine side chains project into two separate and independent pores, they should react independently with MTS reagents and give additive effects of modification; on the other hand, if the two side chains project into a single pore, strong interactions between them might be expected.



Several examples of such strong interactions were indeed observed. The tandem homodimer K231C-K231C, for example, is inhibited by externally applied reagent at rates >20-fold higher than the single-cysteine heterodimer K231C-K231A. Moreover, the final extent of inhibition (~75%) is identical in the two constructs; this result is simply inconsistent with the idea that the cysteine side chains project into separate and independent pores because in that case, the heterodimeric channel should suffer only half the inhibition of the homodimer. Another dramatic experiment demonstrated disulfide cross-linking between the two K231C residues. With this cysteine-substituted channel, current was virtually abolished by mild oxidation conditions, and inhibition was reversed by DTT; in contrast, the tandem C-A heterodimer was insensitive to the same oxidation conditions. This experiment implies forcefully that a disulfide bridge forms between the two 231C residues. Again, the results are harmonious with a link between the residues across a single pore, but they clash with a double-barreled picture in which the two cysteines project into separate pores distant enough to act independently in ion permeation. Further indicators of cysteine-cysteine interaction—MTS reactivity and  $\text{Cd}^{2+}$  block—were presented at several other positions in D4.

These experiments were taken to show that  $\text{ClC}\alpha$ -1 does not adopt a double-barreled structure (17). Instead, the side chains of these residues were proposed to line a single pore in the homodimeric complex. According to this picture, the pore of this twofold symmetric homodimer would lie on the unique axis of symmetry, as in conventional channel architecture.  $\text{ClC}\alpha$ -0 would have to be built likewise, given its close similarity to  $\text{ClC}\alpha$ -1 in both sequence and function.

## What Does It All Mean?

How can we reconcile these two fundamentally different pictures of  $\text{ClC}$  channel structure? The single-channel properties of  $\text{ClC}\alpha$ -0 outlined above, and their recapitulation in  $\text{ClC}\alpha$ -1, point inexorably to two  $\text{Cl}^-$ -permeation pathways operating in parallel, at least for the  $\text{ClC}\alpha$  subfamily. But the strong interactions between the substituted cysteine groups, especially the striking disulfide cross-linking experiment, imply that these D4 residues lie close in space near the twofold symmetry axis of the channel dimer.

These two sets of facts may be straightforwardly reconciled by questioning the central premise leading to the single-pore conclusion: that the D4 cysteine side chains project into the  $\text{Cl}^-$  conduction pathway. The experimental evidence supporting this premise is weak. It is based on a cysteine-scanning study (20) showing inhibition of macroscopic  $\text{ClC}\alpha$ -1 currents upon adding MTS reagents to channels substituted with cysteine in and near the highly conserved “GKEGP” sequence preceding D4. The key argument that these side chains project into the pore is that, in some cases, MTS modification alters selectivity or that cysteine substitution leads to block by  $\text{Cd}^{2+}$ . This is a fallacious argument. In cases with firm structural foundations, channel pores are known to be tightly constructed, and their permeation properties are easily altered by secondary effects of distant molecular manipulations. For instance, in Shaker  $\text{K}^+$  channels, mutations at a residue

in the sixth transmembrane segment produce very large changes in ion selectivity (61), even though this is known, from analogy to the bacterial  $K^+$  channel structure (11), not to be a pore-lining position. (One reason that mutagenesis work on  $K^+$  channels has been so successful in visualizing structure is the standard of extreme caution employed in that field in assigning local effects to point mutations.) Moreover,  $Cd^{2+}$  is known to block the pores of certain cation-conducting channels, but there is neither evidence nor reason to suspect that  $Cd^{2+}$  inhibition of an anion-conducting CIC channel would reflect pore-block; indeed,  $Cd^{2+}$  inhibition of  $CIC\alpha-1$  via gating is more plausible in light of the fact (8) that transition metal cations inhibit  $CIC\alpha-0$  by binding preferentially to a nonconducting conformation (see above), not by plugging the pore.

We suspect that MTS inhibition of the D4 cysteine mutants is a consequence of secondary structural rearrangements, not of chemical modification of side chains lining the anion conduction pore. Certainly, a case for pore-locality of the mutations could not be made in these studies, since changes in gating behavior as profound as a reversal of voltage dependence in some cases occur in these cysteine substitutions (17, 20), as has been seen with many other "permeation mutants" of CIC channels (14, 15, 44, 69). For these reasons, we consider that the cysteine-modification experiments argue only for the location of these residues somewhere near the dimer's axis of twofold symmetry; they imply nothing about the location or character of the pore. In contrast, two-pore architecture naturally accounts for all the experimental results thus far.

So, let us summarize our current opinions on CIC architecture. First, we view  $CIC\alpha$  channels as two-pore, symmetric homodimers. In such a complex, the twin conduction pores must necessarily both be removed from the axis of twofold symmetry, and indeed must be sufficiently distant from each other to permit independent, noninteracting operation (farther, say, than 20 Å, the Debye length at 25 mM ionic strength). We admit to paralysis in choosing between the two mutually exclusive proposals about transmembrane topology in the D4-D5 region; the experimental evidence is fairly split on whether D4 spans the membrane or remains formally on the extracellular side (but perhaps buried within the protein). We accept the contention (17) that the conserved D4 residues are located close to the homodimer's axis of symmetry. To us, this means that this functionally important sequence cannot be directly associated with the pores; instead, we imagine that it is intimately involved (whatever that means!) in inactivation gating, a process known to act on both pores simultaneously. In this view, we would also have to assert that these D4 residues do not act only locally, that mutation leads to global alterations of channel structure and disruption of the channel's linked functions.

The question still remains: Does double-barreled structure apply generally to all CIC channels, or is it a peculiarity of  $CIC\alpha-0$  and  $CIC\alpha-1$ ? Although pore construction would seem to be a property so fundamental to a molecular family as to be general, this is nevertheless a serious and urgent question. Several sightings of single  $CIC\alpha-2$  and  $CIC\beta-3$  channels have been reported in the literature, with records devoid of the double-barreled substate behavior seen with  $CIC\alpha-0$  and  $CIC\alpha-1$ . At this point, however, we remain unconvinced that these

single-channel recordings correspond to the CLC channels claimed. The recordings of the two reports (13, 37) on  $\text{CLC}\beta$ -3 do not resemble each other, and the single channels identified as  $\text{CLC}\alpha$ -2 (83) were not connected to any expressed macroscopic  $\text{CLC}\alpha$ -2 currents. We therefore consider the question about the generality of CLC double-barreled construction to be entirely unresolved and very compelling.

## UNPRECEDENTED GATING MECHANISMS OF CLC CHANNELS

CLC channels present the researcher with a collection of unusual gating behaviors that have been encountered only rarely, if at all, in the huge literature on gating of familiar voltage-dependent and neurotransmitter-activated channels. Because of their novelty and unprecedented character, these phenomena are not yet understood in mechanistic depth, and consequently they pose many fascinating challenges for future work. The three such mechanisms discussed here are (a) the source of gating charge in voltage-dependent CLC channels, (b) coupling of gating to ion permeation, and (c) sensitivity of gating to osmotic conditions.

### Mechanism of Voltage Dependence: Coupling of Gating to Conduction

The fundamental thermodynamic requirement for voltage-dependence of channel gating is that the conformational changes between open and closed states must be linked to the transmembrane movement of electrical charge (84). In electrically excitable membranes, the strong voltage-dependence of  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{K}^+$  channels is achieved mainly by the outward movement of arginine and lysine residues on the fourth transmembrane segment as the channel opens (96). This movement leads by unknown mechanisms to the actual opening of the ion-conduction pore. For classic channels, the processes of gating and ion permeation are independent to a first-order approximation; the gating charge-moving events are similar regardless of the ionic species carrying current through the open channel.

The two voltage-gated CLC channels that have been closely examined— $\text{CLC}\alpha$ -0 and  $\text{CLC}\alpha$ -1—sense transmembrane voltage in strikingly different ways. Here, gating charge is carried by  $\text{Cl}^-$  ion itself, not by charged residues on the protein. The first suggestion for this idea emerged from studies of the effects of external  $\text{Cl}^-$  concentration on  $\text{CLC}\alpha$ -0 gating. Pusch and colleagues (69) showed that an increase in extracellular  $\text{Cl}^-$  opens the channel by shifting the voltage-activation curve to the left (favoring the open state), while minimally affecting its slope. In other words,  $\text{CLC}\alpha$ -0 is a  $\text{Cl}^-$ -activated  $\text{Cl}^-$  channel. They offered the idea that the pore of the closed channel is anion-accessible exclusively from the external solution and that only when  $\text{Cl}^-$  ions occupy the pore can the channel open. Furthermore, the pore-associated activation site was postulated to be located deep within the membrane field, so that binding of external  $\text{Cl}^-$  to that site—and hence channel opening—would be promoted by depolarization. This was

a surprising suggestion, especially in light of previous gating models that invoked a particular carboxyl group on ClC $\alpha$ -1 as the gating charge (19).

Because of the unprecedented nature of this proposal, Pusch and colleagues (69) built a case for direct involvement of pore-associated anions in gating. They showed that only channel-permeant ions, including Br $^-$ , NO $_3^-$ , and (less effectively) I $^-$ , cause this shift in activation. Moreover, they exploited mixtures of permeant ions to correlate a pore property with a gating shift; in Cl $^-$ -NO $_3^-$  mixtures, current through the open channel depends nonmonotonically on the external anion composition, with a minimum at  $\sim$ 30% NO $_3^-$ /70% Cl $^-$ . This “anomalous mole-fraction effect,” usually considered indicative of multi-ion conduction (30), is mirrored by a similar minimum in the voltage of half-maximal activation. These results make a strong argument that anion occupancy of the pore is in some way linked to channel opening, but they do not by themselves finger the permeant anion as the gating charge; for example, Cl $^-$  occupancy might be required only for a conformational change leading to opening, while gating charge is carried by the movement of protein residues, as in a conventional mechanism.

To approach the gating mechanism in more detail, Chen & Miller (9) examined the Cl $^-$  dependence of ClC $\alpha$ -0 activation gating at the single-channel level. Using purified ClC $\alpha$ -0 reconstituted into planar lipid bilayer membranes, they verified that single ClC $\alpha$ -0 channels are strongly activated by external Cl $^-$ . This Cl $^-$  activation results predominantly from an increase in the rate of channel opening, with a much weaker effect on closing rate. Thus, external Cl $^-$  acts upon the not-yet-open channel. Moreover, the study showed that as external Cl $^-$  is reduced toward zero, the channel approaches a state in which it can still open, albeit with very low probability. Most significantly, in this Cl $^-$ -starved condition, channel opening loses nearly all its voltage-dependence. Thus, just as proposed initially (69), Cl $^-$  ion is indeed the gating charge; its movement within pre-open states of the channel confers voltage-dependence to ClC $\alpha$ -0 gating. The number of Cl $^-$  ions involved in channel activation is still uncertain. The small gating charge of  $\sim$ 1 could reflect a single Cl $^-$  ion moving through the entire transmembrane voltage drop, or several ions moving partway. The anomalous mole fraction effects mentioned above would seem to favor the latter possibility, but data on this question are still too sparse for a firm conclusion.

These studies could be carried out only because of the technical feasibility of single-channel studies with ClC $\alpha$ -0 over a wide range of experimental conditions, a capability not yet available with other ClC channels. Nevertheless, it is likely that the gating mechanism of ClC $\alpha$ -1 adheres to the same principles established for ClC $\alpha$ -0. At the macroscopic level, ClC $\alpha$ -1 activation responds to both voltage and Cl $^-$  in ways strongly reminiscent of ClC $\alpha$ -0 (74), and the single-channel behavior is qualitatively similar to that of ClC $\alpha$ -0 in the limited range of conditions so far examined (77).

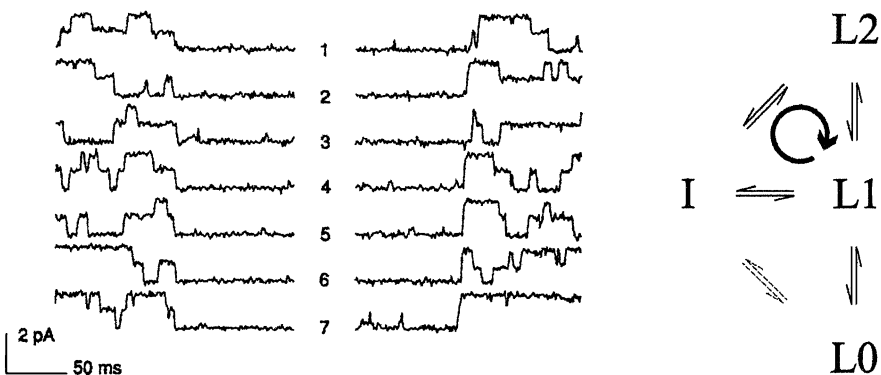
Recently, Rychkov and co-workers (75) extensively examined the effects of anion substitution on ClC $\alpha$ -1 permeation and gating. They studied a broad series of anions and found several distinct classes of behavior: Some anions had minimal

effects on either gating or permeation when substituted for  $\text{Cl}^-$ , while others affected both properties in similar ways. Interestingly, though, a third group of anions including cyclamate and methansulfonate had strong effects on channel gating despite negligible permeability.

### Gating Is a Nonequilibrium Process

One immediate consequence of the above  $\text{Cl}^-$  activation mechanism is that the gating of these channels cannot be at thermodynamic equilibrium. Opening of the channel is inherently coupled to the movement of  $\text{Cl}^-$  ions across the membrane; the free energy of  $\text{Cl}^-$  entering the closed channel from the external solution and leaving the open channel to the internal solution—an irreversible process—is an intrinsic part of the gating reaction. For this reason, the usual conformational equilibrium treatments of gating are inapplicable here. Instead, the kinetic mechanism by which  $\text{Cl}^-$  permeates the pore becomes an inextricable part of any gating model (96), and since details of ClC permeation mechanisms are unknown, satisfactorily quantitative gating models of these channels are a long way off.

Given the mechanism of ClC voltage dependence, the above conclusions about gating irreversibility can be asserted with certainty on thermodynamic grounds alone. But how does nonequilibrium gating show itself experimentally? The distinctive substate behavior of ClC $\alpha$ -0 allows irreversible gating to be observed unambiguously. Because the channel has three distinguishable conductance levels—two open states (L1 and L2) and a long-lived inactivated state (I)—single-channel recordings reveal the life history of the channel as it undergoes state-transitions around a cycle (Figure 4). If channel gating were at thermodynamic equilibrium, then microscopic reversibility would require equal rates of transitions around



**Figure 4** ClC gating violates microscopic reversibility. Left panel: 7 sequential examples of a single ClC $\alpha$ -0 channel (opening upward) entering and leaving the long-lived inactivated state. Right panel: State diagram indicating a clockwise cycle, as in traces 2-7. Data taken from Richard & Miller (71).

the cycle in clockwise and counterclockwise directions. But when Richard & Miller (71) examined these cycles for  $\text{ClC}\alpha\text{-0}$ , they found that in general clockwise cycles ( $\text{L1} \rightarrow \text{I} \rightarrow \text{L2} \rightarrow \text{L1}$ ) predominate over counterclockwise cycles ( $\text{L2} \rightarrow \text{I} \rightarrow \text{L1} \rightarrow \text{L2}$ ). In other words, the channel tends to enter the inactivated state from the low-conductance open state L1, but to leave the inactivated state into the high-conductance state L2. This fact means that channel gating is not at equilibrium; gating must obtain energy from an external source. In the reconstituted system used, the only energy source external to the channel resides in the gradients of transported ions, specifically  $\text{Cl}^-$ . Indeed, the ratio of clockwise to counterclockwise transition rates, a direct measure of external energy input, varies with the magnitude of the  $\text{Cl}^-$  gradient. Other channels are now known to display nonequilibrium gating cycles as well; CFTR channels derive external free energy from ATP hydrolysis coupled to gating (3), and coupling of gating to ion permeation in a mutant NMDA receptor was elegantly modeled by Schneggenburger & Ascher (80).

### Coupling of Gating to Osmotic Conditions

Another novel form of gating has been observed in  $\text{ClC}\alpha\text{-2}$ , one of the  $\text{ClC}$  isoforms implicated in cell volume-regulation. As discussed above, when expressed in *Xenopus* oocytes,  $\text{ClC}\alpha\text{-2}$  currents respond to the osmotic strength of the medium bathing the oocytes—activating in hypotonic media. The pathway by which the channel senses solution tonicity is unknown, but Grunder and colleagues (26) uncovered a remarkable molecular aspect of the phenomenon: that the cytoplasmic N-terminal domain is necessary for osmosensitive gating. When the N-terminus of  $\text{ClC}\alpha\text{-2}$  was replaced with those of either  $\text{ClC}\alpha\text{-0}$  or  $\text{ClC}\alpha\text{-1}$ , basic conduction properties were retained, but osmotic sensitivity was completely lost. Likewise, deletion of 62 N-terminal residues in  $\text{ClC}\alpha\text{-2}$  resulted in a constitutively open channel unresponsive to osmotic conditions. Surprisingly, osmosensitivity could be partially restored by transplanting a large portion of the N-terminal region onto the C-terminal cytoplasmic domain! This unexpected result elicited the suggestion that, in analogy to  $\text{K}^+$  channel inactivation (97), the N-terminal domain acts by a ball-and-chain mechanism, in which the N-terminal “ball” domain occludes the pore, and exposure to low osmotic strength (or downstream consequences of such exposure) removes the ball from its blocking site. This suggestion proceeds from the observation that the osmosensory domain exerts its channel-closing effect regardless of where it resides in the channel sequence, just as the  $\text{K}^+$  channel’s N-terminal peptide plugs the pore regardless of whether it is tethered to the rest of the protein sequence or dissolved free in solution. This logic is understandable, but in our opinion, the absence of any evidence that the  $\text{ClC}\alpha\text{-2}$  N-terminal domain interacts with the channel’s conduction pathway makes this mechanistic proposal premature. We remain similarly skeptical that the cytoplasmic loop connecting D7 and D8 acts as the “receptor” for the N-terminal domain, as proposed from the observation that mutations in this region also abolish osmotically sensitive gating of  $\text{ClC}\alpha\text{-2}$  (36).

## CONCLUSION

We conclude by listing several standing questions about CIC channels. All of these are intrinsically compelling in understanding the unprecedented features of the CIC family.

1. What are the physiological functions of CIC channels?

The ubiquitous expression of CIC channels attests to the many different ends to which cells use these proteins. But except for a small handful of examples, these uses are undocumented. This sparsity of information makes CIC channels attractive targets for investigating the biological consequences of directed gene deletions. The central role of CIC $\alpha$ -0 and CIC $\alpha$ -1 in the excitability of muscle or muscle-derived tissue is firmly established, and CIC $\alpha$ -Kb almost certainly provides a major salt resorption pathway in mammalian kidney. The osmotic sensitivities of CIC $\alpha$ -2 and CIC $\beta$ -3 are clearly established in heterologous expression systems, and it will now be important to discover the physiological contexts in which this unusual property is actually used. The involvement of CIC $\beta$ -5 in acid transport by intracellular vesicles is suggested by recent results; if this role for CIC $\beta$ -5 and perhaps for its close homologue CIC $\beta$ -4 (22) is confirmed by follow-up studies, it is likely to be found well beyond the epithelial setting in which it was first revealed. At this time, we know nothing about the functional properties of any CIC $\gamma$  subfamily members, either in heterologous or endogenous systems. Beyond the simple questions of which CIC channels are “involved in” which functions, we have no information whatever about heterodimeric assembly of CIC homologues, a common means of functional fine-tuning many other types of ion channels.

2. Are all CIC channels double-barreled?

The two-pore property is established in the CIC $\alpha$  subfamily, but we have no information for other CIC channels. From a structure-function standpoint, this question is of obvious importance. There have been several reports of single Cl<sup>-</sup> channels that appear double-barreled, but with conductances much higher than that of CIC $\alpha$ -0 (57, 76); so far, efforts to identify these channels at the molecular level have been fruitless. On the other hand, it will be very important to test the claim (13) that CIC $\beta$ -3 forms a conventional single-barreled channel; verification of this assertion would unequivocally eliminate two-pore construction as a fundamental characteristic of CIC proteins.

3. What do CIC proteins look like?

Until last year, the direct structural investigation of any CIC channel was far out of the realm of possibility. Even the abundantly expressed CIC $\alpha$ -0 could be purified in only minuscule quantities (51). Today's torrent of prokaryotic genome sequences, however, changes this situation dramatically. CIC genes are represented in about half the prokaryotic

genomes examined so far, and overexpression of CIC channels in bacterial expression systems is consequently a plausible experimental goal. Indeed, the first CIC channel for which bacterial overexpression was attempted, the *yadQ* gene of *E. coli*, produced large quantities of pure, functionally active CIC protein. While it is too early to embrace a positive optimism about CIC structure, the dismal feelings of structural hopelessness that have long pervaded the field are no longer warranted. The availability of high-milligram amounts of diverse CIC homologues will soon open the way to protein-level work with an eye toward direct structural information, by solution physical-chemical techniques, spectroscopic probes, and—dare we say it?—crystallization.

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