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Review

Two decades with dimorphic Chloride Intracellular Channels (CLICs)

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ABSTRACT

Plasma membrane channels have been extensively studied, and their physiological roles are well established. In contrast, relatively little information is available about intracellular ion channels. Chloride Intracellular Channel (CLICs) proteins are a novel class of putative intracellular ion channels. They are widely expressed in different intracellular compartments, and possess distinct properties such as the presence of a single transmembrane domain, and a dimorphic existence as either a soluble or membranous form. How these soluble proteins unfold, target to, and auto-insert into the intracellular membranes to form functional integral ion channels is a complex biological question. Recent information from studies of their crystal structures, biophysical characterization and functional roles has provoked interest in these unusual channels.
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1. Introduction

Ion transport across the plasma membrane is involved in numerous cellular functions such as cell-volume regulation, electrical excitability, transepithelial transport, contraction, bone resorption in normal physiology, and acquired diseases. For more than 60 years, the study of ion channels has been dominated by proteins involved in neuronal function and excitability. However, chloride (Cl^-) channels began to attract interest with the discovery of the first non-neuronal chloride channel (ClC) in 1982 in Torpedo electroplax [1]. All living organisms have evolved a variety of ion channel proteins to exploit Cl^- ions towards varied physiological ends. For example, leakage channels in the giant squid axon, first ignored as experimental irritants, were found to be largely mediated by Cl^- channels. In contrast to highly selective cation channels, Cl^- channels may also conduct other anions including halides, pseudohalides (SCN^-) and bicarbonates. In spite of these molecules being transported more efficiently than Cl^-, they are still referred to as Cl^- channels since Cl^- is the most abundant (4–10 mM) anion in organisms. The role of Cl^- channels in diverse cellular functions and diseases has been shown through the study of Knock-Out (KO) animals. Detailed information on the fundamental role of Cl^- channels is presented in other reviews [2–4].

As stated earlier, inquest for Cl^- channels was triggered after the discovery of a ClC and while searching for Cl^- channel proteins. In 1987, Landry, Al Awqati and colleagues isolated the first Chloride Intracellular Channel (CLIC) protein, p64 [5] (now known as CLIC5b), which bound to *R*(+)-[(6,7-dichloro-2-cyclopentyl-2, 3-dihydro-2-methyl-1-oxo-1*H*-inden-5-yl)-oxy] acetic acid 94 (IAA94, a known Cl^- channel inhibitor) from bovine tracheal apical epithelium and kidney cortex microsomal membrane fractions [6,7]. This 64 kDa protein was purified and found to mediate a Cl^- flux upon reconstitution in vesicles [8]. Rat brain p64H1 was the first homologue of p64 to be identified [9] and characterized [10], and its human and murine homologues were named CLIC4. As discussed later, even though these proteins are named CLICs,

Abbreviations: ADP, adenosine diphosphate; *At*, *Arabidopsis thaliana*; CaCC, Ca^{2+} activated Cl^- channel; CHO, Chinese hamster ovary; Cl^-, chloride ion; ClC, chloride channel; CLIC, Chloride Intracellular Channel; CNS, central nervous system; CREB, cAMP response element binding; DHAR, dehydroascorbate reductase; *Dm*, *Drosophila melanogaster*; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; ERK, extracellular signal-regulated kinase; EXC, excretory canal; EXL, EXC like; GHK, Goldman–Hodgkin–Katz; GPCR, G protein-coupled receptor; GST, glutathione S-transferase; H_2O_2, hydrogen peroxide; IAA 94, *R*(+)-[(6,7-dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1*H*-inden-5-yl)-oxy]acetic acid; JEG3, human placental choriocarcinoma cell line; KCl, potassium chloride; KO, Knock-Out; LPA, lysophosphatidic acid; nAChR, n-acetylcholine receptors; NEM, *N*-ethylmaleimide; NHERF2, sodium-hydrogen exchange regulatory cofactor 2; PAH, pulmonary arterial hypertension; PTMD, putative transmembrane domain; RhoA, Ras homolog gene family, member A; ROS, reactive oxygen species; RyR, Ryanodine Receptor; S1P, sphingosine-1-phosphate; SCAM, substituted cysteine accessibility modification; SCN^-, thiocyanate; SH2, src-homology domain type-2; TGF, transforming growth factor; VEGF, vascular endothelial growth factor

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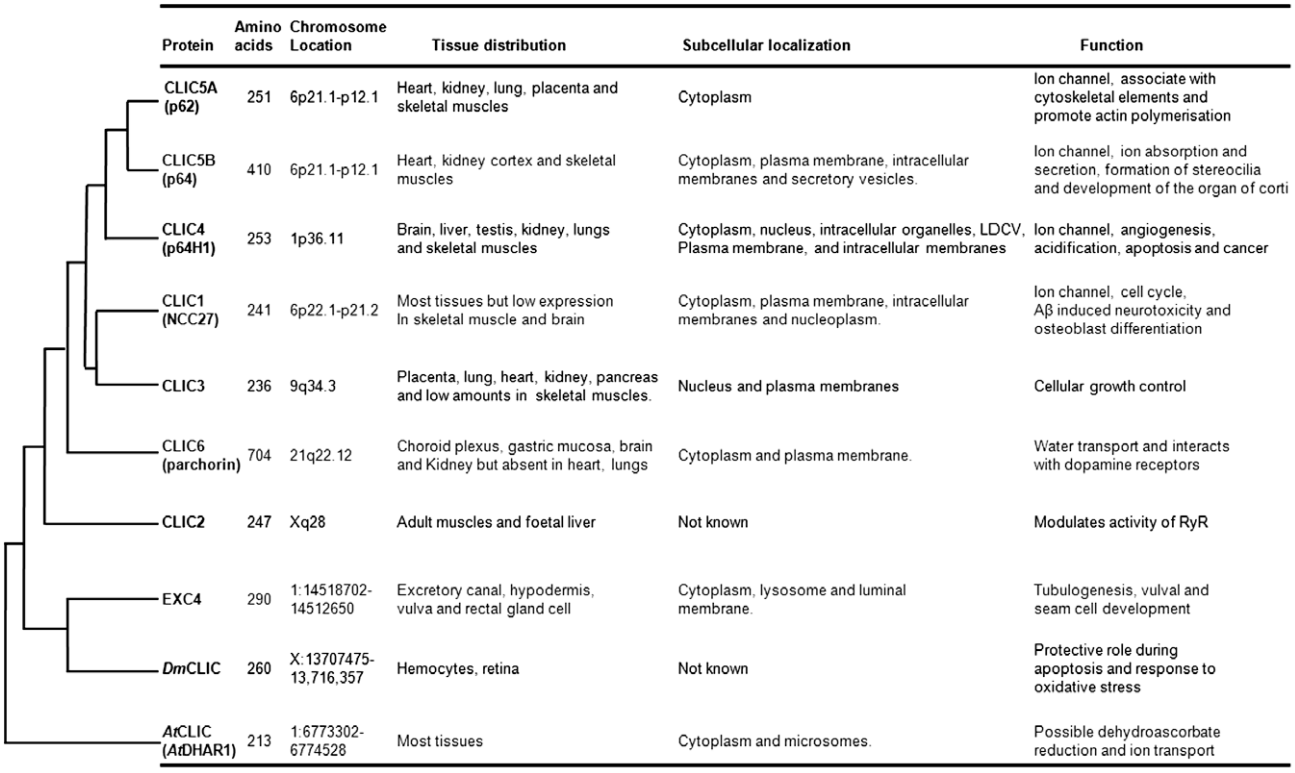
this is a partial misnomer as their selectivity and biophysical properties do not support this nomenclature. Although the molecular identity of CLICs was first deciphered in the late 1980s; cloned and characterized in 1990s [6–8], doubts remained regarding the ability of CLICs to form functional ion channels *in vivo* [2]. CLICs were not included in the larger ion channel family because of the absence of structure–function studies involving mutants with altered biophysical characteristics and lack of consistent ion channel properties. The presence of a single putative transmembrane domain had also led to doubts over their ability to function as ion channels [2]. Recent developments in *in vitro* biophysical characterization, and functional significance, CLICs have now re-emerged as functionally important channel proteins [11,12]. However, one should be cautious about the CLICs functioning as ion channels before conclusive evidence is found in the native system, since previously thought Ca^{2+} activated Cl^- channel (CaCC), hClCa1 does not function as a Cl^- channel but elevates the single channel conductance of CaCC [13].

CLICs are a relatively new class of putative ion channel proteins that differ from the other classes of channels in their primary structure, and in the transmembrane region of their tertiary structure. The CLIC family has six known members (CLIC1-6) in vertebrates [11], three in invertebrates (*Dm*CLIC in *Drosophila melanogaster*, EXC4 and EXL1 in *Caenorhabditis elegans*) [14,15] and at least four genes in *Arabidopsis thaliana* (*At*DHAR1-4) [16] with differential tissue distribution, and function both in the plasma membranes and intracellular organelles (summarized in Fig. 1). They share structural homology with members of the omega glutathione S-transferase (GST) superfamily [12,17] (the structure of CLIC proteins is discussed in Section 2). A unique feature of CLIC proteins that distinguishes them from other ion channels is their ability to exist in two different forms; a soluble globular form and an integral membrane protein, suggested to form functional ion channels. CLIC proteins possibly function as tetramers or higher oligomers and have a predicted channel pore in the N-terminus region [18]. Functional expression of mutant channels altered by site-directed mutagenesis or deletions has led to important insights into their membrane structure [18–20] (the biophysical properties of CLICs will be discussed in Section 3). CLICs play significant roles in diverse processes including bone resorption [21], regulation of cell motility [22], tubulogenesis [14], angiogenesis [23], formation of skeletal muscle and brain [24], β-amyloid induced neurotoxicity [25], and p53-mediated apoptosis [26–29]. Human CLIC1, CLIC4, CLIC5A, CLIC5B, and CLIC6 (rabbit parchorin) can bind to a 133-amino acid domain within AKAP350 through the last 120 amino acids in their conserved carboxyl termini [30], suggesting a role in PKA signaling. In addition, CLIC proteins contain consensus sequences for tyrosine phosphorylation and src-homology domain type-2 (SH2)-binding domain (the functional significance of CLICs is discussed in detail in Section 4).

In the past two decades, a significant amount of work has been done involving CLIC proteins. Recent electrophysiological characterization of CLIC1 [20], CLIC4 [18], and CLIC5 [34] has indicated that these proteins can form ion channels in the artificial bilayers. These channels are either poorly selective for anions (CLIC1) or are non-selective (CLIC4 and CLIC5) with equal permeability to K^+ and Cl^- ions which requires a re-evaluation of the widely accepted 'CLIC' nomenclature. This review is an overview of the CLIC field, which includes known structures of the soluble form, biophysical properties of the membranous form of the channel, and significance of proteins for cellular function.



Protein	Amino acids	Chromosome Location	Tissue distribution	Subcellular localization	Function
CLIC5A (p62)	251	6p21.1-p12.1	Heart, kidney, lung, placenta and skeletal muscles	Cytoplasm	Ion channel, associate with cytoskeletal elements and promote actin polymerisation
CLIC5B (p64)	410	6p21.1-p12.1	Heart, kidney cortex and skeletal muscles	Cytoplasm, plasma membrane, intracellular membranes and secretory vesicles.	Ion channel, ion absorption and secretion, formation of stereocilia and development of the organ of corti
CLIC4 (p64H1)	253	1p36.11	Brain, liver, testis, kidney, lungs and skeletal muscles	Cytoplasm, nucleus, intracellular organelles, LDCV, Plasma membrane, and intracellular membranes	Ion channel, angiogenesis, acidification, apoptosis and cancer
CLIC1 (NCC27)	241	6p22.1-p21.2	Most tissues but low expression In skeletal muscle and brain	Cytoplasm, plasma membrane, intracellular membranes and nucleoplasm.	Ion channel, cell cycle, Aβ induced neurotoxicity and osteoblast differentiation
CLIC3	236	9q34.3	Placenta, lung, heart, kidney, pancreas and low amounts in skeletal muscles.	Nucleus and plasma membranes	Cellular growth control
CLIC6 (parchorin)	704	21q22.12	Choroid plexus, gastric mucosa, brain and Kidney but absent in heart, lungs	Cytoplasm and plasma membrane.	Water transport and interacts with dopamine receptors
CLIC2	247	Xq28	Adult muscles and foetal liver	Not known	Modulates activity of RyR
EXC4	290	1:14518702-14512650	Excretory canal, hypodermis, vulva and rectal gland cell	Cytoplasm, lysosome and luminal membrane.	Tubulogenesis, vulval and seam cell development
***Dm*CLIC**	260	X:13707475-13,716,357	Hemocytes, retina	Not known	Protective role during apoptosis and response to oxidative stress
***At*CLIC (*At*DHAR1)**	213	1:6773302-6774528	Most tissues	Cytoplasm and microsomes.	Possible dehydroascorbate reduction and ion transport

Fig. 1. An overview of CLIC family of chloride channels. Phylogenetic tree of CLICs is shown for seven human CLICs (CLIC1-6, 5A and 5B), two invertebral CLICs (EXC4 and *Dm*CLIC) [14,15] and a plant homologue (*At*CLIC) of CLIC protein [16]. Number of amino acids, chromosome location, tissue, and subcellular localization is given along with the known function for CLIC5A [30–34], CLIC5B [35,36], CLIC4 [10,11,18,26–29,34,37–44], CLIC1 [11,20,25,34,45–54], CLIC3 [11,55–57], CLIC6 [11,58–60], CLIC2 [11,61–65], EXC4 [14,15,19], *Dm*CLIC [15,19], and *At*CLIC [16]. Subcellular distributions of CLIC2 and *Dm*CLIC are not known.

2. Structure of CLICs

To form a functional ion channel, CLICs undergo a structural transition from a water-soluble state which auto-inserts into the plasma membrane in a similar fashion to annexins and other bacterial toxins [66,67]. They are likely to have unique structural arrangements in their dimorphic states. So far, crystal structures of four soluble vertebrate and two invertebrate CLIC proteins have been determined. Each CLIC protein contains a conserved C-terminal domain consisting of ~240 amino acids. CLIC5B and CLIC6 possess an additional large hydrophobic N-terminal domain which is not present in other CLIC proteins. Intriguingly, CLIC proteins share a structural homology with omega class GSTs containing a putative α/β N-terminal binding domain and a compact, mainly α-helical, C-terminal domain [17].

Soluble CLICs are fascinating as they provide insight into the complexity of a structure which does not resemble an ion channel in its soluble form, and contains an omega-GST fold along with an intact glutathione (GSH) binding site (providing the structural homology to CLICs with GST proteins). They also possess an all α-helical C-terminus domain unique to the GST superfamily. The glutaredoxin-like GSH binding site is conserved in all CLICs in the N-terminus region consisting of four beta (β) strands. The glutaredoxin-like active site was shown to participate in reversible dimerization in the presence of strong oxidization conditions, and on re-reduction in the presence of unusually high concentrations of strong reducing agents [46]. The crystal structures of CLIC1 (1.4 Å) [46] and CLIC1 dimer (1.8 Å) [46], CLIC2 (2.0 Å) [68,69], CLIC3 [55], CLIC4 (1.8 Å) [39], EXC4 (1.9 Å), and *Dm*CLIC (1.7 Å) [15] have all been resolved in the past decade. They all show structural homology to each other, and a majority of the structural elements in the vertebrate CLIC structures are preserved in the invertebrate CLICs. They have 10 α-helices (h1, h2, h3, h4a, h4b, h5, h6, h7, h8, and h9), with the exception of EXC4, in which helix h4 is not broken and an additional h10 helix [15], and four β-strands (s1, s2, s3 and s4). EXC4 and *Dm*CLIC also have a divalent metal binding site, and a putative transmembrane domain consisting of an α-helix (h1) and a β-strand (s2) in the soluble structure. CLIC proteins posses a highly negatively charged long loop (Pro147-Gln164) between h5 and h6 of CLIC1 consisting of seven acidic residues. This is a putative site for protein interactions involving CLIC1, and possibly other CLIC proteins.

Structural analysis of CLIC1 showed that it can dimerize in the presence of strong oxidizing agents hydrogen peroxide (H_2O_2), which could be reversed by strong reducing agents dithiothreitol (DTT). The C-termini of monomer and dimer CLIC1 are structurally identical, whereas the N-termini showed major structural rearrangements. These structural rearrangements suggest that CLIC proteins undergo structural modification only at the N-terminal region upon insertion into the bilayers, since it is an energy efficient mechanism. Interestingly, the four β-strands disappear during this structural rearrangement and h1 α-helix extends towards the C-terminus by two α-helical turns. This region is the putative transmembrane domain of the channel [7,39] and indicates that an α-helix can insert into the bilayer, and form a functional channel pore [18] with Cys24 and Cys59 of CLIC1 on the two opposite sides.

It is known that CLICs can auto-insert to form ion channels, but how scrupulously the soluble conformation transits into the membranous form is not known. A phase change from soluble structure to unfolding, insertion, and refolding is a complex process. This complexity originates from many degrees of freedom, and the balance between enthalpic and entropic contribution to the free energy from the polypeptide chain and solvent molecules. In the absence of a crystal structure of the membrane form of CLIC proteins, it is difficult to precisely identify the region involved in the formation of a channel and its pore, selectivity filter, and gate. The structure of ClC showed the presence of two Cl^- binding sites and the absence of a typical pore through the protein that resembles an ion channel. It was further shown that ClC-ec1 was a transporter and not an ion channel [70]. This cautions against predicting the model of a membrane structure of the CLIC proteins. However, on the basis of biophysical and biochemical work carried out by several groups [10,18–20,40,71], sufficient evidence has been gathered to generate a preliminary working model of an ion channel for CLIC proteins. On the basis of Kyte–Doolittle hydropathy analysis [72] and sequence alignments, we have shown that the putative transmembrane domain (PTMD) comprised a region involving α-helix 1 and β-strand 2 of soluble CLIC1, involving Cys24 and Val46 [18,20]. The truncated mutant of CLIC4 (where the whole of the C-terminus was removed retaining the N-terminus, and the PTMD) can auto-insert into the bilayer as a functional ion channel albeit smaller conductance [18]. The decrease in conductance could be attributed to the missing C-terminus, which may either play a role in folding and insertion of CLICs into the bilayer or directly in the formation of the pore region. The finding that truncated channel with an intact N-terminus and transmembrane domain lines the pore, suggests that the C-terminus may be forming a charged channel vestibule that concentrates permeant ions near the entrance to the pore [73]. The structure–function studies showed that Cys24 in CLIC1 is located near the pore region and modification of Cys24 led to channel inhibition [20]. Cys24 and its equivalent residues in other CLIC proteins (with an exception of EXC4 where it is replaced by Asp36) are well conserved, and play an important role in redox regulation of CLIC1, CLIC4 and CLIC5 which could be extended to all other vertebral CLIC proteins. Cys24 was also shown to form a disulfide bond with Cys59 in CLIC1 upon oxidation which interestingly also increased its insertion into the lipid bilayer [46]. Mutating Cys24 did not affect the insertion or channel formation but removed the redox effect on the channel [20]. Similarly, Cys35 in CLIC4 was suggested to be an active site for enzymatic activity [74]. Since equipositional of CLIC1 Cys59 is absent in other CLIC proteins, it is difficult to conclude that the disulfide bond is important for channel insertion. In addition, Cys24 is present on the external side of the PTMD whereas Cys59 is present in the cytosolic side and therefore in functional conditions, CLIC1 cannot form a disulfide bond since it has only one transmembrane domain.

In EXC4 and EXL1, the PTMD was shown to be important not only for the localization of the proteins but also for targeting them to the luminal membranes [19]. The function of the PTMD of *Dm*CLIC, EXC4 and EXL1 were interchangeable. Introduction of a helix breaking mutation in h1 disrupts the localization of EXC4 to the luminal membrane. Deletion of the N-terminal region from CLIC5A showed diffused channel distribution as compared to the full length channel in the human placental choriocarcinoma cell line (JEG3) cells [33]. Concomitantly, this verifies the presence of a single transmembrane domain of the CLIC proteins in the N-terminal region. Understanding the membranous structure is important, and therefore a more systematic structure–function analysis is required for CLICs in the absence of a crystal structure. Structure–function studies involving the Cys-reactive compounds *N*-ethylmaleimide (NEM) and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) showed that CLIC proteins insert into planar bilayers in a distinct orientation [18,20] as shown earlier by antibodies against epitope-tagged CLIC1 in CHO-K cells [71]. The N-terminus is always located in the luminal side and the C-terminus towards the cytosolic side. Both truncated CLIC1 and CLIC4, with just one Cys located on top of the PTMD, were blocked by NEM and DTNB from

the luminal side in bilayers indicating that the N-terminus is exposed to the luminal side [18,20]. Similarly intact octa-His tag on CLIC1, CLIC4 and truncated versions of CLIC4 were blocked by the addition of nickel in the luminal side but not from the cytosolic side [18]. CLIC proteins have only one transmembrane domain; therefore, a single molecule cannot form a functional ion channel pore. Accordingly, it is possible to predict that minimum of four molecules are required to form a functional channel in the membrane (Fig. 2).

Residues lining the pore of CLIC1 and other proteins have been predicted on the basis of structure–function studies and helical wheel projections (Fig. 2A and B). Interestingly, there are two residues with positively charged side arms (Arg29 and Lys37) extended into the pore region forming two rings at the top and centre of the channel pore (Fig. 2C and D). The single hydrophobic residue Val33 located in the middle of the channel pore could form a gate for CLIC1 in a similar manner to nicotinic acetylcholine receptors (nAChR) [75]. The two hydrophilic threonines (Thr40 and Thr44) located towards the cytosolic face of the pore (Fig. 2C) may also play a crucial role in the selectivity of the channel. The total rise of the alpha helix of TMD is 30 Å (calculated on the basis of each 360° turn is 10.8 Å) which is sufficient to span the membrane to form a functional pore (Fig. 2C). The predicted channel pore needs to be probed extensively with substituted cysteine accessibility modification (SCAM).

3. Biophysical properties of CLICs

Traditionally, intracellular ion channels are studied by reconstituting crude membrane preparations from intracellular organelles, purified proteins from membranes or recombinant proteins in artificial bilayers or liposomes. Their activity is either determined by measuring the efflux of anions from liposomes, by fluorescence or via electrophysiological techniques. Due to technical limitations, recording ion channels directly from the intracellular membranes is challenging, making it difficult to study these channels in their native form or physiological environment. It is possible to drive intracellular ion channels to the plasma membrane by overexpression in cells but a population of these channels is still transported to the specific intracellular membranes, due to the presence of a specific localization signal. Alternatively, engineered protein sequence might assist their targeting to the plasma membrane and allow the functional studies but this approach has its own physiological limitations. Native intracellular channels have been reconstituted in the planar bilayer system, and this is currently the most effective method to study these channels [11,76]. However, with the advent of new techniques, it is now feasible but extremely challenging to isolate the intracellular organelles and record ion channel activity by patch clamp [76,77].

CLIC proteins have one putative transmembrane domain in the N-terminus region. The single transmembrane domain lines the

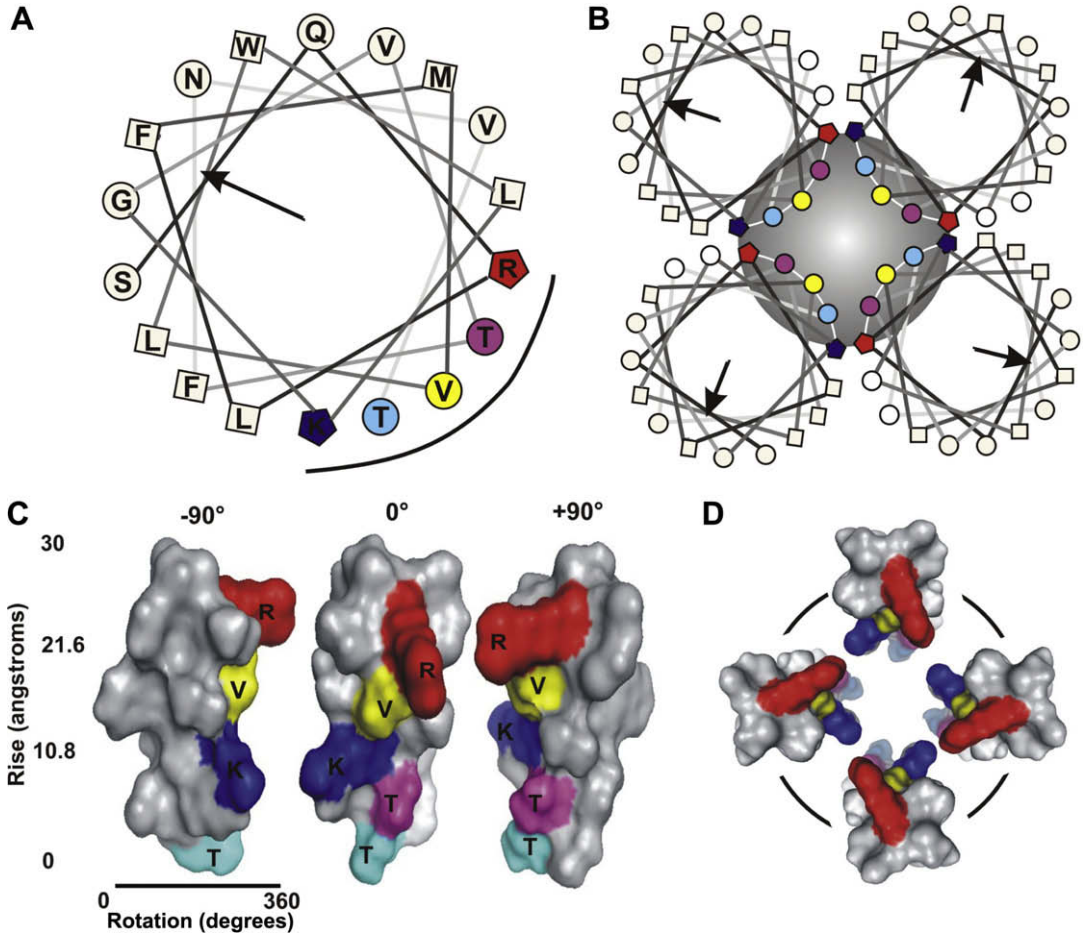


Fig. 2. Putative transmembrane domain of CLIC1. (A) Helical wheel projection of the PTMD of CLIC1 from N to C-terminus. Arrow indicates the direction of the hydrophobic movement and the arc indicates the predicted pore-lining residues. Squares, circles and polygons indicate relatively hydrophobic, hydrophilic or positively charged residues, respectively. (B) Cartoon of the predicted tetrameric pore using helical wheel A. (C) Alpha helical projection of PTMD. The residues lining the pore are indicated with the corresponding colours to helical wheel projection. Rise (angstroms (Å)) of TMD was calculated from the alpha helical projections. (D) View from the luminal side of the predicted pore involving four monomers of CLIC1 from the N-terminus. Positively charged residues (R (red) and K (blue)) form two selectivity rings in the pore region indicating their importance in maintaining selectivity.

Table 1
Biophysical properties of CLIC channels. The specific experimental systems and corresponding conductance of CLIC1 [20,25,46,50,52–54,71,78], CLIC2 [68], CLIC4 [10,18,39,40], CLIC5A [34], CLIC5B [6,79,81], EXC4 [15] and *Dm*CLIC [15] are summarized in the table. Single channel conductance of CLICs differs between groups. CLIC3 and CLIC6 are not shown to form functional ion channels. Limited information is available on the selectivities of the channels as shown in the table but the channels are either poorly anion-selective or non-selective. Single channel conductances were recorded either in symmetrical (*) or under *cis*:*trans* gradient (**). Conductance of CLIC5A was reported to be same under DTT, GSH/GSSG or H_2O_2. (***) CLIC5A poorly discriminates between Na^+ or K^+ or Cl^- ions.

Protein	Conductance	Selectivity	Method
CLIC1 (NCC27)	20 pS (130 mM NaCl)*	Anion-selective $P_{SCN^-} > P_{F^-} > P_{Cl^-} > P_{NO_3^-} = P_{I^-} = PHCO_{3^-} > P_{acetate}$	Transfected CHO-K1 cells
	33 pS (140 mM KCl)*	Anion-selective	Nuclear patch (CHO-K1 cells)
	8 pS (130 mM NaCl)*		
	8 pS, 17 pS (140 mM KCl)*		Transfected CHO-K1 cells
	32 pS (140 mM KCl)*		Tip dip (PC)
	29 pS (140 mM KCl)*		Transfected CHO-K1 cells
	60 pS (300:50 mM KCl)**	Anion-selective	Planar bilayers (Asolectin or PE/PS)
	120 pS (300 mM KCl)*	$P_{Cl^-} > P_{K^+}$	
	31 pS (substates of 8 pS, 14 pS, 21 pS) (140 mM KCl)*		Tip dip (PC)
	28 pS (140 MM KCl)*		Tip dip (DPPC)
	6 pS (127.5 mM *N*-methyl glucamine Cl:140 mM NaCl)**		Microglia and BV-2 cells
	24 pS, 25 pS, 38 pS (500:50 mM KCl)**	Non-selective $P_{I^-} > P_{SCN^-} \geqslant P_{Cl^-} P_{NO_3^-} \geqslant P_{Br^-} \geqslant P_{F^-}$	Planar bilayers (POPE, POPS and cholesterol)
CLIC2	48 pS (140 mM KCl)*		Tip dip (PC)
CLIC4 (p64H1)	1 pS (300:140 mM CholineCl)**		Patch clamp HEK-293 cells
	10 pS (300:140 mM CholineCl)**	Poorly-selective	Planar bilayers (PE and PS)
	30 pS, 58 pS, 86 pS (140 mM KCl)*		Tip dip (PC)
	2-15 pS (500:50 mM KCl)**	Non-selective $P_{Cl^-} = P_{SCN^-} = P_{NO_3^-} = P_{I^-} = P_{Br^-} P_{F^-}$	Planar bilayers (POPE, POPS and cholesterol)
CLIC5A (p62)	3 pS, 5 pS, 7 pS, 11 pS, 20 pS, 29 pS, 116 pS (500:50 mM KCl)**	Non-selective*** $P_{Na^+} = P_{K^+} \geqslant P_{Cl^-}$	Planar bilayers (POPE, POPS and cholesterol)
CLIC5B (p64)	26 pS, 100 pS, 400 pS (140 mM KCl)*		Planar bilayers (Asolectin)
	100-150 pS (140 mM KCl: 140 mM K-gluconate)**		Planar bilayers (soyabean phospholipid)
	42 pS (140 mM KCl)*	Anion-selective $P_{Cl^-} > P_{K^+}$	Planar bilayers (Asolectin)
EXC4	38 pS (140 mM KCl)*		Tip dip (PC)
*Dm*CLIC	31 pS, 57 pS, 87 pS (140 mM KCl)*		Tip dip (PC)

pore (Fig. 2), targets the protein to the membrane and also plays an active role in ion transport. Even though it is established that some CLICs can form functional ion channels, biophysical studies of CLICs are still in their infancy. This is chiefly due to limited electrophysiological techniques for examining intracellular ion channels, and the unavailability of a method to directly access intracellular organelles. Most of these studies have been carried out by several groups in artificial bilayers utilizing recombinant or over expressed proteins. The first evidence of a CLIC1-mediated current was shown in CLIC1 transfected CHO-K1 cells through cell and nuclear patch clamp [52]. Later, CLIC1 channels were recorded from transfected CHO-K1 cells, and they exhibited a single channel conductance of 8 pS (at 130 mM symmetrical NaCl) with slight inward rectification [53]. Endogenous CLIC1 channels (which were inhibited by IAA-94) have been observed following nuclear fragmentation during the G2/M phase of mitosis [53]. These native channels had similar biophysical properties as the expressed proteins in cells, implicating CLIC1 as an endogenous cellular ion channel. The purified CLIC1 protein has been reconstituted by several independent groups [20,50,54,78] to show that CLIC1 forms functional ion channels in the absence of any ancillary protein. The single channel conductance of reconstituted CLIC proteins differ between independent studies (summarized in Table 1), which was attributed to the lipid environment [20]. CLIC1 and CLIC4 can insert into monolayers containing different lipids or lipid mixtures, but consistent channels were always obtained in well-defined planar bilayers containing cholesterol. Similarly, low pH and oxidation were shown to enhance the binding of CLIC1 and CLIC4 to artificial lipid bilayers [15,39,46]. CLIC4 was earlier localized to specific cholesterol rich domains of membranes such as caveolae [42]. Channel formation in cholesterol rich bilayers suggests that CLICs may only be functional in specific cholesterol rich microdomains in membranes. These domains may facilitate the insertion and proper refolding of CLICs to form functional channels. CLIC2 [68], CLIC4 [10,18,39,40], CLIC5A [34], CLIC5B [6,79,80]. EXC4 [15] and *Dm*CLIC [15] were also reported to form functional ion channels but the conductance of these channels varies in different systems and within the same system [34]. The single channel conductance of CLIC1, CLIC2, CLIC4, CLIC5A, CLIC5B, EXC4 and *Dm*CLIC are summarized in Table 1 along with their recording conditions.

The single channel conductance of CLIC1 varied from 6 pS to 120 pS [20,25,46,49,50,52–54,71,78], CLIC4 from 1 pS to 86 pS [10,18,39,40], *Dm*CLIC from 30 pS to 87 pS [15], CLIC5A [34] from 3 pS to 120 pS and CLIC5B from 26 pS to 400 pS [6,79–81] (Table 1). The difference in single channel conductance is an open question, and could be attributed to the presence or absence of cholesterol in bilayers, redox conditions or pH of the recording solution. However, these reasons could not single handedly explain the differences in the conductances of CLIC5A under the same conditions [34]. Two distinct substates of CLIC channels were reported by several independent studies [15,18,20,34], and similar substates were shown in ion channels reconstituted from rat brain microsomes [82] and sheep heart mitochondria [83]. As stated earlier, this was interpreted in terms of a model with a minimum of four conducting "protomers" displaying different gating mechanisms, depending on the number of protomers open at any given instant. If a similar model is to be predicted for CLIC1 and CLIC4, a minimum of 16 subunits are required per channel (four per "protomer", since each subunit contains a single TMD, Fig. 2B and D). Similarly CLIC5A and CLIC5B will have multiple "protomers" functioning together to give rise to large conductance channels (116 and 400 pS,

respectively). This model can explain the association of groups of highly cooperative subunits into even larger structures.

So far, all electrophysiological studies carried out in planar bilayers have indicated that CLIC proteins predominantly form non-selective ion channels [16,18,20,34] with different conductance levels (Table 1). Selectivity is occasionally determined by measuring either the relative permeability or the relative conductance of a channel for a range of ions. To measure the relative permeability, reversal potentials for currents and sub conductance levels are measured in solutions of known ionic composition using the Goldman–Hodgkin–Katz (GHK) equation. The relative conductance is measured by obtaining the amplitude of single channel currents in comparable concentrations of a range of permeant ions. The channel is best described when either relative permeability or relative conductances are known. Unfortunately very limited data are available for CLIC proteins covering both aspects. Several groups have measured the selectivity of recombinant CLIC1, CLIC4 and CLIC5 by reconstituting them in artificial bilayers or by patch clamp (Table 1). Their selectivities differ remarkably; they are either non-selective in the case of *At*DHAR1 [16], CLIC1 and CLIC4, or fail to differentiate between cations and anions as observed for CLIC5 [18,20,34]. The selectivity of CLICs significantly increased from non-selective to more anion-selective when a large cation such as Tris was used [20]. These independent observations prove that CLICs are predominantly non-selective channels. Alternatively, reconstituted CLICs in the bilayers were missing the critical cellular components interacting with the protein. These protein interactors may be playing a key role in determining the selectivity of the channel. Further studies are required to identify these components, and decipher their functional roles.

How CLICs facilitate the passage of ions across the bilayer, their protein architecture and shape, and how these conformations are related to the pore across the bilayer still need to be addressed. It has been established that K^+ and Cl^- channels are multi-ion pores, which use the mutual repulsion between neighboring ions to lower the barriers for ions diffusing through the channel. A helical wheel alignment of four subunits reveals two distinct rings of positively charged residues at the neck and the bottom of the putative pore (Fig. 2). The arginine and lysine residues lining the pore region (Fig. 2C) could bind to anions and provide an opportunity for counter ions to cross the membrane without encountering repulsive forces as shown in the neuronal background Cl^- channels in the rat hippocampal neurons [84,85]. In the absence of a crystal structure, the pore size is usually determined by using ions of different size; depending on the permeability of an ion, pore size is measured. In case of CLICs, this approach was used with the limitation of availability of large anions. Iodide ions (2.2 Å) were more permeable through CLICs as compared to fluoride ions (1.36 Å). In the presence of large cations, the selectivity of CLIC proteins significantly increased, consistent with the predicted model for the channel pore [18,20]. The poor-selectivity can be attributed to a wide pore lacking specific ion binding sites, but the maximum conductance values of CLIC1 (120 pS), CLIC4 (86 pS), *Dm*CLIC (87 pS), EXC4 (30 pS), CLIC5A (78 pS) and CLIC5B (400 pS) in potassium chloride (KCl) are inconsistent with a wide water-filled pore (similar to porins). Similar behavior was observed in the neuronal "background" Cl^- channels, where it was suggested that anions and cations crossed the membrane as counter ions [84,85]. This indicates that CLICs might be one of the components of neuronal "background" chloride channels.

4. Function

Several studies have shown that CLICs behave as atypical ion channels and function as intracellular ion channels with limited physiological roles. CLIC proteins have been implicated in several important functions such as membrane trafficking, cytoskeletal function, apoptosis, cell cycle control, mitosis, and differentiation. However, these roles are not attributed to the soluble or membranous form of the protein. Regulation of CLIC insertion into membranes from a soluble pool could be a mechanism for the regulation of ion permeability in the cytoplasm. How CLICs perform these functions, their signaling pathways, and the form (soluble or membranous) involved in each of these functions are not well understood.

CLIC1 protein has been extensively studied and its functional roles have been elucidated in different systems. The expression of CLIC1 changes in different stages of cell cycle indicating that it is involved in cell-volume regulation [53]. CLIC1 is widely expressed in epithelial and non-epithelial cells where it exhibits tissue specific distribution and sub-cellular localization. In columnar epithelial cells, CLIC1 is expressed in apical domains [51], whereas in placental trophoblasts, it is distributed in the cytoplasm [32]. This vast and diverse distribution indicates the distinct roles played by CLIC1 and other CLIC proteins, such as in endocytosis, exocytosis, cell signaling, acidification, cell cycle and possibly during pulmonary arterial hypertension (PAH) [86]. In human endometrium, CLIC1 is localized to the luminal and glandular epithelium, and this distribution changes within proliferative and secretary phases [87] during 'plasma membrane transformation'. CLIC1 is up-regulated in Alzheimer's brain where Aβ promotes channel formation in the plasma membrane to mediate Cl^- currents [47]. This regulation and translocation of CLIC1 is NADPH oxidase-derived reactive oxygen species (ROS) regulated. CLIC channels were also shown to be regulated by redox potential from the luminal side [20] and CLIC1 under physiological conditions is present in the cytosol and intracellular organelles in the cerebellar granule neurons (Singh et al., unpublished data). Oxidative stress and ROS generation in the central nervous system (CNS) are believed to be responsible for neurodegeneration in Alzheimer's disease and other neurological disorders. Translocation of CLIC1 by redox regulation or Aβ stimulation [25] and formation of ion channel in the membranes could qualify CLIC1 as a novel potential therapeutic target in Alzheimer's disease. Even though CLIC1 is highly conserved and expressed widely, CLIC1 KO mice (*clic1*$^{-/-}$) did not show any embryonic lethality but had a mild bleeding disorder [88]. The average bleeding time of KO mice was significantly longer despite having 15% higher basal platelet count. The rate of adenosine diphosphate (ADP)-stimulated platelet aggregation was lower in *clic1*$^{-/-}$ and was dependent on P2Y12 but not P2Y1 receptors [88]. Bleeding disorder and defect in ADP/P2Y12 -induced platelet activation in *clic1*$^{-/-}$ points to the role of Ras homolog gene family, member A (RhoA) signaling in the function of CLICs, because ADP activates RhoA downstream of Gq, and $G_{12/13}$ pathways, and regulates granule release and fibrinogen receptor activation in platelets [89].

CLIC4 is a key player in p53- and cMyc-mediated apoptosis and it translocates to the nucleus under metabolic stress, growth arrest and apoptosis [28]. Over-expression of CLIC4 results in changes in the mitochondrial membrane potential, cytochrome *c* release, and caspase activation resulting in apoptosis [27]. TNFα increases CLIC4 expression and promotes its translocation to the nucleus, independent of p53 [26,28]. Expression of CLIC4 is also elevated in myofibroblasts that form stroma in breast cancers [22], and it has been shown that CLIC4 expression and its association with Schnurri-2 as well as its translocation to the nucleus are all promoted by transforming growth factor (TGF-β) [41]. This nuclear localization is an essential component of pro-apoptotic function of CLIC4 and growth arrest activity in which TGF-β signaling plays an essential role [41]. The role of CLIC4 in cancer and apoptosis has been well established, and CLIC4 might be a potential target for

drugs and cancer therapies [29,90]. In mitochondrial DNA (mtDNA) depleted cells, CLIC4 was shown to be expressed and regulated by membrane potential through cAMP response element binding (CREB)-regulated p53 transcriptional activity, predicting the role of CLIC4 in membrane potential generation [91]. Recently, an invertebrate CLIC, EXC4 has been implicated in the cell-hollowing tubulogensis of the excretory cell of *C. elegans* [14], and CLIC4 has been shown to be involved in vascular endothelial growth factor (VEGF)-induced tubulogenesis in mammalian endothelial cells [92]. Normal levels of CLIC4 expression promote endothelial cell proliferation and regulate endothelial morphogenesis whereas increased expression promotes proliferation, network formation, capillary-like sprouting, and lumen formation in human umbilical venous endothelial cells [23]. Upon stimulation of G_{13}-coupled, RhoA-activating receptors by lysophosphatidic acid (LPA), thrombin or sphingosine-1-phosphate (S1P), soluble CLIC4 undergoes rapid transient translocation to discrete domains at the plasma membrane that contain RhoA-activating G protein-coupled receptors (GPCRs) in complex with their scaffolds, e.g., sodium-hydrogen exchange regulatory cofactor 2 (NHERF2) [74]. It was shown that CLIC4 translocation was dependent on $G_{\alpha 13}$-mediated RhoA activation and F-actin integrity, but not on Rho kinase activity implying that active RhoA-GTP accumulation is responsible for the translocation. Surprisingly, CLIC4 translocation to the plasma membrane did not modulate Cl^- currents and an N-terminal HA-tag of membrane-targeted CLIC4 failed to stain with anti-HA antibody, indicating that N-terminus is not located towards the external side or the protein is associated but is not inserted in the membrane as required to form an ion channel. These results strongly support a model in which soluble CLIC4 acts as a Cys-dependent transferase, similar to its omega-GST homologue [74]. CLIC4 KO (*clic4*$^{-/-}$) mice exhibit defective angiogenesis, and it was shown that CLIC4 is involved in tubulogenesis by supporting acidification of vacuoles [93]. Role of CLICs in RhoA signaling [74] may also offer an alternative explanation for the defective angiogenesis and tubulogenesis in CLIC4 KO mice [93], considering the known role of RhoA in exocyst function [94], e.g., vesicle fusion and actin dynamics.

Besides CLIC1 and CLIC4, other CLIC proteins are shown to play crucial physiological roles. CLIC2 proteins are widely distributed in the heart and skeletal muscles and modulate Ryanodine Receptors 1 and 2 (RyR1 and RyR2). CLIC2 facilitates the population of submaximal conductance levels by RyR Receptors [64] by facilitating [3H] ryanodine binding to the skeletal sarcoplasmic reticulum (SR) and purified receptors [95]. This effect of CLIC2 was abolished in the presence of a CLIC2 specific antibody [61]. RyR receptors are predominantly present in the calcium storage/release organelles [96], and in order to directly modulate RyR, CLIC2 should be localized in the vicinity of these receptors. The functional roles of CLIC2 other than modulating RyR are not known. It was also shown to have low glutathione peroxidase activity similar to the GST structural family (GSTA2-2 and GSTT2-2). CLIC2 is located on chromosome region Xq28 [65], the region involved in several hereditary disease conditions such as mental retardation and X-linked epilepsy. This could make CLIC2 an important candidate of medical relevance if it is expressed in substantial amounts in central nervous system. CLIC3, interacts with extracellular signal-regulated kinase (ERK7) [57], a MAP kinase family member; it is present on the placental and fetal membranes, and its distribution is regulated during trimesters [56]. It may be involved in Cl^- movement and regulation of cellular processes. Since ERK7 is known to inhibit DNA synthesis, it may be possible that CLIC3 is involved in transcription regulation either via directly interacting with ERK7 or by conducting ions, and regulating osmolarity and intracellular pH in the cells.

The *clic5* gene undergoes alternative splicing at the first exon, giving rise to CLIC5A and CLIC5B. CLIC5A is known to interact with cytoskeletal elements and its channel activity is regulated by F-actin. On formation of F-actin, CLIC5A activity is inhibited either through direct blocking of the channel pore or by removing the channel from the membrane [34]. CLIC5A protein was also absent in F-actin-containing stress fibers, indicating that F-actin prevents channel insertion or localization in its vicinity. Interestingly, CLIC5A promotes the assembly of an F-actin containing complex *in vitro* and colocalized with ezrin in the apical microvilli [33]. CLIC5B (p62) appears to be the human orthologue of bovine p64, and is expressed in osteoclasts and bone marrow cells where it is necessary for Cl^- conductance, acidification and bone resorption. It interacts with the SH2 and SH3 domains of c-Src and is associated with a kinase activity [97]. CLIC1, and CLIC5 also interact with F-actin, and actin polymerization regulates the ion channel activity of these channels [34], indicating that CLICs are specifically activated when the cell undergoes physiological changes such as cell cycle, apoptosis or tubulation. Mice homozygous for a spontaneous recessive mutation of the *clic5* gene (*jitterbug*), exhibit impaired hearing and vestibular dysfunction due to dysmorphic stereocilia and progressive hair cell degeneration [31]. CLIC5 was shown to associate with a radixin–actin cytoskeletal complex in inner ear stereocilia where it may help form or stabilize connections between the plasma membrane and actin bundle of stereocilium [31]. In stereocilia, CLIC5 may play a role in cytoskeletal assembly and maintenance, protein localization, or signal transduction.

CLIC6 has two isoforms (A and B) and is the longest amongst the CLIC family with 704 amino acids having decapeptide repeats [58]. The C-terminus of CLIC6 has INTCLCCHANNEL finger print composed of five peptide domains and was also shown to participate in dimerization [59]. CLIC6 was localized in water and hormone secreting cells; and forms a multimeric complex with D2-like receptors and scaffolding proteins [59,60]. This implies that CLIC6 could be involved in the regulation of water and hormone secretion in cells.

5. Future prospects

CLIC proteins are new molecules in the ion channel field and there are several unsolved questions, which require to be addressed. Solving the crystal structure of the membranous form of CLIC proteins remains the single most important goal for the future studies of CLIC biophysics in addition to determining the physiological roles of the channel form. It will be extremely important to obtain the structure of CLICs that are relevant to functional channels, with predictable electrophysiological properties, and not the structure of proteins that simply insert into the membrane, giving rise to inconsistent ion channels. Meanwhile, it is essential to carry out structure–function studies using site directed mutagenesis to gain insight into the molecular determinants of the functional ion channel. Systematic structure–function studies will reveal the gating region and significant residues involved in channel activity. The nature of the ion channels formed by CLIC proteins and their functional relevance is the major priority. These channels display a unique non-canonical architecture and unusual properties, and remain an appealing puzzle to solve in the ion channel field. Since they are homologues of GST proteins, it will be interesting to study whether these proteins are evolved GSTs with auto-inserting properties. One key question is whether CLICs and GSTs are functionally related and if they are related, what is the physiological significance of this relation. Do CLICs have enzymatic activity similar to GSTs? The putative transmembrane domain of CLICs is absent in GST proteins and this could be the reason for the absence of any channel like activity amongst the latter. In order to test this idea, it will be interesting to study whether the replacement of equipositional residues of GSTs with putative

transmembrane segment of CLICs could confer ion channel function to the chimeric proteins.

CLICs are synthesized without a signal sequence and also bypass the secretory pathway to directly insert into the membranes akin to many toxins, which is unique for eukaryotic proteins. They exist in soluble and membranous forms but how these proteins unfold from the soluble state, refold and insert into the membranes is an open question. It has been recorded that several factors such as redox effects, lipid charge, low pH, and membrane composition are critical factors for this change in phase transition. Changes due to pH were well documented where β-strand 2 undergoes structural transitions to form an α-helix, but the roles of other factors are to be investigated. It is important to clearly assign functions to the soluble and membranous forms of the channel. How these two forms work interdependently as well as in a regulated manner, and how the balance between two states is modulated are yet to be studied. It is possible that CLICs exist in soluble form and in response to certain pro-apoptotic conditions insert into the membranes where they may or may not function as ion channels. Apart from CLIC2 and CLIC4, majority of biological functions of CLICs are associated with cellular processes occurring at the plasma membrane. Are CLICs also involved in the functions of intracellular membranes? To answer this question, it is important to develop and improvise techniques to study the functional role of intracellular membrane associated proteins such as CLICs.

CLICs can form functional redox-regulated ion channels with multiple conductances, and substate levels, consistent with pores formed by oligomers which can assemble with variable stoichiometry also observed in antiapoptotic Bcl-2 family [98]. It has been shown that CLICs are regulated by cytoskeletal elements as well as redox potential, which provide an insight into the functional relevance of ion channels *in vivo*. It is not known whether CLICs can form functional ion channels *in vivo* and what are their regulatory mechanisms. There is an imperative need to explore the biophysical properties of the channel. This could be achieved by incorporating highly efficient screening techniques such as directly recording ion channels in the native membranes by patching mitochondria or other intracellular organelles.

KO models or CLIC1, CLIC4 and CLIC5 were not lethal even though these proteins are widely distributed. This could be due to considerable abundance of other CLIC family members, and they may be compensating for the removed CLIC protein. Systematic removal of all the CLIC proteins could elucidate the possible role of these proteins. Information on protein interactors of CLICs and their role in channel regulation is limited. It is extremely important to investigate the protein interactors *in vitro* and *in vivo* using biochemical approaches [99], and study the functional significance of these interactions. It will be interesting to study the role of CLICs in cytoskeletal filament polymerization and anchoring to the membrane. The consistent channel activity is associated with specific lipids, but how lipids regulate the protein insertion and channel formation is not known. CLICs are implicated in several pathophysiological conditions and it is important to consider them as potential drug targets similar to other ion channels. Extensive information is available on CLIC function, their physiological roles, and genetics. But the gap between the identification of CLICs, the functional importance and the possible drug targets is increasing. Identification of channel modulators, blockers and other drugs acting on CLICs can offer promise for the treatment of several disorders and diseases such as cancer, cystic fibrosis, Alzheimer's and arthritis.

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