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Anion Channels of Mitochondria

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Abstract

Mitochondria are the “power house” of a cell continuously generating ATP to ensure its proper functioning. The constant production of ATP via oxidative phosphorylation demands a large electrochemical force that drives protons across the highly selective and low-permeable mitochondrial inner membrane. Besides the conventional role of generating ATP, mitochondria also play an active role in calcium signaling, generation of reactive oxygen species (ROS), stress responses, and regulation of cell-death pathways. Deficiencies in these functions result in several pathological disorders like aging, cancer, diabetes, neurodegenerative and cardiovascular diseases. A plethora of ion channels and transporters are present in the mitochondrial inner and outer membranes which work in concert to preserve the ionic equilibrium of a cell for the maintenance of cell integrity, in physiological as well as pathophysiological conditions. For, e.g., mitochondrial cation channels K_{ATP} and BK_{Ca} play a significant role in cardioprotection from ischemia–reperfusion injury. In addition to the cation channels, mitochondrial anion channels are equally essential, as they aid in maintaining electro-neutrality by regulating the cell volume and pH. This chapter focusses on the information on molecular identity, structure, function, and physiological relevance of mitochondrial chloride channels such as voltage dependent anion channels (VDACs), uncharacterized mitochondrial inner membrane anion channels (IMACs), chloride intracellular channels (CLIC) and the aspects of forthcoming chloride channels.

Keywords

Anion channels; Chloride intracellular channels (CLICs); Inner membrane anion channel (IMAC); Mitochondria; Mitochondrial permeability transition pore (mPTP); Uncoupling protein (UCP); Voltage dependent anion channel (VDAC)

1 Introduction

Typical ionic concentration and composition of the cytosol dramatically differ from the extracellular environments. All the cells from prokaryotes to eukaryotes maintain a cytosolic pH of ~7.2, with concentration of potassium ions (K^+) many fold higher than sodium (Na^+)

and calcium (Ca^{2+}) ions (Bose et al. 2015; Prindle et al. 2015). In addition to cations, several organic and inorganic anions such as halides exist in cytosol to counter cations and also play an active functional role in cells. Intracellular ions exist in either free or remain bound to cytoplasmic proteins; however, they are classically divided by intracellular membranes, and are enclosed in intracellular organelles. The distribution of intracellular ions in different cellular compartments as well as cytosol is chiefly governed by Gibbs–Donnan effect (Sollner 1955), and is responsible for maintaining physiological functions of the cell.

Cell membranes possess a wide array of ion transporters and channel proteins in order to maintain physiological ionic concentrations (Jentsch et al. 2004). The ionic gradients and membrane potentials across cellular membrane (plasma and intracellular organelle) are key drivers of several physiological processes such as neuro-transmission, cardiac conduction, and muscle contraction and relaxation (Jentsch et al. 2004). Intracellular ion transport is important for cell cycle-regulation, energy production, cellular signaling, and apoptosis (Leanza et al. 2013). Ion transport across the plasma membrane has been discussed in several reviews (Feske et al. 2015; Bates 2015; Singh et al. 2012a; Toro et al. 2014), and therefore in this chapter, emphasis is on the ion channels across mitochondrial membranes.

Several models of energy production in the mitochondrion indicate that the presence of an ion channel on mitochondrial membrane would lower efficiency and effectiveness of the process (O'Rourke 2007). In addition, theoretical opening of any ion channels, for example, a $\text{mitoBK}_{\text{Ca}}$ will readily depolarize mitochondrial membrane within few nanoseconds, hence supporting the earlier hypothesis that mitochondria should not possess ion channels (Singh et al. 2012a; O'Rourke 2007). At the same time, it is well-recognized that membrane potential of inner mitochondrial membrane (IMM) is ~ -180 mV (mV) (Colombini 2012; Colombini and Mannella 1818; O'Rourke et al. 2005). However, when compared to the electric potential of plasma membrane (~ -70 mV), mitochondrial membrane seems to be a better capacitor in storing charge. To further signify the effectiveness of mitochondrial membrane potential (ψ_{mito}), we can precisely calculate the charge stored by these cellular capacitors. It is known that the IMM is ~ 7.0 nm thick (Perkins et al. 1997) thus the voltage gradient across the IMM is 0.180 V per 7.0×10^{-7} cm. This is equivalent to 257,142.90 V per cm which is significantly higher than high-voltage transmission electricity supply lines employing voltage gradient of about 200,000 V per km (and higher to the plasma membrane voltage gradient of 200,000 V per cm as well) (Lodish et al. 2000). Majority of the membrane potential across the mitochondrial inner membrane is generated by proton transport which is directly coupled with ATP production.

Increasing evidence indicate that both outer mitochondrial membrane (OMM) and IMM possess ion channels (Szabo and Zoratti 2014). As stated earlier, until 1980s, scientific community was highly skeptical and divided over existence of typical ion channels in mitochondria (Colombini and Mannella 1818; O'Rourke 2000; Colombini 1979). However, isolation of voltage dependent anion channel (VDAC) from paramecium, and reconstitution in lipid bilayers in 1976 set the ground for mitochondrial ion channels (Schein et al. 1976). This was immediately followed by characterization of permeability transition pore (PTP) (Hunter et al. 1976), and in the last 40 years several ion channels of OMM and IMM were established (Szabo and Zoratti 2014). More recently, mitochondrial ion channels are shown

to be the key modulators involved in protection from ischemia–reperfusion (IR) injury, apoptosis, and several other pathophysiological disorders (O’Rourke 2007; O’Rourke et al. 2005; Szabo and Zoratti 2014; O’Rourke 2000). Even though anion channels (VDAC) were the first one to be described in mitochondria (Colombini 1979), majority of the physiological functions are attributed to the cation channels (Szabo and Zoratti 2014). This is chiefly due to non-availability of specific pharmacological agents for anion channels and lack of molecular identity of mitochondrial anion channels residing in IMM.

Much like plasma membrane anion channels, mitochondrial anion channels are proteinaceous pores which allow passive diffusion of negatively charged ions down their electrochemical gradient. Anion channels may conduct other anions (F^- , Br^- , I^- , or NO_3^-) better than Cl^- but they are often referred as Cl^- channels, as Cl^- is the most abundant anion, predominantly permeating under physiological conditions (Ashley 2003). Apart from VDAC which resides in the OMM (Colombini and Mannella 1818; Colombini 1979), chloride intracellular channel 4 (CLIC4), and CLIC5 (Ponnalagu et al. 2016a), molecular identity of other inner mitochondrial anion channels is not known. Also classification of anion channels is still ambiguous (Roelfsema et al. 2012). Previous attempts to classify them on the basis of their localization (plasma membrane vs. intracellular membranes), single-channel conductance, and/or regulatory mechanism could not comprehend the diversity of anion channels (Verkman and Galietta 2009). We believe that mitochondrial anion channels can be systematically classified on the basis of their localization, outer membrane anion channels and inner membrane anion channels (IMACs), as well as on the basis of their selectivity. As anion channels are known to be permeable for other ions, we propose to classify them as anion-selective anion channels [ASACs, non-selective for anions], cation-selective anion channels (CSACs, prefer cation over anions), and non-selective anion channels [NSACs, not selective for cations or anions]. These channels will be discussed in detail in following sections.

In this chapter, we have presented an overview of mitochondrial anion channels, investigated their mitochondrial localization as well as distribution, and also show how some of the known anion channel inhibitors affect mitochondrial reactive oxygen species (ROS) generation.

2 Anion Channels of Outer Mitochondrial Membrane

2.1 Voltage Dependent Anion Channels

VDAC, an intrinsic membrane channel, is the first channel to be reconstituted and studied at the single-channel level (Colombini and Mannella 1818; Schein et al. 1976). It is a highly conserved protein, homologous to bacterial porins and is localized in the OMM (Colombini 2012; Mertins et al. 2014; Neumann et al. 2010) as also observed in the mitochondria of rat cardiac myogenic cell line, H9C2 (Fig. 1). Geographical distribution of VDAC1 by stimulated emission depletion (STED) nanoscopy also indicates their unique clusters at nano-resolution in H9C2 cells (Fig. 1) as observed earlier in U2OS cells transfected with VDAC (Neumann et al. 2010). It is the principle mode of transport of ions and metabolites across the OMM, and also a probable contact point between the inner and outer membrane (Colombini 1979). VDAC had a serendipitous discovery; it occurred as a result of failed

attempts in characterizing voltage-gated calcium sensitive channels from *Paramecium aurelia* (Colombini and Mannella 1818). It is widely distributed in many species across the taxon, yeast have two (POR1 and POR2), and vertebrates have three isoforms (VDAC1, VDAC2, and VDAC3) (Szabo and Zoratti 2014). Humans, like all other vertebrates canonically encode three different isoforms. It is a well-characterized mitochondrial anion channel in terms of structure and function. Amongst the various isoforms, VDAC1 was the first one to be discovered. VDAC1 and VDAC2 have very strong poreforming characteristics, while VDAC3 forms smaller conductance channels (Cecchetto et al. 2014) and consequently modulates the physiological functions of other proteins.

2.1.1 Structural Insights—VDAC, a small, >30 kDa protein consisting of 280 amino acid residues, shows high sequence homology (>80 %) within the same species. Using X-ray crystallography and nuclear magnetic resonance (NMR), three independent studies (Hiller et al. 2008; Ujwal et al. 2008; Bayrhuber et al. 2008) have revealed that VDAC forms β -barrel structures similar to bacterial porins, comprising of 19 unique β -strands connected with 18 loop-like connections, and a short N-terminal α -helix between residues 7 and 17. All the β -barrel strands are oriented in the anti-parallel fashion except strands 1 and 19 which are parallel to each other and close the β -barrel structure (Ujwal et al. 2008). The height and width of the β -barrel were reported to be 35 Å and 40 Å, respectively (Ujwal et al. 2008), and the path for solute passage measures ~15 Å by 1 Å. The N-terminal β -barrel strands (1–4) contribute to conformational stability of VDAC whereas Glu73 is a key residue determining the structural stability of the protein. This residue is conserved in all the VDAC isoforms present in humans. The N-terminal helix folds up inside the β -barrel wall to reach the midpoint of the hydrophobic portion of the membrane. The positively charged residues of the helix face the interior of the β -barrel, while the negatively charged residues interact with the most conserved residues in the β -barrel wall of VDAC proteins (Bayrhuber et al. 2008). As a result the N-terminus is a site for many molecular interactions. The preceding N-terminal region is lightly attached to the β -barrel wall and orients towards the pore outlet (Bayrhuber et al. 2008). Each loop contributes a specific role in channel-gating, ion-selectivity, pore size, and structural stability of VDAC. The long loops fold into the pore lumen and determine the ion-selectivity and the gating of the channel. The short loops provide structural support by linking with the adjacent β -strands. The stability of β -strands is also important for the voltage-gating, and protein–protein interactions of VDAC. Similar to other porins, the C-terminal region extends to the intermembrane space (IMS) (Bayrhuber et al. 2008; Schulz 2002). Various studies indicate the ability of VDAC to form oligomers (Malia and Wagner 2007). Using crystallographic symmetry operators, a model for dimeric form of VDAC was constructed which indicated that the dimeric interface residues reside in β -strands, β 1 (Ile-27, Leu-29), β 2 (Glu-50, Thr-51), β 18 (Leu-257, Leu-259), and β 19 (Leu-277) (Bayrhuber et al. 2008).

2.1.2 Electrophysiological Properties—The channel properties of VDAC have been studied mainly by using planar bilayer systems. VDAC forms a large voltage dependent pore with maximum conductance of 4–5 nS in symmetrical 1 M KCl (Szabo and Zoratti 2014; Colombini 2004; Bera and Ghosh 2001). At lower holding potentials of 20–30 mV, VDAC exists in fully open state and exhibits weak anion-selectivity, whereas at higher holding

potentials, it is cation selective, and the conductance observed is approximately half of the original conductance shown by the “closed” state of the channel (Szabo and Zoratti 2014). Exposed charge residues of the pore are the key determinants for anion selectivity. Amino-terminal sequence determines the voltage-induced gating of VDAC (Mannella 1997; Shoshan-Barmatz et al. 1797). Although channel activity is observed in bilayers, a consistent conductance is not yet recorded through patch clamping studies (Kinnally and Antonsson 2007). VDAC possesses a binding site for Ca^{2+} (Gincel et al. 2001) and also transports calcium (Ca^{2+}), ATP, and superoxides. The open state of the channel poorly conducts Ca^{2+} ($P_{\text{Cl}^-}/P_{\text{Ca}^{2+}} \sim 25$), while partially closed state still prefers Cl^- over Ca^{2+} , with a $P_{\text{Cl}^-}/P_{\text{Ca}^{2+}}$ as 1–4.5 (Szabo and Zoratti 2014; Tan and Colombini 1768). Mitochondrial VDAC-like conductance was also observed in excised patches of plasma membrane (Bahamonde et al. 2003) of neuroblastoma cells. Thus, suggesting VDAC as a molecular correlate of the plasma membrane maxi Cl^- channels (Bahamonde et al. 2003). In another study, electrophysiological and immunocytochemical evidences showed the activation of VDAC in plasma membrane of neurons during apoptosis (Elinder et al. 2005). These observations are supported by the identification of a VDAC isoform (pl-VDAC) containing a leader sequence for its trafficking to the plasma membrane (Buettner et al. 2000; De Pinto et al. 2010) and also the presence of VDAC in caveolae (Bahamonde et al. 2003).

2.1.3 Regulation of VDAC—VDAC is regulated by many cellular and extracellular factors. It gets phosphorylated by Ser-Thr kinases GSK3 β (Das et al. 2008; Sheldon et al. 2011), tyrosine kinase (Salvi et al. 2005), protein kinase A (PKA) (Banerjee and Ghosh 2006), and nima-related kinases (NEK) (Chen et al. 2009; Chen et al. 2010). Phosphorylation regulates the apoptotic activity of VDAC via modulating its interaction with cytoskeletal components (Kerner et al. 1818). However, phosphorylation does not affect the current magnitude and the open probability in the positive clamping potentials, but lowers both in the negative clamping potentials (Bera and Ghosh 2001). Further, it has been shown that phosphorylation of VDAC by JNK3 leads to channel closure and thereby causes cell death (Gupta and Ghosh 2015). VDAC has been shown to interact with multiple factors ranging from pyridine dinucleotides (Lee et al. 1996; Zizi et al. 1994), lipids including cholesterol (Betaneli et al. 2012; Rostovtseva and Bezrukov 2008), Bcl-xL (Malia and Wagner 2007; Arbel et al. 2012), creatine kinases (Beutner et al. 1996; Brdiczka et al. 1994), Ca^{2+} , etc. Interaction of VDAC with tubulin (Sheldon et al. 2011) results in its increased sensitivity to voltages causing a channel closure at potentials as low as 10 mV. It also has been known to be inhibited by gelsolin, a Ca^{2+} -dependent actin-binding regulatory protein, thereby exerting anti-apoptotic effects (Kusano et al. 2000).

2.1.4 Functions—VDAC has been extensively studied with respect to its role in apoptosis. It is known to interact with Bax and Bak to form a large pore and stimulate efflux of cytochrome c (Shimizu et al. 2000; Shimizu et al. 1999; Banerjee and Ghosh 2004) which is a hallmark of apoptosis. Although, VDAC2 is shown to be pro-apoptotic, facilitating sensitivity to t-Bid (Yamagata et al. 2009), its deletion in lymphocytes was fatal (Cheng et al. 2003) but was rescued by deletion of Bak, reasserting VDAC2's role in apoptosis. There are contradictory reports arguing VDACs (VDAC1 and VDAC3) role in apoptosis; mouse embryonic fibroblasts (MEFs) lacking either one of these isoforms showed no response to

their apoptotic response to Bax overexpression and also showed no role of VDAC in cytochrome c release (Baines et al. 2007). Bcl-2 family members like BclxL inhibit VDAC's activity and exhibit their anti-apoptotic activity (Arbel et al. 2012) via interaction with their N-terminal segment (Malia and Wagner 2007; Abu-Hamad et al. 2009). Overexpression of plant VDACs in jurkat cells (T cell line) induced apoptosis that can be blocked by Bcl-2 and the VDAC inhibitors (Godbole et al. 1642). VDACs have also been widely studied in cancers due to their association with hexokinase 1 (HK1) and hexokinase 2 (HK2) which are over-expressed in glycolytic cancers (Wolf et al. 2011). HKs via binding to VDAC1 provide a metabolic benefit to cells, and suppress apoptosis, causing a cell proliferative advantage and increased resistance to chemotherapy (Shoshan-Barmatz et al. 1848). However, the association of VDAC and HK is also regulated by cyclophilin D (CypD) (Machida et al. 2006) and its acetylation status which is in turn modulated by deacetylases like sirtuin-3 (Shulga et al. 2010; Verma et al. 2013). VDAC is prone to oxidative damage when its tyrosine residues get converted to 3-nitrotyrosine by reactive nitrogen species (RNS) including NO and peroxynitrite, produced upon oxidative stress during aging. Therefore, increased VDAC nitration leads to VDAC-dependent, rapid, and massive cytochrome c release. These functions signify the very important physiological role of VDAC in cell life and death. VDAC has been arguably considered to be a component of mitochondrial permeability transition pore (mPTP) (Zoratti and Szabo 1995), as the electrophysiological property of VDAC resembled to that of mPTP (Szabo et al. 1993). It was also predicted to be in the dimeric state in mPTP complex (Szabo and Zoratti 1993). Molkentin and his group further showed that mitochondria from the VDAC null mutant mice were susceptible to Ca^{2+} and oxidative stress induced mPTP formation instigating a debatable role of VDACs in mPTP formation (Baines et al. 2007). A recent study showed the SGP7 (mitochondrial AAA protease) which is an essential component of mPTP complex interacts with VDAC (Shanmughapriya et al. 2015). These studies further suggest that VDACs role in forming mPTP complex is dispensable and probably regulates mPTP formation via interacting with modulators like Bax, Bak, and SGP7.

3 Anion Channels of Inner Mitochondrial Membranes

As stated above, it was widely believed that the inner membrane of the mitochondria is unlikely to contain ion channels, as any movement of ions across it would degenerate the proton motive force driving ATP synthesis (O'Rourke 2007). Although, later, multiple conductances were recorded in IMM, no specifics on molecular identity of these channels are known (Szabo and Zoratti 2014). The realization for the existence of ion channel in inner membrane came from early studies on mitochondrial swelling, which were induced by movement of cations and anions into the matrix compartments (O'Rourke 2007).

3.1 Inner Membrane Anion Channel: Historical Perspective and Ion Channel Properties

Around the 1960s, Azzi et al. promoted the idea of Mg^{2+} governing the permeability of the mitochondrial membrane to univalent cations, which switches to anion, upon increasing pH (Azzi and Azzone 1965; Azzi and Azzone 1967). However, mechanism for this ion extrusion was uncertain. In 1969, Brierley observed that the permeability of the inner membrane to anions is very low at acidic pH in heart mitochondria, which further increased under alkaline

conditions (Brierley 1969; Garlid and Beavis 1986). These findings supported the concept of Azzi et al., about the existence of an Mg^{2+} and pH dependent anion transport pathway in IMM (Garlid and Beavis 1986). Furthermore, the transport of several anions like Cl^- , Br^- , I^- , SCN^- , NO_3^- , PO_4^- , HCO_3^- , and SO_4^{2-} across the mitochondrial membrane was demonstrated (Garlid and Beavis 1986; Stockdale et al. 1970), thus indicating the presence of an IMAC in the mitochondria (Garlid and Beavis 1986).

3.1.1 Electrophysiological Properties of IMAC—Initial patch clamp studies performed on IMM revealed a slightly anion-selective channel with a mean conductance of ~107 pS under symmetrical (*cis:trans*) 150 mM KCl condition (Sorgato et al. 1987). It was named as mitochondrial centum picosiemens (mCS) channel. This was followed by observations of multiple anion channel conductances, on mitoplasts or mitochondrial membrane vesicles (Ballarin and Sorgato 1995; Borecky et al. 1997; De Marchi et al. 1977; Hayman et al. 1993; Kinnally et al. 1992; Klitsch and Siemen 1991) isolated from different tissues (brown adipose tissue, heart, and liver). Patch clamp studies on mitoplasts isolated from brown fat adipose tissue showed a ~108 pS conductance in symmetrical 150 mM KCl solution (Borecky et al. 1997; Klitsch and Siemen 1991). This channel was a candidate for IMAC as the channel currents were inhibited by purine nucleotides, Mg^{2+} as well as variations in pH (Borecky et al. 1997). In both liver and cardiac tissues, Sorgato et al. found a ~100 pS voltage-sensitive anion channel (Sorgato et al. 1989). In yet another study, two distinct anion channel currents were observed in sheep mitoplasts with one showing conductance of 100 pS (150 mM KCl) and the other of 50 pS (Hayman et al. 1993). Remarkably, both species of channel activity lasted for 10–15 min and then exhibited irreversible inactivation which could be a result of desensitization of these channels (Sun et al. 2002). P_{Cl^-}/P_{K^+} values for both the channels were also different. Small conductance channel was shown to be non-selective between anions. Although the channel conductances were similar to that observed by Sorgato et al. (Sorgato et al. 1987), neither of these channels were sensitive to ATP, Mg^{2+} , or pH changes, a property exhibited by IMAC.

Interestingly, three classes of IMM channel activities were recorded in rat cardiac mitochondria, using patch clamp studies (Kinnally et al. 1992; Kinnally et al. 1991). The “107 pS activity” was slightly anion-selective and voltage-dependent (opens with positive potentials towards matrix); a Ca^{2+} or voltage-dependent “multiple conductance channel” (MCC) activity exhibited a single-channel conductance ranging from ~40 to over 1,000 pS, and a “low-conductance channel” (LCC) showing conductance of ~15 pS that was receptive to pH and Mg^{2+} changes. Thus, MCC was considered responsible for the Ca^{2+} -induced permeability transition observed in mitochondrial suspensions. LCC was suggested to be similar to IMAC currents with anion selectivity in the order of $SCN^- > NO_3^- > Cl^- > P_i$. All of the IMM channels reported so far are highly regulated and have a very low open probability under physiological conditions.

IMAC currents were also observed even in yeast (both wild type and VDAC less strain), but the properties of these channels differed (Ballarin and Sorgato 1996). Here, two kinds of channel activity were recorded: a small conductance channel of ~45 pS, which decreased at negative holding potentials, and large conductance channel, whose conductance increased at positive holding potentials (~400 pS at -40 mV; ~800 pS at 40 mV). Unlike IMAC, these

channels were unaffected by matrix Ca^{2+} and Mg^{2+} . However, both showed ATP-dependence, indicated by the complete inhibition of the activity of the small channel, and the loss of voltage-dependency for large conductance channel in the presence of nucleotides. Another study revealed the existence of phosphate carrier (PIC) protein, as mitochondria purified from *Saccharomyces cerevisiae* upon reconstitution in liposomes showed anion-selective single-channel currents in patch clamp experiments (Herick et al. 1997). It exhibited a frequent conductance of $\sim 40 \pm 10$ pS, and was inhibited by higher concentration of phosphate. Although PIC had similar conductance to that of small conducting channel earlier isolated from yeast, its activity was inhibited by Mg^{2+} and Ca^{2+} unlike the later one. This further indicated the existence of multiple IMM anion channel conductances in yeast.

In addition, pH-regulated plant inner mitochondrial anion channel (PIMAC) mediated flux of Cl^- , NO_3^- , succinate, malate, and oxaloacetate was shown in potato tuber mitochondria (Beavis and Vercesi 1992), as well as in durum wheat seedlings and Jerusalem artichoke tubers (Laus et al. 2008). Like yeast IMACs, they exhibit different properties than the mammalian IMAC, because of the lack of inhibition by recognized inhibitors of mammalian IMACs. Maize IMAC shows temperature independent open probability in contrast to that of mammalian IMAC, but this level of activity varied with the cold tolerance levels of the maize population. Although these studies point out the presence of IMAC in mitochondria, more data is required to establish the molecular identity of these channels.

3.1.2 IMAC Modulators—Light scattering on swelling mitoplasts was used to describe IMAC in initial studies (Garlid and Beavis 1986; Beavis and Vercesi 1992; Halle-Smith et al. 1988; Powers and Beavis 1991; Zernig et al. 1990). IMAC has been shown to be modulated by palmitoyl-CoA ($\text{IC}_{50} \sim 2.5 \mu\text{M}$) (Halle-Smith et al. 1988), Mg^{2+} (Garlid and Beavis 1986; Beavis and Vercesi 1992), ($\text{IC}_{50} \sim 38 \mu\text{M}$), pH ($\text{pIC}_{50} \sim 7.7$) (Beavis and Vercesi 1992; Beavis 1992), *N,N'*-dicyclohexylcarbodiimide, mercurial (chloromercuribenzenesulfonate and mersalyl), and amphiphilic amines such as propranolol and triorganotins (Powers and Beavis 1991). Tributyltin (TBT), a potent triorganotin, inhibits malonate transport via IMAC at 0.9 nmol/mg and 95 % efficiency (Powers and Beavis 1991). IMAC like other chloride channels is reversibly inhibited by stilbene derivatives such as 4,4'-Diisothiocyanato-2,2'-stilbenedisulfonic acid disodium salt (DIDS) (Beavis and Davatol-Hag 1996). Other inhibitors include amiodarone, amitriptyline, dibucaine, Ro 5-4864, and thiol cross linkers, like N-ethylmaleamide. Even inhibitors of adenine nucleotide translocase (ANT) block the anion transport by IMAC (Powers et al. 1994). The plant IMACs unlike that of mammalian are not inhibited by matrix Mg^{2+} , mercurials, or *N,N'*-dicyclohexylcarbodiimide but by other well-known IMAC inhibitors such as propranolol ($\text{IC}_{50} = 14 \mu\text{M}$), TBT ($\text{IC}_{50} = 4 \text{ nmol/mg}$), and the ANT inhibitors like erythrosin B and Cibacron Blue 3GA. Ca^{2+} also modulated the activity of IMAC in mitochondrial swelling experiments (Zernig et al. 1990). In addition, benzodiazepines that inhibit mitochondrial benzodiazepine receptor (mBzR) present in the OMM also blocked IMAC activity but the mechanism of inhibition is unclear. Even though the functions of these channels are poorly understood, their tight regulation suggests their important role in maintaining physiology of the cell by possibly regulating mitochondrial function.

3.1.3 Functions—IMACs are mainly implicated in volume homeostasis. IMAC has been proposed to play a role in postischemic electrical and contractile dysfunction in the heart (O'Rourke 2007; Akar et al. 2005; Aon et al. 1762). In adult cardiomyocytes, it was observed that inhibitors of IMAC prevented reverse oscillatory mitochondrial depolarizations induced by substrate deprivation (O'Rourke 2000) and oxidative stress (Aon et al. 2003). In spite of multiple conductance observed in the IMM, it is challenging to assign specific physiological roles due to its ambiguous molecular identity.

3.2 Chloride Intracellular Channel Proteins

These are unique class of intracellular channel proteins consisting of single trans-membrane domain, and exist in dimorphic state of both soluble and integral membrane form (Ashley 2003; Singh 2010). There are seven paralogs of CLIC identified in mammals to date, namely CLIC1–CLIC6 (Singh 2010) which includes CLIC5A and CLIC5B (isoforms of CLIC5). They are also conserved among prokaryotes and widely distributed across other eukaryotic species, including three isoforms, identified in invertebrates (*DmCLIC* in *Drosophila melanogaster*, EXC4 and EXL1 in *Caenorhabditis elegans*) (Berry et al. 2003; Littler et al. 2008) and four in plants (*AtDHAR1-4* in *Arabidopsis thaliana*) (Singh 2010; Littler et al. 2010; Elter et al. 2007). CLICs, as the name specifies, are localized to intracellular organelles (Singh 2010) specifically nuclear membrane (Valenzuela et al. 1997; Ulmasov et al. 2007), secretory vesicles of hippocampal neurons (Chuang et al. 1999), caveolae (Edwards and Kahl 2010), trans-Golgi network (Edwards and Kahl 2010), endoplasmic reticulum, (Duncan et al. 1997; Ponnalagu et al. 2016b) and mitochondria (Ponnalagu et al. 2016a; Edwards and Kahl 2010; Ponnalagu et al. 2016b; Fernandez-Salas et al. 1999).

3.2.1 Structural Insights—CLIC proteins show high sequence and structural similarity to members of omega glutathione-S-transferase (GST) family of proteins (Singh 2010). They contain ~280 amino acid residues, with a conserved C-terminal domain. CLIC5B and CLIC6 contain an additional hydrophobic N-terminal domain (Singh 2010; Littler et al. 2010). Until now, crystal structures of only the soluble forms of CLIC are obtained. CLIC4 and CLIC1 show high structural similarity due to 67 % sequence homology. Mammalian CLICs also share structural similarity to invertebrates. All CLICs contain 10 α -helices (h1, h2, h3, h4a, h4b, h5, h6, h7, h8, and h9) and four β -strands (s1, s2, s3, and s4) except in EXC4, where helix h4 is unbroken but has an additional h10 helix (Littler et al. 2008). Soluble structures of EXC4 and *DmCLIC* indicate that the putative transmembrane domain is formed by an α -helix (h1) and a β -strand (s2) (Littler et al. 2008). In mammalian CLICs the putative transmembrane domain (C24-46) comprises the pore, and plays a role in ion transport and targeting the protein to the membrane (Singh 2010; Singh and Ashley 2006; Singh and Ashley 2007). For CLIC1 it is shown that modification of Cys24 inhibits the channel activity (Singh and Ashley 2006). Cys24 and its equivalent conserved residues in other CLIC proteins play a key role in the redox-regulation of CLIC1, CLIC4, and CLIC5 (Singh 2010). Also, it is observed that CLIC proteins insert into planar bilayers with the N-terminus located in the luminal side and the C-terminus towards the cytosolic side (Singh and Ashley 2006; Singh and Ashley 2007). Auto insertion of CLIC1 to the bilayers increased with the formation of disulphide bond between the residues Cys24 and Cys59 upon oxidation, indicating the role of Cys24 in integration of soluble form of CLIC to membranes. However,

the residue Cys59 is not conserved in all other CLICs, and also it is not very clear how these residues form disulphide bonds in membrane form as they are oriented at opposite directions. Therefore, the role of disulphide bonds in insertion of CLICs is still questionable. As CLICs have single transmembrane domain, it is predicted that at least four molecules of them will be necessary to form a functional ion channel in the membrane (Singh 2010). Conformation flexibility of putative transmembrane increased in an acidic pH of ~5.5, thereby increasing the auto insertion into the membrane and exhibiting enhanced single-channel conductance (Stoychev et al. 2009).

3.2.2 Mitochondrial CLIC Proteins—Even though, CLICs were named as intracellular ion channels, their cellular localization is not systematically explored chiefly due to lack of specific antibodies. However, information on localization of some of the CLIC proteins is available (Singh 2010). Out of six CLIC proteins, CLIC4 was characterized as a putative mitochondrial ion channel protein (Szabo and Zoratti 2014), and was demonstrated to reside in mitochondria of keratinocytes (Fernandez-Salas et al. 2002), where it is involved in Ca^{2+} -induced differentiation of keratinocytes (Suh et al. 2007). Hence, CLIC4 is also known as mitochondrial CLIC4 (mtCLIC4). Furthermore, R (+)-Methylindazole, R (+)-[(6, 7-Dichloro-2-cyclopentyl-2, 3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)-oxy] acetic acid (IAA-94)-sensitive CLIC-like currents were also observed in cardiac mitochondria (Misak et al. 2013) suggesting their presence in cardiac mitochondria. We have recently showed that CLIC4 and CLIC5 are cardiac mitochondrial proteins, where CLIC4 is localized to the OMM and CLIC5 to the IMM of rat cardiac mitochondria (Ponnalagu et al. 2016a; Ponnalagu et al. 2016b). CLIC1 and CLIC2 are chiefly localized to the endoplasmic reticulum of adult cardiomyocytes (Ponnalagu et al. 2016a; Ponnalagu et al. 2016b).

In agreement to these studies here we further show mitochondrial localization of CLIC4 in H9C2 cardiac cells (Fig. 2a–h). We also investigated localization of CLIC5 and found it to be localized to the mitochondria of H9C2 cells (Fig. 3a–h). Interestingly, bioinformatic analysis of CLICs revealed the absence of any mitochondrial targeting sequence (MTS) (Ponnalagu et al. 2016a), further indicating a unique unconventional mechanism for their mitochondrial localization in H9C2 cells. To understand geographical distribution of CLIC4 and CLIC5 in cardiac cells, we acquired super resolution images by using a custom-built STED microscope (Rodríguez et al. 2012). Figures 2i–n and 3i–n revealed a unique cluster distribution of CLIC4 (80 ± 10 nm, $n=500$ clusters) and CLIC5 (98 ± 12 nm $n=500$ clusters) in H9C2 cells. These results indicate that even though CLIC4 and CLIC5 localize to mitochondria of H9C2 cells, they follow a distinct distribution either due to differential localization within the mitochondria as observed in adult cardiac mitochondria (Ponnalagu et al. 2016a; Singh et al. 2012b) or due to unique functional roles.

3.2.3 Electrophysiological Properties—Channel activity of CLIC proteins have been carried out in artificial bilayers (Singh and Ashley 2006; Tulk et al. 2002; Warton et al. 2002) using either recombinant proteins or ectopic expression in mammalian cells (Valenzuela et al. 1997; Tonini et al. 2000). CHO-K1 cells transfected with CLIC1 showed a single-channel conductance of 8 pS in cell and nuclear patch clamping studies (Valenzuela et al. 1997). Conductance of all CLICs varied depending on the lipid composition, recording

conditions, and redox environment of the bilayers (Singh 2010; Singh and Ashley 2006). All the CLICs form NSAC as they do not differentiate between cations and anions; however, they turn into anion-selective in the presence of large cations like Tris (Singh 2010; Singh and Ashley 2006). Arg29 and Lys37 in the putative transmembrane domain have been shown to play a role in ion channel function. Lys37 altered the single-channel conductance whereas Arg29 affected the open probability of a single-channel in response to variation in membrane potential (Averaimo et al. 2013). Ionic conductance of CLIC1 and CLIC5 is regulated by actin (Singh et al. 2007), but not for CLIC4. Single-channel biophysics of CLICs in their native environment is not yet reported.

3.2.4 Functions—CLICs are multifunctional proteins. They involve in membrane trafficking, cyto-skeletal function (Berryman et al. 2004), apoptosis (Fernandez-Salas et al. 2002; Suh et al. 2004), cell cycle control (Valenzuela et al. 2000), tubulogenesis (Berry et al. 2003), VEGF-mediated angiogenesis of endothelial cells (Tung et al. 2009), mitosis, and differentiation (Suh et al. 2007). CLIC2 was reported to modulate the function of ryanodine receptors (RyR1 and RyR2) (Board et al. 2004; Takano et al. 2012). In humans, the transcriptional analysis showed the downregulation of CLIC2 in dilated cardiomyopathy patients (Diaz et al. 2010) indicating its possible role in cardiac physiology. CLIC4 also showed a role in regulating the endothelial function, where CLIC4 deficient animals were shown to be resistant to pulmonary hypertension (Wojciak-Stothard et al. 2014). Diaz et al. showed blocking chloride channels using IAA-94, prevented the cardio protective effects of ischemic pre-conditioning (IPC) as there was increased myocardial infarction (MI) due to ischemic/reperfusion (IR) injury in both the heart as well as isolated adult cardiomyocytes (Diaz et al. 1999). It was also shown that other chloride channel blockers like DIDS, 5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), and 4-acetamido-4'-isothiocyanato-stilbene-2,2'-disulfonic acid (SITS), protected hearts from MI further suggesting a probable cardio protective role of IAA-94 specific targets, CLICs (Zheng et al. 2013; Wang et al. 2015).

Overexpression of mtCLIC4 in keratinocytes induced its mitochondrial localization leading to reduced mitochondrial membrane potential and thereby triggering apoptosis (Fernandez-Salas et al. 2002). It was also demonstrated that RNAi suppression of CLIC4 enhanced H₂O₂-induced glioma cell apoptosis (Xu et al. 2013). Recent study also showed that abrogation of CLIC5 increased the ROS generation of cardiac mitochondria in mice (Ponnalagu et al. 2016a). Thus, these studies provide evidence of CLIC in modulating mitochondrial function which is known to play a role in regulation of apoptosis, cardioprotection from IR injury, and their direct involvement in maintaining cellular physiology.

4 Other Chloride Transporters or Modulators in Mitochondria

Apart from the above mentioned anion channel conductances observed in both IMM and OMM, there are still other upcoming anion channels or modulators maintaining mitochondrial ion flux which needs to be further studied. We have discussed some of them in the following section.

4.1 Mitochondrial Permeability Transition Pore

mPTP is a non-selective channel present in the mitochondrial inner membrane which allows a solute of up to 1.5 kDa to pass through it resulting in dissipation of electrochemical proton gradient, inhibition of ATP synthesis further leading to cell death (Szabo and Zoratti 2014; Bernardi et al. 2006). The conductance of this channel varies from 0.9 to 1.3 pS, with mild anion-selectivity and can also switch to cation-selective channel (Szabo and Zoratti 2014), therefore they are considered as non-selective channels present in the mitochondrial membrane. Opening of the channel is reliant on mitochondrial Ca^{2+} concentrations and matrix alkalization. Earlier it was shown that PTP is inhibited by submicromolar concentration of an immunosuppressant drug, cyclosporine A (CsA) (Fournier et al. 1987; Crompton et al. 1988; Broekemeier et al. 1989; Davidson and Halestrap 1990) thus indicating the presence of CypD as the molecular component of the channel. Multiple studies have predicted that mPTP comprises of VDAC (Szabo et al. 1993; Szabo and Zoratti 1993), ANT (Kokoszka et al. 2004), CypD, and hexokinase. As discussed earlier in Sect. 2.1.4, interaction of SGP7 and VDAC was found to be essential for formation of mPTP (Shanmughapriya et al. 2015). A latest study has also suggested the likelihood of F_1F_0 ATPase itself as forming a PTP (Halestrap 2014; Bonora et al. 2013). Further, studies on identifying the molecular correlate of mPTP are still underway, and usage of a multidisciplinary approach will certainly provide much desired information (Singh et al. 2012a; Singh 2010; Rodríguez et al. 2012; Singh et al. 2012b; Singh et al. 2009). Activity of the channel is modulated by CsA, matrix acidification, adenine nucleotides, and divalent cations apart from Ca^{2+} (Szabo and Zoratti 2014). In spite of its unclear molecular identity, multiple studies signify its key role in IR injury (Akar et al. 2005; Halestrap et al. 2004), neurodegeneration (Schinzel et al. 2005) in pathological disorders, apoptosis, and progression of cancer (Szabo and Zoratti 2014).

4.2 Maxi-Chloride Channel

This section summarizes the evidence of the presence of Maxi Cl^- channel in mitochondria (De Marchi et al. 1777). Patch clamp analysis of liver mitoplast revealed a channel activity similar to monomeric state of PTP. The occurrence of this activity is not found to be consistent in liver mitochondria but was observed more frequently in human colon tumor 116 (HCT116) cells. The conductance of the channel recorded in HCT116 cells was ~400 pS. This channel was considered to be anion-selective in fourfold KCl gradient and at reversal potentials in the range of -26 to -31 mV. The ratio of permeability coefficients reported was $P_{\text{Cl}^-}/P_{\text{K}^+} \approx 7-18$. As the channel activity represented the monomeric form of PTP, VDAC was considered as a probable candidate for this channel activity. The channel activity did not differ upon VDAC deletion, thus, ruling out the possibility of VDAC as its molecular component. The channel occasionally switched to cation-selective state. Interestingly, the activity was inhibited by only DIDS and SITS but not by IAA-94, NPPB, or niflumic acid. Properties of this channel were similar to swelling-activated and voltage-inactivated maxi-chloride channels reported earlier (Sabirov et al. 2006). Although molecular structure of the channels is unknown but is considered to have a strong link with PTP.

4.3 Uncoupling Proteins

UCP is a ~33 kDa protein, primarily acting as an H⁺ carrier in the inner membrane of mitochondria. There are five isoforms of UCP identified in mammals. Anion channel transport was associated with them as it was also demonstrated to play a role in mitochondrial swelling (Nicholls and Lindberg 1973), and Cl⁻ transport was confirmed in vesicles reconstituted with UCP (Huang and Klingenberg 1996; Jezek et al. 1990). There are six transmembrane domains and in case of UCP1 and UCP2, it is shown that transmembrane 2 (TM2) contributes to anion conductance (Yamaguchi et al. 2004). Also, arginine residues of TM2 were shown to be crucial for anion transport (Hoang et al. 2015). The channel exhibited an inward-rectification with a single-channel conductance of 75 ±6 pS at positive holding potentials, and 84 ±8 pS at negative holding potentials. Divalent cations like Mg²⁺ and Ca²⁺ aided in the occurrence of these channels. The permeability to ions decreased in the order of Cl⁻>Br⁻>F⁻>SCN⁻>I⁻>NO₃⁻>gluconate. Like all other uncharacterized mitochondrial anion channels, the channel activity was sensitive to nucleotides (Huang and Klingenberg 1996). Although, UCP is required to dissipate the proton gradient to provide energy for oxidative phosphorylation, these studies speculate the potential of UCP behaving as an anion channel.

4.4 Vacuolating Cytotoxin A

VacA, the vacuolating cytotoxin A, as the name implies forms large vacuoles in cells and is one of the major virulence factors released by *Helicobacter pylori* (Rassow 2011). VacA toxin is the prominent mediator in *H. pylori*-induced apoptosis of cells (Cover and Blanke 2005; Boquet et al. 2003), and is also known to form anion channels (Iwamoto et al. 1999; Czajkowsky et al. 1999; Szabo et al. 1999) of low conductance of ~10 pS. This conductivity is completely inhibited by the chloride channel blocker NPPB (Szabo et al. 1999; Tombola et al. 2000). Previous studies also demonstrated that VacA intoxicates mammalian cells by reducing mitochondrial membrane potential and inducing apoptosis (Willhite and Blanke 2004). Based on these studies a model has been hypothesized to link the channel activity with its function: VacA after entering mammalian cells localizes to the mitochondria, and modulates mitochondrial membrane permeability by forming a channel resulting in cytochrome c release. Thus, VacA joins the ranks of anion channels playing a role in intrinsic pathway of apoptosis (Willhite and Blanke 2004).

5 Pharmacological Evidence of Cardiac Mitochondrial Anion Channels

Most of the chloride channels in mitochondria have been identified and characterized using pharmacological inhibitors. However the specificity of these pharmacological agents is not yet established, and is shown to be non-specific at different concentrations which is a limitation for their usage. Nevertheless, these pharmacological modulators have played a significant part in deciphering the molecular identity and physiological functions of these channels. Thus, in this section we investigated the presence of putative chloride channels in cardiac mitochondria of *Rattus norvegicus* and their respective roles in modulating mitochondrial ROS generation (Murphy 2009) using these pharmacological inhibitors of chloride channels.

Mitochondrial Ca^{2+} capacity and ROS generation play a key role in multiple pathologies, including cardiac IR injury, neurodegenerative diseases, diabetes, cancer, and premature aging (Sena and Chandel 2012). In a recent study, mitochondrial Ca^{2+} capacity was used to identify components of mPTP (Shanmughapriya et al. 2015). Here, we have incorporated widely used canonical anion channel blockers, and studied their impact on mitochondrial ROS generation to identify possible mitochondrial anion channels present in the heart. Cl^- channel inhibitors used are IAA-94 (Landry et al. 1989; Weber-Schurholz et al. 1993), Anthracene-9-carboxylic acid (A9C) (Al Khamici et al. 2015), NPPB (Malekova et al. 2007), DIDS (Malekova et al. 2007), glibenclamide (Sheppard and Welsh 1992), and 4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxy]butanoic acid (DCPIB) (Bourke et al. 1981; Decher et al. 2001) that are known blockers of CLICs (IAA-94 and A9C both), calcium-activated chloride channel, CLC's, cystic fibrosis transmembrane conductance regulator (CFTR), and swelling-activated chloride channels, respectively. Further, earlier studies have shown the presence of these channels in the heart (Misak et al. 2013; Duan 2013; Baumgarten et al. 2005).

Electron transport chain complex II/III were activated with its substrate, succinate and the ROS generated was measured using amplex red as an indicator of H_2O_2 produced, in fluorescence spectrophotometer F-2710 as described previously (Singh et al. 2012b). It was observed that there was significant reduction in the rate as well as total ROS generation (relative to DMSO control) after addition of inhibitors such as A9C (100 μM), IAA-94 (100 μM), and NPPB (50 μM) (Fig. 4a–c, g and h). This further indicated the possible presence of CLICs, and calcium-activated chloride channel in the cardiac mitochondria isolated from *R. norvegicus*. There was no change in the rate of ROS production in case of 300 μM DIDS (CLC inhibitor) (Fig. 4d, g). Also, there was a non-specific immediate spike in the rate of ROS production observed for most of the inhibitors. 100 μM of each glibenclamide, and DCPIB showed significant reduction in the rate of ROS production (Fig. 4e–g), but no significant reduction in the total ROS produced was observed relative to vehicle control (DMSO) at the end of 45 min (Fig. 4h). This could be either due to reversible inhibitory effect of these channel inhibitors or unhealthy condition of the mitochondria in the presence of a drug. These results further strengthen the existence of Cl^- channels in the mitochondria, and also provide evidence for their role in modulating mitochondrial function. Thus, molecular identity of other anion channels needs to be further elucidated, as they could be a probable candidate for developing new therapeutic tools.

6 Concluding Remarks

Chloride channels always remained in rear seat as they are considered as “unimportant leaks” associated with cation channels in excitable cells (Jentsch et al. 2004; Ashley 2003; Jentsch et al. 2002). This is mainly due to lack of specific tools/ligands/modulators to characterize them. In spite of lack of efficient tool to identify channel recordings in intracellular organelles, several mitochondrial anion channel conductances (summarized in Table 1) have been reported but the molecular correlate of these channels is unknown or debatable. So far, molecular identities of only VDAC, CLIC4, and CLIC5 have been well established in mitochondria and many mitochondrial anion channels are yet to be identified.

Moreover, ongoing and upcoming studies signify the potential role of mitochondrial ion channels as regulators of mitochondrial morphology as well as physiology, contributors of cancer and cardiac-related diseases, and key participants in cell life and death. Despite their significant functions in cell fate determination, little effort has been put in developing specifically targeted therapeutics agents to modulate their activity, mainly because of lack of molecular identity and structure of these channels. Therefore, efficient tools/modulators should be generated in identifying the molecular structure of these channels, as this will aid in providing specific targets for development of novel therapeutics.

Enormous evidence supports the existence of multiple physiologically relevant anion channel conductance in the mitochondria. Hence, these channels cannot be ignored as unimportant leaks and more efforts should be carried out towards understanding the molecular identity and physiological function of these channels.

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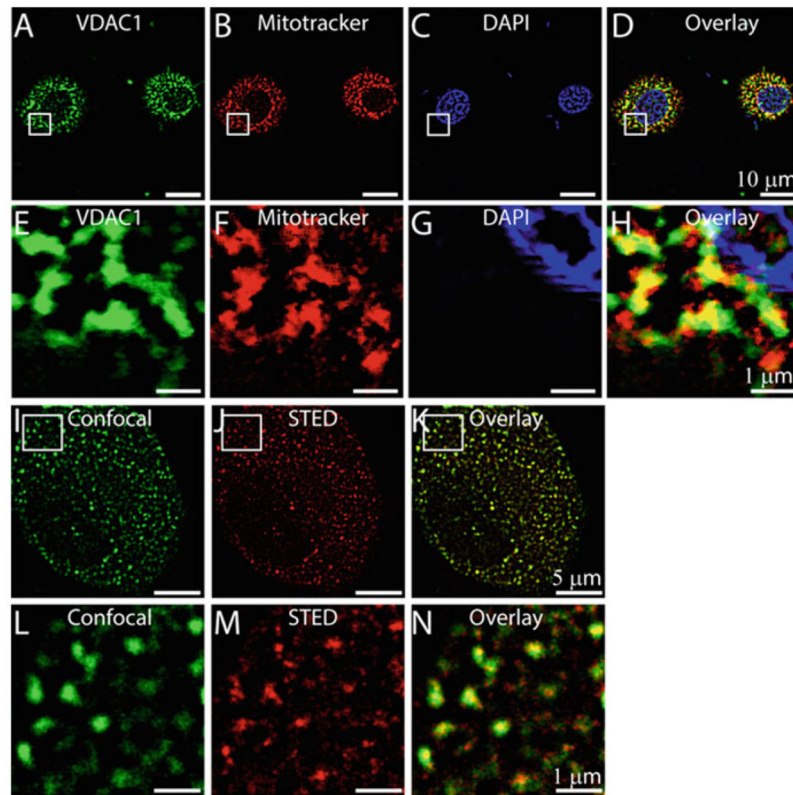


Fig. 1. Localization of VDAC1 in H9C2 cells. H9C2 cells loaded with mitotracker (**b**) were labeled with anti-VDAC1 antibody (**a**), and stained with a nuclear marker, DAPI (**c**). (**d**) is merge image of **a**, **b**, and **c** showing colocalization of VDAC1 to the mitochondria of H9C2 cells. **e**, **f**, **g**, and **h** are enlarged images of the squared regions **a**, **b**, **c**, and **d**, respectively. STED microscopy of H9C2 cells showing expression of VDAC1 (**j**, **k**). Confocal image of H9C2 cells labeled with anti-VDAC1 antibody (**i**) Corresponding STED image (**j**), overlay of **i** and **j** highlights increased resolution of VDAC1 localization to mitochondria with STED microscopy (**k**). **l**, **m**, and **n** are enlarged images of the squared region in **i**, **j**, and **k**, respectively

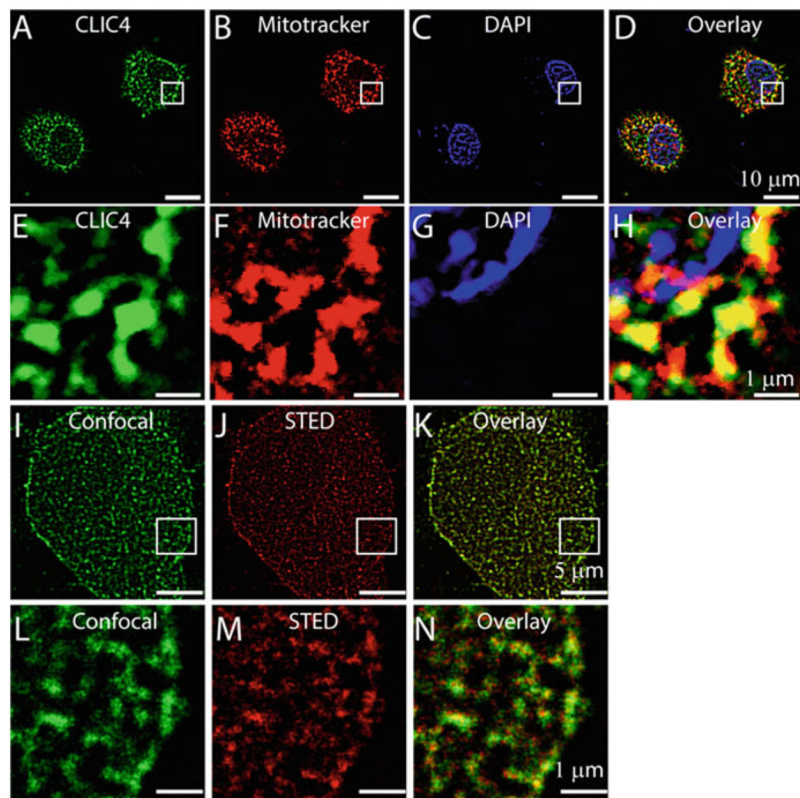


Fig. 2. Localization of CLIC4 in H9C2 cells. H9C2 cells loaded with mitotracker (**b**) were labeled with anti-CLIC4 antibody (**a**), and stained with DAPI (**c**). **d** is merge image of **a**, **b**, and **c** showing colocalization of CLIC4 to the mitochondria of H9C2 cells. **e**, **f**, **g**, and **h** are enlarged images of the squared regions in **a**, **b**, **c**, and **d**, respectively. STED microscopy of H9C2 cells showing expression of CLIC4 in the mitochondria (**j**, **k**). Confocal image of H9C2 cells labeled with anti-CLIC4 antibody (**i**). Corresponding STED image (**j**), overlay of **i** and **j** highlights increased resolution of CLIC4 localization to mitochondria with STED microscopy (**k**). **l**, **m**, and **n** are enlarged images of the squared region in **i**, **j**, and **k**, respectively

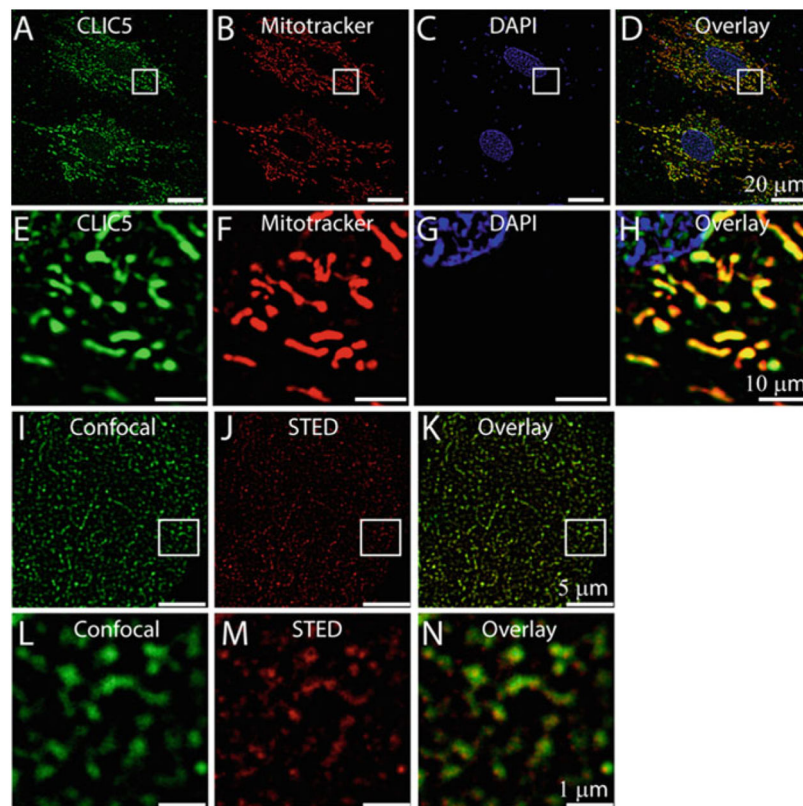


Fig. 3. Localization of CLIC5 in H9C2 cells. Mitotracker-labeled (b) H9C2 cells were incubated with anti-CLIC5 antibody (a), and stained with DAPI (c). d is merge image of a, b, and c showing colocalization of CLIC5 to the mitochondria of H9C2 cells. e, f, g, and h are enlarged images of the squared regions in a, b, c, and d, respectively. STED microscopy of H9C2 cells showing expression of CLIC5 in the mitochondria (j, k). Confocal image of H9C2 cells labeled with anti-CLIC5 antibody (i) Corresponding STED image (j), overlay of i and j highlights increased resolution of CLIC5 localization to mitochondria with STED microscopy (k). l, m, and n are enlarged images of the squared region in i, j, and k, respectively

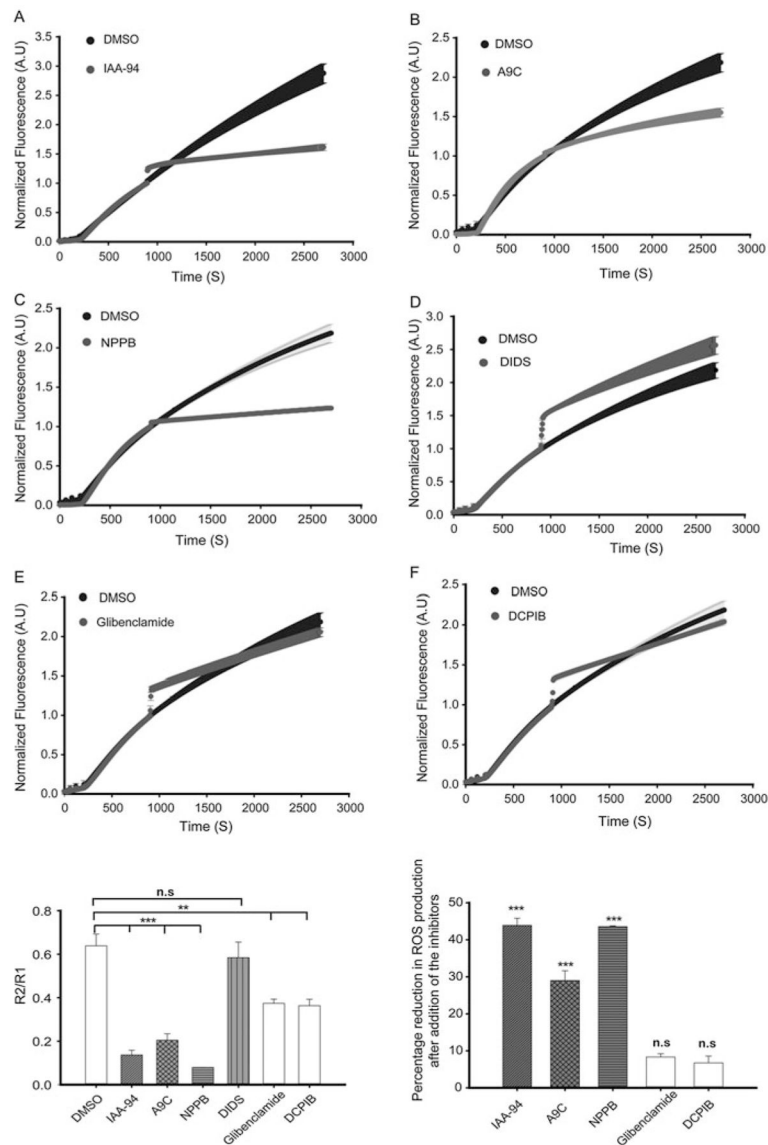


Fig. 4. Differential modulation of the rate of ROS generation in complex II/III of cardiac mitochondria by various chloride channel inhibitors. Cardiac mitochondria isolated from *R. norvegicus* and the ROS generated by complex II/III was measured as described earlier. Profile representing the rate of ROS production by cardiac mitochondria in the presence of DMSO (*black*) vs. 100 μ M IAA-94 (*gray, a*), 100 μ M A9C (*gray, b*), 50 μ M NPPB (*gray, c*), 300 μ M DIDS (*gray, d*), 100 μ M glibenclamide (*gray, e*), and 100 μ M DCPIB (*gray, f*). R1: Initial rate of ROS production which is 15 min prior to addition of the inhibitors, R2: rate of ROS production calculated after 15 min of addition of inhibitors. R2/R1 significantly decreased in presence of the chloride channel inhibitors IAA-94, A9C, NPPB, glibenclamide, and DCPIB (**g**). IAA-94, A9C, and NPPB showed significant reduction in the total ROS produced (relative to DMSO) at the end of 45 min whereas negligible change in ROS production relative to DMSO control was observed in case of glibenclamide and

DCPIB (**h**). One way ANOVA was used to calculate the statistical significance (***) $p < 0.0005$, ** $p < 0.005$). *IAA-94* R(+)-Methylindazole, R(+)-[(6,7-Dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)-oxy]acetic acid, *A9C* anthracene-9-carboxylic acid, *NPPB* (5-nitro-1-(3-phenylpropylamino)benzoic acid, *DIDS* 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid, *DCPIB* 4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]butanoic acid ($n = 4$, independent preparations with each pharmacological agent used)

Table 1

Mitochondria ion channels, their localization, selectivity, and conductance

Name	Location	Selectivity	References
VDAC1	OMM	Cl selective at the open state of channel [$P_{Ca} = 0.94 \pm 0.05$, $P_{Cl} = 22.6 \pm 0.2(\times 10^{-16} \text{ L/s})$], also permeable to calcium at the closed state [$P_{Ca} = 4 \pm 1$, $P_{Cl} = 5 \pm 1(\times 10^{-16} \text{ L/s})$], conductance of 2.4–4.3 nS at $V_m = 10 \text{ mV}$	Szabo and Zoratti (2014), Tan and Colombini (1768), and De Pinto et al. (1987)
VDAC2	OMM	Cl selective, conductance varied as it showed lower conductance ($1.55 \pm 0.01 \text{ nS}$ at 5 mV) and higher conductance ($1.71 \pm 0.01 \text{ nS}$ at 12 mV). On an average conductance reported were $3.79 \pm 0.10 \text{ nS}$.	Xu et al. (1999) and Menzel et al. (2009)
VDAC3	OMM	Cl selective at a holding potential of 12 mV, a small conductance of 0.68 nS–0.71 nS at tenfold KCl gradient	Checchetto et al. (2014), Colombini (2004), and Xu et al. (1999)
CLIC4	OMM	15 pS (500/50 mM KCl), 30 pS (140 mM KCl), 1 pS (300:140 mM choline Cl), 43 (50 mM choline chloride)	Ponnalagu et al. (2016a), Duncan et al. (1997), Ponnalagu et al. (2016b), Singh and Ashley (2007), and Proutski et al. (2002)
IMAC	IMM	P_{Cl}/P_K is 4.5, 107–110 pS in symmetrical 150 mM KCl.	Sorgato et al. (1987), Klitsch and Siemen (1991), and Sorgato et al. (1989)
CLIC5	IMM	Varied from ~3–116 pS (500/50 mM KCl) when reconstituted in planar bilayers, 26 pS, 100 pS and 400 pS (140 mM KCl), 100/280 pS (140 mM), 42 pS (140 mM KCl)	Ponnalagu et al. (2016a), Ponnalagu et al. (2016b), Singh et al. (2007), Landry et al. (1989), Weber-Schurholz et al. (1993), and Edwards et al. (1998)
mPTP	IMM	0.9–1.5 nS (150 mM KCl), average conductance of ~1.3 nS in patch clamp studies, low anion selectivity, and sometimes exhibit cation selectivity	Szabo and Zoratti (2014), De Marchi et al. (2006), and Petronilli et al. (1989)
Maxi Cl channel	IMM	400 pS (fourfold KCl gradient) in patch clamp studies under reversal potentials of –26 to –31, $P_{Cl}/P_K \approx 7$ to 18	De Marchi et al. (1777)
UCP	IMM	~75 pS at positive holding potential (symmetrical 100 mM KCl) in patch clamp studies. ~84 pS at negative holding potential, inward rectifying Cl^- currents were observed.	Huang and Klingenberg (1996)