

## Review

# Diversity of Cl<sup>-</sup> Channels

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**Abstract.** Cl<sup>-</sup> channels are widely found anion pores that are regulated by a variety of signals and that play various roles. On the basis of molecular biologic findings, ligand-gated Cl<sup>-</sup> channels in synapses, cystic fibrosis transmembrane conductors (CFTRs) and ClC channel types have been established, followed by bestrophin and possibly by *tweety*, which encode Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels. The ClC family has been shown to possess a variety of functions, including stabilization of membrane potential, excitation, cell-volume regulation, fluid transport, protein degradation

in endosomal vesicles and possibly cell growth. The molecular structure of Cl<sup>-</sup> channel types varies from 1 to 12 transmembrane segments. By means of computer-based prediction, functional Cl<sup>-</sup> channels have been synthesized artificially, revealing that many possible ion pores are hidden in channel, transporter or unidentified hydrophobic membrane proteins. Thus, novel Cl<sup>-</sup>-conducting pores may be occasionally discovered, and evidence from molecular biologic studies will clarify their physiologic and pathophysiologic roles.

**Key words.** ClC; CFTR; bestrophin; *tweety*; volume regulation; Ca<sup>2+</sup> activation; pharmacology; knockout mouse.

## Introduction

Cl<sup>-</sup> channels are expressed as selective anion pores, have been found in a variety of physiologic preparations and are ubiquitously distributed, including in oocytes. Cl<sup>-</sup> channels allow the passive diffusion of negatively charged ions along electrochemical gradients or the transport of positive charges, and they conduct other anions, such as HCO<sub>3</sub><sup>-</sup>, I<sup>-</sup>, SCN<sup>-</sup> and NO<sub>3</sub><sup>-</sup>. The physiological roles of Cl<sup>-</sup> channels in the plasma membrane and in the vesicular membrane of intracellular organelles were vigorously investigated in the 1980s. Measurement of Cl<sup>-</sup> current in various cells revealed that Cl<sup>-</sup> channel gates were regulated by membrane voltage, cell volume, extracellular

ligands, intracellular ions (such as Ca<sup>2+</sup>, H<sup>+</sup> or anions) or phosphorylation of residues by various protein kinases. Therefore, one method of classification of Cl<sup>-</sup> channels was based on their functions. To summarize the diversity of function, the following list may be conducted on the basis of phenotype *in vivo*: (i) ligand-gated transmission in the post-synaptic membrane, (ii) stabilization of resting membrane potential in (skeletal) muscle, (iii) depolarization of smooth-muscle cells or possibly of retinal pigment epithelium, (iv) cell-volume regulation in various cells, (v) fluid transport in epithelia and (v) neutralization of H<sup>+</sup> ions in lysosomal vesicles. Identification of a molecule usually leads to a breakthrough in elucidating its function.  $\gamma$ -aminobutyric acid (GABA or glycine receptors are composed of heteromeric subunits forming a Cl<sup>-</sup> channel that plays roles in post-synaptic membranes, as mentioned above in

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(i). The most exciting topic in  $\text{Cl}^-$  channel research was the cloning and functional expression of the first member of the CIC family, by Jentsch et al. [1]. Interestingly, mutations in the CIC family have revealed that the CIC family covers functional roles in diverse processes, as mentioned above in (ii) to (vi). Furthermore, the study of mice lacking some CICs has taught us that morphogenesis is an additional role of  $\text{Cl}^-$  channels in the normal development of organs [2]. These aspects of  $\text{Cl}^-$  channels have been extensively discussed in a recent comprehensive review by Jentsch et al. [2]. In addition, Nilius [3–6] has extensively reviewed volume-sensitive and  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels.

One method of classification of channel molecules is based on properties found by electrophysiologic analysis. Expression of CIC has revealed  $\text{Cl}^-$  channels with conductance less than 10 pS. There are, however, three types of  $\text{Cl}^-$  channels, as determined by their conductance *in situ*: small (<10 pS), middle (10–100 pS), and large (>100 pS [maxi- $\text{Cl}^-$ ]) conductance channels. Some  $\text{Cl}^-$  channels have different states of conductance, namely double- or multi-barreled type (table 1). Explanation of the relationship between variable magnitude of conductance and molecular structure is disclosed by extensive studies with mutations in CIC channels [7–9]. The molecular structure of  $\text{Cl}^-$  channels is also diverse. The number of transmembrane segments (TMSs) of  $\text{Cl}^-$  channels is variable; for example, CFTR [10] and CIC [2, 11] have 10 or 12 TMSs. The CICA family, which has 5 TMSs, encodes middle-conductance,  $\text{Ca}^{2+}$ -

activated  $\text{Cl}^-$  (CaC) channels [12, 13]. Newly found CaC channels include bestrophin (the vitelliform macular dystrophy [VMD] protein), which defines a new family of chloride channels [14] with 4 TMSs, and *tweety*, which encodes 5–6 TMSs [15]. The Isk channel encodes 1 TMS, forming a  $\text{Cl}^-$  and  $\text{K}^+$  channel pore expressed in *Xenopus* oocytes [16].

Functional transporters or other channels occasionally allow anion conductance. An example is an amino acid, glutamate transporter expressed in *Xenopus* oocytes, in which a  $\text{Cl}^-$  current has been observed after the binding of glutamate to the transporter [17]. Aquaporin-6 (AQP-6) encodes an  $\text{Hg}^{2+}$ -sensitive water channel that is simultaneously expressed as a unique acid-dependent  $\text{Cl}^-$  channel [18].

Crystal structural analysis performed on CIC channels has shown a unique homodimeric composition [19]. Interestingly, bacterial CIC shows a characteristics of  $\text{Cl}^-/\text{H}^+$  transporters as well as  $\text{Cl}^-$  channels [20]. Such a complex structure-function relation is found only in anion carrying protein, not in cation channels.

On the other hand, functional  $\text{Cl}^-$  channel pores can be artificially designed and synthesized as 20–30 amino acids [21]. These peptides assemble to make a functional pore consisting of a cluster of 4 or 5 molecules. The proposed structure of the peptide shows an  $\alpha$ -helix possessing hydrophilic alignment on one side and a hydrophobic cluster of amino acids on the other [21, 22]. As suggested by studies of peptide channels, construction of a non-selective anion or cation selective pore is easier

Table 1. Single-channel conductance and endogenous  $\text{Cl}^-$  current.

		Refs.
CIC0 (two opening states)	8, 20 pS	2
CIC1	1.5 pS	2
CIC2	2.5 pS	2
CIC3–CIC5	unknown owing to endosomal channels	2
GABAA (multiple states)	12, 17–20 and 27–30 pS	118
Amino acid transporter	0.7 pS	2
CFTR	6–10 pS	119
CLCA1	25–35 pS	
p64 protein	40 pS	120
Glycine receptor (multiple states)	12–14, 18–23, 24–36, 42–49, 59–72, 80–94 and 105–112 pS	2, 25
TTYH3 (two opening states)	260, 50 pS	15
Bestrophin	<6 pS	16
MCLC	70 pS	80
Endogenous current		
<i>Xenopus</i> oocytes	steeply outward-rectified by intracellular Ca	
CHO-K1	steeply outward-rectified	
	weakly outward-rectified by swelling	121
HEK	steeply outward-rectified (large conductance)	122

than construction of a Na<sup>+</sup> or K<sup>+</sup> selective pore. Thus, the Cl<sup>-</sup> channel may be formed occasionally by unrelated clusters of TMSs that form a hydrophilic pore [23].

In this review, explanations of the molecular functions of Cl<sup>-</sup> channels are given, according to the above criteria, and consideration of the Cl<sup>-</sup> channel pore suggests the possibility of the future discovery of novel Cl<sup>-</sup> channels. Discussion of ligand-gated Cl<sup>-</sup> channels is omitted because of detailed reviews elsewhere [24, 25].

## Diversity of Cl<sup>-</sup> channel function

### Ligand-gated transmission in the post-synaptic membrane

There are a number of articles and reviews regarding the ligand-gated Cl<sup>-</sup> channel GABA and glycine receptor [26].

### Stabilization of membrane potential

Excitation of the membrane is generally achieved by an influx of the cations Na<sup>+</sup> and/or Ca<sup>2+</sup>. The subsequent recovery of membrane potential is driven by the efflux of K<sup>+</sup> or by the influx of Cl<sup>-</sup>. The ClC-1 channel is a voltage-dependent type of channel that is a known example of a membrane potential stabilizer and is expressed in skeletal muscle. It has a single-channel conductance of 1 pS and contributes approximately 75% of the resting conductance of the muscle membrane, with a high open probability at negative membrane potential. The ClC-1 channel suppresses depolarizing inputs and stabilizes the membrane potentials [27–29]. Mutations in the ClC-1 channel cause either recessive or dominant congenital myotonia, in which the mutant ClC-1 channels act as dominant negative subunits of the ClC-1 channel pore [30]. Homodimers or heterodimers of mutants with wild-type subunits result in a dramatic shift in voltage dependency. A loss of membrane potential stabilization by the mutant leads to prolonged depolarization of excitable membrane, resulting in a myotonic phenotype. The ClC-2 channel is activated by hyperpolarization, acidic pH and swelling of the cell [31, 32]. Postsynaptic GABA<sub>A</sub> and glycine receptors are ligand-gated Cl<sup>-</sup> channels that yield hyperpolarizing or depolarizing currents, depending on the intracellular Cl<sup>-</sup> concentration [33]. Since the activity of ClC-2 determines intracellular Na<sup>+</sup> and Cl<sup>-</sup> concentration [34], iClC-2 is important in GABA and andglycine-induced hyperpolarization and depolarization. Activation of ClC-2 may help the ligand-gated Cl<sup>-</sup> channels to yield hyperpolarizing currents. ClC-2 is thus assumed to play a role in membrane stabilization with hyperpolarizing transmission by GABA and glycine in neurons. The mapping of an epilepsy susceptibility locus close to the *CLCN2* gene suggested that *Clcn2*<sup>-/-</sup> mice might suffer from spontaneous seizures [35,

36]. However, the lack of ClC-2 in mice has resulted in retinal degeneration or male infertility without a lowered threshold of seizure by chemicals [37]. This is one example of the unexpected physiological role of Cl<sup>-</sup> channels revealed from work with knockout animals.

### Depolarization

In contrast to excitable membrane in which voltage-dependent cation channels play an important role in suppressive depolarization, in non-excitable cells, such as smooth muscle cells, the opening of Cl<sup>-</sup> channels drives the membrane potential V<sub>Cl</sub>, which is approximately -20 mV and which is excitable depolarization. When excitatory ligand binds to smooth-muscle cells, a rise in intracellular Ca<sup>2+</sup> occurs in response to the second messenger. The CaC channel has been recorded in a variety of electrophysiologic preparations and is ubiquitously distributed across all cell types [38], although not always fully evident. In isometric tension recordings, niflumic acid, a blocker of the CaC channel, inhibited spontaneous contractions. The CaC channel has received further attention in the study of secretory, epithelial, smooth-muscle and neuronal cell types, but the properties and phenotypic functions of the CaC channel are not as well characterized as those for Ca<sup>2+</sup>-activated K (CaK) channels [38–40].

CaC and CaK channels are frequently coexpressed and coactivated by elevation of Ca<sup>2+</sup> concentration within the cell. Simultaneous activation of CaC and CaK channels may help stabilize plasma membrane potential. For example, vascular smooth-muscle cells possess both channels: one of the CaK channels is a large-conductance channel, called the maxi-K channel when encoded by *Drosophila slow poke*, but the molecular nature of the CaC channel is not known. Spontaneous transient inward currents (STICs) are associated with the simultaneous opening of a CaC channel after the periodic release of Ca<sup>2+</sup> from intracellular stores. STICs are often co-incident with spontaneous transient outward currents carried by CaK channels [38]. In the absence of functional maxi-K channels when a defect in the b-subunit occurs, smooth-muscle cells tend to be constricted, resulting in high blood pressure [41]. Assuming that the remaining CaC function is exaggerated in the absence of the maxi-K channel, CaC would contribute to excitation of smooth muscle, leading to contractions [38, 41].

Molecular identification of CaC channels is difficult, since CaC channels are usually detected in cells used for expression of exogenous complementary DNA (cDNA). Thus, variable molecules have emerged at candidates of CaC channels. Proposed structures of various candidates of Cl<sup>-</sup> channels are shown in figure 1.

mClCA1 is isolated from airway epithelia expressing a CaC channel current. An extensive comparison has been made between the currents evoked by mClCA1

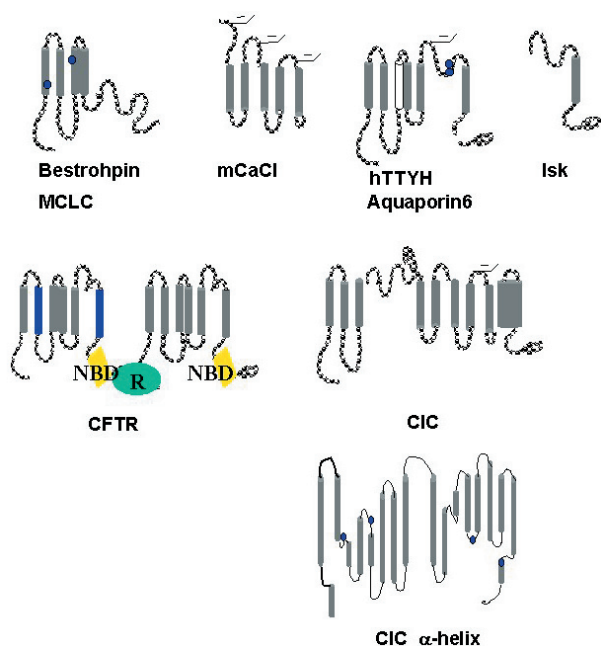


Figure 1. Proposed structures of  $\text{Cl}^-$  channels by their hydrophobicity are illustrated. Bestrophin is a promising molecule encoding  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels with four transmembrane segments (TMSs). mClCa with 5 TMSs and hTTY with 5–6 TMSs and Isk with 1 TMS are illustrated. CFTR encodes 12 TMSs with nucleotide binding domains (NBDs) and a regulatory domain (R). The CIC channel is assumed to be 10–12 TMSs. However, crystallographic analysis suggests that the 18  $\alpha$ -helices of CIC are folded complexes. Closed circles filled with blue indicate positions of amino acids related to anion permeability.

expression and native CaC channel currents in various smooth-muscle cells [12, 13, 42, 43]. Dithiothreitol, a blocker of mCLCA1, had no effect on CaC channels in murine portal vein cells; again, channel kinetics and channel modulation differed. Therefore, most subsequent studies concluded that mCLCA1 alone does not comprise the CaC channel [44] but is a subunit that aids the expression of endogenous CaC.

Proteins encoded by these genes might be involved in tumorigenesis. hCLCA2 is critical for endothelial adhesion of cancer cells as a complex with integrin b4 [45–47]. This is a novel and interesting mechanism because several studies have suggested that  $\text{Cl}^-$  channels play a role in cell growth and apoptosis, as described below.

Recently, molecular identification of (probably small conductance) CaC channels was achieved [14]. VMD (Best disease; MIM 153700) is an early onset, autosomal dominant disorder in which accumulation of lipofuscin-like material within and beneath the retinal pigment epithelia is associated with a progressive loss of central vision. The gene mutated in VMD, *VMD2*, was identified in 1998 [48, 49]. Bestrophin, a VMD gene product, is homologous to at least 3 other proteins within the

human genome, 4 in the *Drosophila* genome and 24 in the *Caenorhabditis elegans* genome. An aberrant electrooculogram is noted when depolarization of retinal pigment epithelial cells is reduced, suggesting that bestrophin encodes a channel contributing to membrane depolarization. Induction of the light peak requires a 'light-peak substance' that is secreted by the neurosensory retina [50]. Transduction of the signal that induces the light peak requires signaling across the retinal pigment epithelial cell from a proposed receptor at the apical surface of the cells to activate one or more  $\text{Cl}^-$  channels in the basolateral plasma membrane of the retinal pigment epithelial cell. Bestrophin is involved in the small- to middle- conductance  $\text{Cl}^-$  channel in the basolateral membrane, contributing to depolarization of the membrane and leading to granule secretion. Bestrophin encodes 4 or 6 TMSs and induces a larger  $\text{Cl}^-$  current in HEK cells after an increase in intracellular  $\text{Ca}^{2+}$  released from the caged compound activated by light. Human, *Drosophila* or nematode bestrophins (1 and 2) have different types of current-voltage relationships [14]. Furthermore, the mutant in VMD showed a dominant negative subunit and coexpression of mutant and wild-type heteromeric bestrophin, which provides less current than the wild-type homopolymer [14]. Although HEK cells still possess endogenous  $\text{Cl}^-$  channels, these results strongly indicate that bestrophin encodes CaC channels. Bestrophin is bound to protein phosphatase 2, and this mechanism is considered to be functionally regulated by phosphorylation/dephosphorylation by this enzyme [51]. Exogenous bestrophin increases the magnitude of the  $\text{Cl}^-$  channel current in HEK cells or in HeLa cells after exposure to hypotonic solution. Bestrophin may encode channels responsible for volume-sensitive  $\text{Cl}^-$  channel current proposed to be <6 pS or may help to express these channels in the retinal pigment epithelial cells [52]. The functional role of CaC *in vivo* will be clarified in future analyses of these molecules.

### Cell-volume regulation

When cells are exposed to hypotonic media, the cells gain volume by an influx of water, and an increase in intracellular Ca becomes evident [53]. This increase leads to loss of KCl via activation of  $\text{Cl}^-$  and  $\text{K}^+$  channels, resulting in a loss of intracellular osmolarity as equilibrium with extracellular hypotonicity is reached. Subsequently, swollen cells recover normal volume (regulatory volume decrease). In early experiments,  $\text{Ca}^{2+}$ -dependent channels were considered to play a central role in volume regulation, but studies have since identified other volume-sensitive  $\text{Cl}^-$  channels (VDCCs) or  $\text{K}^+$  channels that are not directly activated by cytosolic  $\text{Ca}^{2+}$ . Direct coupling of membrane tension with channel opening is important, when stretch-activated  $\text{Cl}^-$  or  $\text{K}^+$  channels, channels that are open by suction of patch pipettes, are found in the



swollen cell. However, stretch-activated Cl<sup>-</sup> channels are not found in many cells. Swelling of the cell not only stretches the membrane surface but triggers unidentified, presumably cell-specific, signal transduction, resulting in the opening of VDCCs [53–55]. Candidates for signal transduction toward VDCCs include calmodulin [56], tyrosine kinase [57] and small molecular G proteins [58] (fig. 2), but study of the mechanisms await the molecular identification of the channel molecules.

Again, the molecular identification of VDCC has been difficult because of the absence of identification of the cell line of VDCCs (endogenous VDCCs in table 1). Various proteins are suspected to be molecular candidates for VDCCs. The three initial candidates, P-glycoprotein (P-gp) [59], pICln [60] and CIC-3, have since been discarded. On the basis of the well-known function of

CFTR, hypothesized to be a bifunctional multiple-drug transporter, P-gp would adopt two mutually exclusive functional states, either as an adenosine triphosphate (ATP)-dependent drug transporter or a VDCC [61, 62]. However, this is unlikely because other laboratories could not repeat the original experiments on which this hypothesis was based.

The second protein that has been proposed for VDCCs is the ubiquitously expressed pICln [62]. The electrophysiologic and pharmacological properties of pICln-associated Cl<sup>-</sup> conductance in *Xenopus* oocytes differ from those of VDCC currents. pICln has a constitutive cytosolic and nuclear location. pICln interacts with splice factors, suggesting a role in gene expression and embryonic development [63, 64].

The CIC-3 protein [65], a member of the CIC family, was suggested recently as a molecular candidate for VDCCs. However, some characteristics of CIC-3-induced currents, such as single-channel kinetics, biophysical properties such as rectification, modulation by PKC and the large current amplitude under isotonic conditions, are at variance with those of VDCCs [66, 67]. In addition, CIC-3 is mainly located in endosomes, and no changes in VDCCs have been detected in cardiac muscles in mice lacking the CIC-3 gene [68]. Rather, disruption of the CIC-3 gene leads to morphologic abnormalities, suggesting that CIC-3 is important in maintaining pH and Cl<sup>-</sup> concentration within endosome vesicles, as discussed below. Recently, CIC-3 was again reported to be a fundamental molecular component of VDCCs in epithelial cells [69], in smooth muscle cells [70] and on the basis of antisense oligonucleotide experiments [69]. Some associated proteins related to recycling of the plasma membrane and endosomes have been isolated [71]. When the recycling is regulated by signal transduction induced by swelling, the complex of CIC-3 and an associated protein is a leading candidate in VDCCs [72].

Several large-conductance Cl<sup>-</sup> channels (maxi-Cl<sup>-</sup>) with single-channel conductance between 250 and 430 pS, behaving like VDCCs, have been described [73–77], including a Cl<sup>-</sup> channel with a conductance of 400 pS that is activated by osmotic cell swelling and that is sensitive to Gd<sup>3+</sup>. This channel is ATP conductive, with a  $P_{ATP}/P_{Cl}$  of 0.09, and is a candidate for the volume- and voltage-dependent ATP-conductive pathway for swelling-induced ATP release (maxi-Cl<sup>-</sup><sub>ATP</sub>) [77]. In contrast to the general role of anionic transport by Cl<sup>-</sup> channels, maxi-Cl<sup>-</sup><sub>ATP</sub> can transmit a cationic excitatory signal to an adjacent cell. Swelling of the cell possessing maxi-Cl<sup>-</sup><sub>ATP</sub>, by mechanical stress, releases ATP, which then activates the influx of Ca<sup>2+</sup> into adjacent cells having a purinergic ATP receptor. This phenomenon is evident in the mechanism of renal tubuloglomerular feedback in macula densa cells [77, 78] (fig. 3). The higher the luminal Cl<sup>-</sup> concentration,

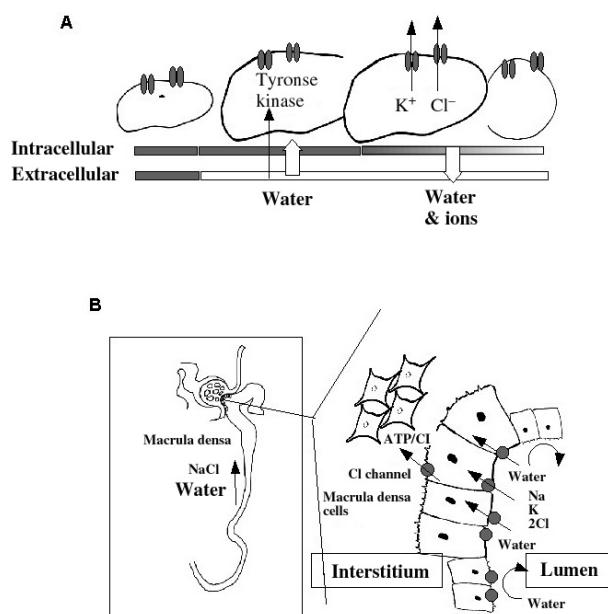


Figure 2. Mechanism of cell swelling and a possible role of VDCC in tubulo-glomerular feedback. (A) Mechanism of cell volume regulation and activation of VDCC is shown. The cell under the aniso-osmotic surrounding is illustrated at the top of the panel. The tonicity of extracellular and intracellular solution is expressed on the bottom. Cells swell in hypotonic (indicated as a white column) extracellular solution by an influx of water via aquaporin channels. The influx of water per se or change of volume induces several lines of signal transduction, which then activate Cl<sup>-</sup> (VDCC) and K<sup>+</sup> channels. Efflux of these ions lowers the concentration of ions in the cell interior reaching equilibrium to extracellular tonicity, and then cell volume is restored. (B) Macula densa is an apparatus located between a glomeruli and its own distal tubule (inset). Larger cells get together to make a specific apparatus. The cells have high water permeability surrounded by the water-impermeable ascending limb of Henle. When Na/K/2Cl and water enters into the cells from the lumen, the cells swell, resulting in an activation of basolateral VDCC. This Cl<sup>-</sup> channel is a class of large-conductance channel permeable to ATP. The released ATP then contracts mesangial cells through the purinergic receptor. The contraction of mesangial cells reduces the glomerular filtration rate, lowering the luminal NaCl and water levels, comprising the 'tubulo-glomerular' feedback loop.

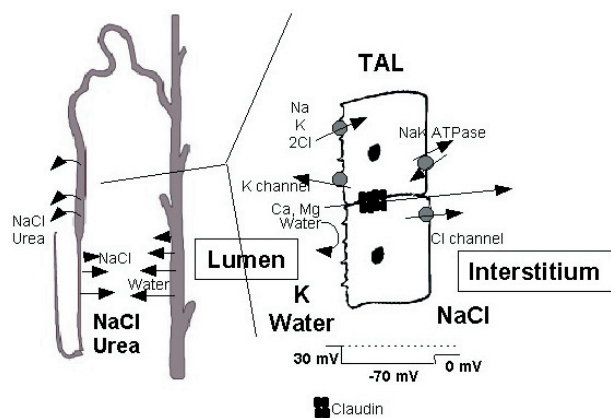


Figure 3. Fluid transport and  $\text{Cl}^-$  channel. Ascending loop of Henle (thick ascending limb, TAL) is an important part of the tubular segment (left panel), playing a cardinal role in construction of high osmolality in the renal medulla by unidirectional transport of NaCl. Luminal  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$  are transported via the Na/K/2Cl transporter into cells without water.  $\text{Na}^+$  and  $\text{Cl}^-$  are transferred into the basolateral space and thereby concentrated in osmolality.  $\text{K}^+$  is back-leaked by  $\text{K}^+$  channels resulting in lumen-positive (compare with interstitium) transtubular potential. This lumen-positive potential drives cations,  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ , through the cell junction (claudin) under the electrochemical gradient. Thus, all 3 modes of transport, Na/K/2Cl transporter, K channel and Cl channel ( $\text{ClC}_K$ ) are cardinal in the construction of the high osmolality. Lack of these transport modes results in Barter's syndrome, where diluted urine, hypokalemia and hypocalcemia are noted.

the larger the volume of macula densa cells that have maxi- $\text{Cl}^-_{\text{ATP}}$  in the basolateral membrane. Although macula densa cells are a continuous part of the water-impermeable loop of Henle, the luminal membrane of these cells has high water permeability, and the cells change volume easily. The basolateral maxi- $\text{Cl}^-_{\text{ATP}}$  activated by an increase in volume releases ATP and  $\text{Cl}^-$  toward adjacent smooth-muscle-like mesangial cells. Contraction of mesangial cells reduces renal blood flow, leading to a lower amount of luminal solute involving  $\text{Cl}^-$  concentration. This feedback mechanism is suggested to be central in the renal response to salt overloading, and the identification of maxi- $\text{Cl}^-_{\text{ATP}}$  molecules would be a clue to elucidating the mechanism of hypertension. A possibility of ATP conductivity of  $\text{Cl}^-$  channels is suggested, but not evident, in brain and lung epithelial cells [2, 78].

We have reported that *Drosophila tweety* may encode a large-conductance CaC channel that has been detected on occasion *in situ* in endothelium, neurons or smooth-muscle cells [15]. These channels have five or six TMSs [15, 79]. There are three members of the family of *tweety* homologs (TTYH1–3) in mammals. When TTYH1–3 are expressed in Chinese hamster ovary (CHO) cells, a large current is evoked by addition of a  $\text{Ca}^{2+}$  ionophore or in 0.1 mM  $\text{Ca}^{2+}$  in a pipette fill, which is sensitive

to 10–100 mM DIDS but resistant to NPPB or niflumic acid. A spliced variant of TTYH1 was activated in the expressed cells exposed to hypotonic solution. A single-channel recording of TTYH3 showed  $\text{Cl}^-$  conductance of 260 pS in a cytosolic  $\text{Ca}^{2+}$  concentration of  $>1$  mM, while a 250-pS endogenous  $\text{Cl}^-$  channel can also be found after prolonged depolarization in CHO cells. Mutants of TTYH3 reveal altered selectivity to anion, suggesting that TTYHs may encode large-conductance  $\text{Cl}^-$  channels. The *tweety* locus is adjacent to the *flightless* locus and is regulated by the same promoter, but whether *tweety* is directly related to flying in the fly is not known [79]. The *tweety* gene is constantly expressed during development from the larva to the adult fly. There are two *tweety* families in *Drosophila*, and knockout of these genes has proved to be lethal. Thus, experimentation with tissue- or time-specific knockout of these genes is essential to identification of their properties *in vivo*.

Another  $\text{Cl}^-$  channel, the MID-1-related  $\text{Cl}^-$  channel (MCLC), is a protein of 541 amino acids and four putative TMSs [80]. MID-1 encoded a yeast *Saccharomyces cerevisiae* membrane protein of two TMS that is expressed as a stretch-activated cation channel in mammalian CHO cells [81]. Using the MID-1 sequence as a probe, human MCLC has been cloned by BLAST search. MCLC is located in intracellular compartments, including the endoplasmic reticulum and the Golgi apparatus, and provides a 70-pS permeable  $\text{Cl}^-$  channel in a planar lipid bilayer. MCLC is also considered to be a new class of VDCC expressed in intracellular compartments [80].

### Fluid transport

The mechanism for contribution of  $\text{Cl}^-$  channels to NaCl fluid transport across epithelial cells is well documented and is summarized elsewhere [2, 82, 83]. One of the most striking examples put forward is the case of the  $\text{ClC-Kb}$  channel. The renal thick ascending loop of Henle is an essential part of the osmotic concentration gradient in the interstitial space, since NaCl is transported from the lumen to the interstitium without movement of free water (fig. 3). Luminal  $\text{Na}^+$  entering the cell via Na/K/2Cl transport (NKCC) is reabsorbed by the basolateral  $\text{Na}^+$ / $\text{K}^+$  pump, whereas  $\text{K}^+$  is recycled back through apical  $\text{K}^+$  channels (ROMK and Kir1.1). The accumulated  $\text{Cl}^-$  is transported via the  $\text{ClC-Kb}$  channel through the basolateral membrane by means of the electrochemical gradient. Finally, NaCl accumulates in the interstitium, and  $\text{K}^+$  remains in the lumen, where  $\text{K}^+$  creates positive lumen potential and drives other cations, such as  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , to the interstitial space through the paracellular pathway. Barttin is a binding protein of the  $\text{ClC-Kb}$  channel, with a two-membrane spanning domain that plays a role in the membrane expression of this channel. The channels  $\text{ClC-Ka}$  and  $\text{ClC-Kb}$  are constitutively open when co-expressed with their subunit Barttin [84].

Both channels are slightly outwardly rectifying, inhibited by extracellular acidosis and potentiated by an increase in extracellular calcium. Functional loss of NKCC, Kir1.1, CIC-Kb or Barttin is a cause of Bartter syndrome, in which nephrogenic diabetes insipidus, hypokalemia, hypomagnesemia and hypocalcemia have been observed as variations in phenotype [82–84].

A similar schema can be illustrated for the cells of the Stria vascularis, which makes inner-ear fluid. The CIC-Ka and CIC-Kb channels are involved in the recycling of Cl<sup>-</sup> that accompanies the secretion of K<sup>+</sup>. A high concentration of K<sup>+</sup> is necessary for hair cells to sense mechanical stimuli. Therefore, congenital deafness accompanied with diabetes insipidus is associated with genetic mutation in the CIC-Ka and CIC-Kb channels and in barttin protein [2, 4, 82, 83].

Another important Cl<sup>-</sup> channel is encoded by the cystic fibrosis transmembrane conductance regulator (CFTR) [85, 86]. Cystic fibrosis (CF), the most common lethal genetic disease among caucasians, is complicated by abnormal epithelial solute and fluid transport due to mutations in CFTR. CFTR belongs to the ATP-binding cassette (ABC) family of transporters, containing 12 predicted transmembrane helices and five cytoplasmic domains consisting of two nucleotide-binding domains (NBDs), a regulatory (R) domain containing numerous consensus phosphorylation sequences (fig. 1). The ABC superfamily regulates other processes in addition to its own role. For example, the sulfonyleurea receptor, SUR, regulates K<sup>+</sup> channels. Likewise, CFTR regulates Na channel (ENaC) and outward-rectified Cl<sup>-</sup> channel (ORCC) by excretion of ATP, although these are still controversial [87–89]. Work with truncation or mutation suggests that the first transmembrane domain (TMD-1) of CFTR, especially predicted  $\alpha$ -helices 5 and 6, forms an essential part of the Cl<sup>-</sup> channel pore, whereas the first NBD domain is essential for its ability to regulate ORCCs [88]. All three functions, Cl<sup>-</sup> channels, ORCC and Na<sup>+</sup> channel together contribute isotonic transport of fluid across the respiratory epithelia [89].

#### Neutralization of H<sup>+</sup> ions in lysosomal vesicles

The lysosome is an intracellular vesicular organelle that plays a role in protein degradation and has a low interior pH. To maintain this low pH, the vesicle possesses H<sup>+</sup>-ATPase-accumulating H<sup>+</sup> ions. Accumulation of H<sup>+</sup> ions is back-leaked by the electrochemical gradient until cationic deflection due to charge accumulation is diminished. To neutralize the voltage deflection and maintain pH, an acid-activated Cl<sup>-</sup> channel is required in order to accumulate Cl<sup>-</sup> in the interior of the vesicle. CIC-5, located in renal proximal tubules, is a well-known example of a Cl<sup>-</sup> channel that plays a role in protein degradation [2, 90–94]. A functional defect in this gene causes progressive renal failure with nephrocalcinosis. A

defect in this channel apparently does not show abnormal metabolism of Cl<sup>-</sup>, water or Na<sup>+</sup> ions. Thus, loss of Cl<sup>-</sup> permeability per se does not induce progressive loss of nephron function. Megalin, which is closely related to metabolism of 1,25(OH)<sub>2</sub>D<sub>3</sub>, is degraded in the lysosomes of renal proximal tubules [94]. Similarly, several proteins, such as parathyroid hormone (PTH) and micromolecular proteins are not degraded in the proximal tubules. Lack of degradation of megalin and PTH in mice lacking CIC-5 is believed to lead to a high luminal concentration of these calcitropic proteins, resulting in microalbuminuria, hyperphosphaturia and hypercalciuria [93]. Hypercalciuria, however, has not been observed in CIC-5 knockout mice used in different studies [94].

A defect in the Cl<sup>-</sup> channel in lysosomes (CIC-3) may further cause morphologic disorders. CIC-3 knockout mice are viable but smaller. They survive >1 year, but show severe degeneration of the CA1 region of the hippocampus and the retina, resulting in a complete loss of photoreceptors. Electrophysiological analysis of hippocampal slices from juvenile CIC-3 knockout mice revealed no major functional abnormalities, except for a slight increase in the amplitude of miniature excitatory postsynaptic currents [68]. Another model lacking CIC-3 revealed significant kyphosis, in addition to brain anomalies, and no major change in swelling-activated Cl<sup>-</sup> permeability [95]. Therefore, CIC-3 is an endosomal Cl<sup>-</sup> channel that is related to the degradation of proteins and that plays a role in morphogenesis in expressed tissue.

A defect in CIC-7 in the endosomes of osteoclasts reveals a striking phenotype, osteopetrosis, in which bone remodeling, reabsorption and calcification are not processed inherently. Osteoclasts generate and secrete H<sup>+</sup>, by means of H<sup>+</sup>-ATPase, into the surface of bone in order to digest mineral crystals. Ruffled border, a closed space on the reabsorption surface where secreted H<sup>+</sup> accumulates, has a pH of 4.0–6.0. Osteoclasts lacking CIC-7 are unable to maintain a low pH. The mechanism is the same as that for maintenance of low pH in lysosomes [96].

AQP-6 is a member of water channel but is activated by a low pH, to express Cl<sup>-</sup> channel in renal collecting segments. AQP-6 is located in endosomes, suggesting that the functional role of this channel is similar to the above CICs, although mice lacking this gene have not yet been studied [11, 97].

#### Do Cl<sup>-</sup> channels play a role in tumorigenesis?

Cl<sup>-</sup> channels are thought to contribute to cell growth. Glioma Cl<sup>-</sup> channels are upregulated in glioma cells. Some Cl<sup>-</sup> channel blockers induce suppression of gene p21 [98]. Similarly, blocking of VDCC-related Cl<sup>-</sup> channels prevents apoptosis of various cells [99]. As described above, the hCICA2 family may be related

to tumorigenesis. hCICA1 is also related to carcinoma cells [100]. These data may suggest a novel role for Cl<sup>-</sup> channels in biology, but evidence at the level of a single gene is needed.

### Blockers of Cl<sup>-</sup> channels

A number of reagents block Cl<sup>-</sup> channels. Most of these reagents, however, are not specific for one class of Cl<sup>-</sup> channels. Disulfonic stilbenes, for example, are widely used blockers of any Cl<sup>-</sup> channel but only some anion transporters. Niflumic acid is employed to block endogenous CaC current in *Xenopus* oocytes. But niflumic acid is also used to inhibit cation current [101]. Table 2 summarizes widely used reagents for blocking most Cl<sup>-</sup> channels, where IC<sub>50</sub> (mean inhibitory concentration) less than 100 mM is described. Classic anion channel blockers inhibit CaC and VDCC at micromolar concentrations. In general, CIC is resistant to blockade with classical blockers. CIC-1 alone can be inhibited by 9-AC, DPC and niflumic acid in the micromolar range. CIC-2 is inhibited by these reagents at millimolar concentrations

but by Zn<sup>2+</sup> ion at micromolar concentrations. CFTR is an ATP-binding cassette family, blocked by sulfonylureas such as glibenclamide at over 100 mM. DIDS inhibits CFTR but only from inside the cell. Thiazolidinone derivatives are potent inhibitors of CFTR at less than 10 mM. [102] In a search for more specific reagents, chlorotoxin was found and has been advertised as a selective Cl<sup>-</sup> channel blocker toxin [103]. However, it may be ineffective [104]. Direct CFTR activator have been characterized. These include the xanthenes and the flavonoids, of which the isoflavonoid genistein is the most potent activator. Interestingly, phenylglycine (2-[(2-1H-indol-3-yl-acetyl)-methylamino]-N-(4-isopropylphenyl)-2-phenylacetamide) or sulfonamide [6-(ethylphenylsulfamoyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid cycloheptylamide] prolongs the single channel open probability of DF508CFTR (most frequent mutant in CF patients). These reagents may be useful in treating of CF patients [105].

Unique reagents are being developed to discriminate between CaC channels and VDCCs. The antimalarial drug mefloquine and the antidepressant fluoxetine (Prozac), considered to be selective 5-hydroxytryptamine reuptake

Table 2. Cl<sup>-</sup> channel blockers.

#### 1. Classical Blockers

Disulfonic stilbenes (irreversibly binding)	DIDS, SITS [123]
Disulfonic stilbenes (reversibly binding)	DNDS
Arylamino benzoates	DPC [124, 125]
Fenamates	NPPB, FFA, NFA [126]
Anthracene carboxylates	9-AC
Indanylalkanoic acids	IAA-94
Clofibrinic acid derivatives	CPP, CPPS(-)CPB [127]

#### 2. CIC blocker

DIDS  
Metal ions Zn<sup>2+</sup>, Cd<sub>2</sub> [2]  
**CICKa:** 2-(p-chlorophenoxy)-3-phenylpropionic acid [128]

#### 3. CFTR blocker

Sulfonylureas glibenclamide, tolbutamide [129]  
DIDS (from inside)  
Suramin [130]  
Thiazolidinone derivatives [102]  
Scorpion venom [103]

#### 4. Other blockers

**p64:** *ts-tm*-Calix(4)arene, DNDS [109]  
**VDCC:** tamoxifen [131], clomiphene [132]  
**CaC:** antimalarial mefloquine, fluoxetine [133]  
Macrolide antibiotics [107]

DIDS (4'-diisothiocyanostilbene-2,2'-disulfonic acid); (SITS (4-acetamido-4-isothiocyanostilbene-2,2'-disulfonic acid); DNDS(4,4'-dibenzamidostilbene-2,2'-disulfonic acid); DPC (diphenylaminocarboxylate); NPPB (5-nitro-2-[3-phenylpropylamino]benzoic acid); FFA (flufenamic acid); NFA (niflumic acid); 9-AC (anthracene-9-carboxylate); DPC (diphenylaminocarboxylate); IAA (indanyloxyacetic acid); CPP, [2-(p-chlorophenoxy)propionic acid]; CPPS(-)CPB [S(-)2-(4-chlorophenoxy)propionic acid].



inhibitors, block CaC channels and have IC<sub>50</sub> values of approximately 5 mM [106]. VDCCs are inhibited specifically by antioestrogens such as clomiphen, nafoxidine and tamoxifen, and by antimalarials such as mefloquine. A large-conductance, volume-dependent Cl<sup>-</sup> channel is blocked by Gd<sup>3+</sup> [77]. Macrolide antibiotics, erythromycin and clarithromycin may inhibit Cl<sup>-</sup> current in some cells [107].

### Structure and permeation of Cl<sup>-</sup> channels

There are several Cl<sup>-</sup> channel structures, suggested by their hydrophobic analysis (fig. 1). Mechanisms of Cl<sup>-</sup> channel permeation have been successfully studied by means of X-ray analysis of ClC channel crystals. This sophisticated method has revealed a complex structure of ClC channels and a binding motif of Cl<sup>-</sup> ions [19, 108]. The structure revealed a complex fold of 18  $\alpha$ -helices per subunit with at least two Cl<sup>-</sup> ions bound in the center of each protopore [109, 110]. These studies have shown that a lone Cl<sup>-</sup> ion bound to the center of the ClC pore is pushed out by a second ion that enters the pore and takes its place. The pushing of an ion by another is favorable for energy barriers that reduce the largest free-energy gap. This center portion is the narrowest part of the pore, formed by residues Tyr-445 and Ser-107 [19] and stabilizes Cl<sup>-</sup> ions from the water shell, which prevents the passage of uncharged solutes through this gate. A critical glutamic acid residue was identified whose side chain seems to occupy a third Cl<sup>-</sup> ion binding site in the closed state and that moves away to allow Cl<sup>-</sup> binding [111, 112]. ClC has double-barreled pores, the pathways of which are determined by mutation analysis [7–9]. Compared with the structure of the K<sup>+</sup> channel with six TMSs, the structure of ClC is far more complex and not predicted by hydrophobic analysis. Interestingly, crystallography shows prokaryotic ClC to be a H<sup>+</sup>/Cl<sup>-</sup> transporter rather than an anion channel [20]. Furthermore, recent data support this finding, so that ClC0, 1, 2 and Ka/b function as ion channels, and ClC3–7 as Cl<sup>-</sup>/H<sup>+</sup> exchangers [113].

The crystal structures of other Cl<sup>-</sup> channels are unavailable, including that for CFTR. Extensive studies with mutants suggested that the TMS6 and NBD domains are important in the permeation pathway [114]. However, the conducting pore of CFTR [115], glycine receptor [116] or even a cationic channel can be functionally and structurally reproduced in artificial membranes, by means of synthetic peptides. The peptide is designed to make a cylinder with an uneven distribution of hydrophobic and hydrophilic alignment (fig. 4). At least 18–20 amino acids are required in order to penetrate the lipid bilayers. The sequence requires positively charged amino acids in the entrance of the cylinder. Four or five oligomers combine

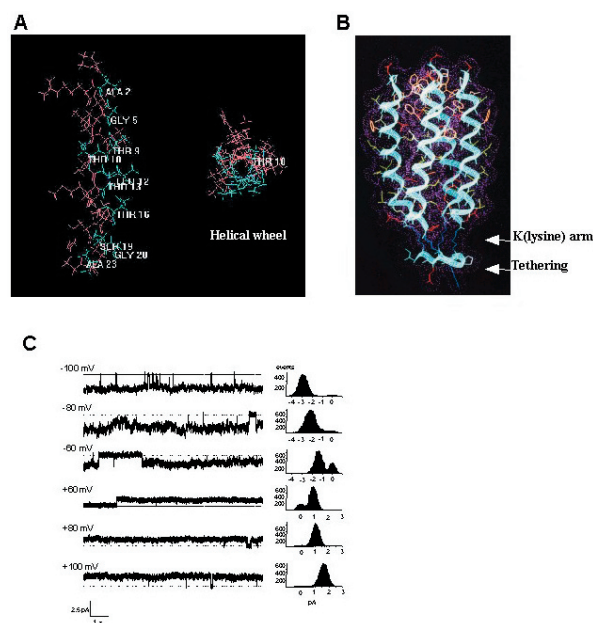


Figure 4. Functional peptides tethered to form anion pores. (A) Design of peptide sequences is based on the alignment of positively charged amino acids (blue) calculated by computer-based analysis of helical wheel and three-dimensional prediction. (B) Four peptide oligomers are tethered by peptides with artificial chains (lysine). Structural analysis reveals an inside pore formed by the alignment of charged amino acids. (C) The tethered peptide channels or peptides alone can express as an anion pore. The peptide is administered on one side of the bath solution of artificial membrane. Electrical recording was performed with asymmetrical anion but symmetrical cation solutions (KCl). Once expressed, single-channel anionic conductance, as indicated by the event-current relations on the right, is observed stably and continuously. The current-voltage relation shows the reversal potential of  $E_{Cl}$  but not  $E_K$ .

to form a conducting pore. To enhance the formation of oligomers, tethering of the peptide by an artificial chain (lysine) and ring ( $\beta$ -turn structure) effectively reproduces the anion-conducting pore in the artificial membrane [20–22]. The computer based structure of TMS2 of glycine receptor (the sequence of amino acids from the second transmembrane of the glycine receptor) suggested that Cl<sup>-</sup> ions without a water shell are bound to the center of the pore [21, 22]. Using this technique, homo- and heteropolymerization of TMS2 and TMS6 of CFTR have been probed to form anion-conducting pores resembling the single-channel conductance of CFTR. However, TMS1, -3, -4 and -5 fail to form ion-conducting pores [115]. The artificial peptide Cl<sup>-</sup> channel may be used to ameliorate poor secretion of fluid in lung epithelia of patients with cystic fibrosis [115]. Interestingly, under the rule of amino acid sequences, many unexpected peptides are potential Cl<sup>-</sup> channels. In other words, many possible Cl<sup>-</sup> channel pores are hidden in various TMSs, as indicated by calculation of cylinder conformation with uneven hydrophobicity. Several molecules have been thought to be Cl<sup>-</sup> channels; they be subunits of

ubiquitously expressed Cl<sup>-</sup> channels or may be due to pore formation by unexpected TMS [21, 22, 116, 117]. Cl<sup>-</sup> channels are classified by their physiologic roles. Studies of the family of ClC channels have revealed a majority of their functions. Additional evidence of novel Cl<sup>-</sup> pores by various molecules with different TMSs has been growing. Functional evidence that is based on the study of molecular expression of defective molecules *in vitro* or *in vivo* requires a molecule-based classification of the diversity of Cl<sup>-</sup> channels in the future.

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