Chapter 9

Lifting the Fog over Mitochondrial Chloride Channels

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Abstract

The current through mitochondrial chloride channels was first described in 1987. Subsequently, several types of ion channels permeable to chloride and other anions were found in the mitochondria of different origins. The increasing number of electrophysiological studies, however, yielded only more ambiguity rather than order in the field of chloride channels. This uncertainty was slightly reduced by two different studies: experiments that showed a significant role of chloride channels in the process of mitochondrial membrane potential oscillations and experiments that localized chloride intracellular ion channel (CLIC) proteins in cardiac mitochondrial membranes. Our recently published single-channel electrophysiological experiments are well in line with the channel activity of recombinant CLIC proteins. The experimental evidence seems to be inevitably, though slowly converging on a connection between single-channel activity and the identity of the mitochondrial chloride channel protein.

Keywords: chloride channel, mitochondria, cardiomyocyte, inner membrane anion channel—IMAC, chloride intracellular ion channel—CLIC

1. Introduction

Ion channels permeable to anions were underrated for a long time concerning their role in the life of a cell. The role of chloride channels in mitochondria was particularly underrated. This chapter focuses on the chloride channels of the inner mitochondrial membrane. The different electrophysiological descriptions of these channels that have appeared since 1987 are compared. In addition to single-channel current measurements, measurements of mitochondrial membrane potential oscillations in whole cardiomyocytes provided information about the role of the mitochondrial chloride channel. At the molecular level, super-resolution fluorescence

imaging and Western blot analysis yielded invaluable information about the localization of chloride intracellular channel (CLIC) isoforms in the mitochondrial membrane. The results from these different fields of research are discussed and combined to identify a connection between measured chloride channel activities and the identity of the corresponding proteins.

2. First chloride channels on (electrophysiological) stage

One of the earliest studies of anion channel activity was reported in 1979 by White and Miller [1]. The authors used membrane vesicles from the electric organ of Torpedo californica fish and fused these vesicles into a planar lipid bilayer to measure the single-channel current. The fascinating story about the discovery of these channels was recounted by Miller [2]. Indeed, the reconstitution of intracellular ion channels in planar lipid bilayers in combination with the single-channel patch-clamp approach was extensively used to characterize ion channels of different origins and cellular localizations. The following years were fruitful with discoveries of new chloride channels. At present, chloride channels are classified into several groups; some are based on the genetic information known about the channel proteins, and others are defined only according to the described single-channel electrophysiological properties [3]. The first member of the 'chloride channel' (ClC) family was cloned more than 20 years ago [4]. Since then, several other members have been identified [5]. When the ClC homologues from *Escherichia coli* and *Salmonella typhimurium* were crystallized, the dimeric structure of these channels was revealed; each of the two monomers has its own conducting pore [6]. This 'double-barrel' structure is reflected at the level of electrophysiological measurements by the presence of two open-channel levels with equal conductances of approximately 10 pS each [1, 7–9]. Another chloride channel group contains ligand-regulated channels, such as glycine and γ-aminobutyric acid (GABA) receptors [10, 11]; these two channels have a neuronal origin, and they were described at the single-channel level in 1983. The cAMP-activated cystic fibrosis transmembrane conductance regulator (CFTR) group represents a separate chloride channel family localized in epithelial cells [12, 13]. The CFTR channel was purified, reconstituted into liposomes and measured at the single-channel level in 1992 [14]. The last of the chloride channel groups to be genetically distinguished was the group of chloride intracellular ion channels (CLICs); the first member of this group was described at the single-channel level in 1987 [15]. We return to CLICs later, in part 6. In addition, finally, there are channels that have unknown encoding genes—swelling-activated chloride channels [16] or calcium-activated chloride channels [17, 18]; both channel types were described at the beginning of the 1990s. In the following sections, we focus on the chloride channels localized in the inner mitochondrial membrane.

3. A multitude of chloride channels

One of the presumptions of Mitchell's chemiosmotic hypothesis is that the inner mitochondrial membrane is impermeable to ions other than protons [19]; however, with the development

of experimental approaches that allowed the detection of ion transport through the mitochondrial membrane, evidence showing that the mitochondrial membrane is crowded with ion transporters accumulated (rev. in [20]). At the beginning of the 1980s, the commonly used methods were rather indirect: the uptake of the radioactive isotope $^{36}Cl^-$ was measured [21], or the light scattering from swollen mitochondria was monitored [22, 23], although the biophysical approach for single-channel current measurement was already available [24–26].

3.1. Anion fluxes in mitochondria

The anion transport through inner mitochondrial membranes of mammalian origin was described for the first time in 1979 by Selwyn et al. [27]. This chloride uniporter was later named 'inner membrane anion channel' (IMAC) [28]. IMAC was characterized by light-scattering measurements of swollen mitochondria in the presence of rotenone, which inhibits complex I of the respiratory chain [28–32]. Beavis and Garlid showed that the transport of anions was strongly dependent on pH when Mg^{2+} ions were depleted from the mitochondria by the A23187 ionophore; in the presence of Mg^{2+}, the transport rate was low and could be increased by alkaline pH [22]. A role for IMAC in the regulation of mitochondrial volume after pathological swelling has been suggested [28]. The group of Beavis looked for the putative identity of IMAC and searched for pharmacological similarities between IMAC and adenine nucleotide translocase (ANT) [33]. They found that several nucleotide analogues (e.g. Cibacron Blue) partially inhibit the flux of small anions and block the flux of malonate [33], whereas at low doses, these compounds stimulate the flux. The most important difference between IMAC and ANT was the effect of the selective ANT inhibitor carboxyatractyloside, which did not affect IMAC in any way [33]. The researchers concluded that IMAC is not identical to ANT. Later, in 1996, Beavis and Davatol-Hag studied the effect of several stilbene-2,2′-disulfonates [34], which are known as nonspecific chloride channel inhibitors [3, 34]. 4,4′-Diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) is most often used to inhibit chloride channels. These researchers showed that DIDS, which was applied from the side of the intermembrane space, partially inhibited the flux of chloride by 30%. The inhibitory effect was more pronounced for malonate transport. A possible mechanism of IMAC inhibition by large anionic molecules (DIDS, nucleotide analogues) has been proposed [33] where these molecules bind to a binding site within the conductive pathway. This hypothesis is based on the fact that neither of the compounds could completely block the flux of small ions, which is in contrast to the inhibition of flux of larger anions such as malonate. The conductive pathway seems to be large enough to enable the entrance (at least partial) of complex compounds such as DIDS, as IMAC is also permeable to large anions [22]. The most recent publications concerning IMAC, which was measured by the light-scattering technique, appeared in 2004. One of these studies evaluated the combined effect of temperature and Mg^{2+} on IMAC [35], and the second one measured the activation of IMAC by fatty acids [36]. Schonfeld et al. [36] showed that fatty acids activated the chloride flux through IMAC. The positive effect of fatty acids on activity was confirmed by single-channel measurements. The authors suggested that long-chain fatty acids directly remove the Mg^{2+} ions from the binding sites of the IMAC protein or that these fatty acids form complexes with Mg^{2+} ions, thus lowering their matrix concentration. However, the described single-channel experiments provide questionable support for the proposed mechanism of activation. On the

other hand, the A23187 ionophore [22] caused the depletion of the Mg^{2+} matrix pool [36], leading to IMAC activation. The inhibitory effect of Mg^{2+} ions on IMAC activity was not only pH-dependent but also temperature-dependent [35]. At 25°C, the flux through IMAC was blocked by Mg^{2+} ions at physiological pH. Researchers have opined that the activation of IMAC in physiological processes is unlikely and that its role under these conditions is unclear [37, 38]. Beavis and Powers estimated that at physiological Mg^{2+} concentrations and 37°C, IMAC activity is ~7% of its maximum. The authors concluded that other factors regulating the activity of IMAC are probably involved and are waiting to be discovered [35].

3.2. Electrophysiology measurements of the inner mitochondrial membrane

The largest advance in the study of anion transport in mitochondria occurred in 1987 when the current through giant mitoplasts, that is, mitochondria deprived of the outer membrane [39], was first measured by a relatively novel (at the time) electrophysiological patch-clamp method [25]. To obtain the giant mitoplasts necessary to allow the stable connection of the patch pipette to the mitoplast membrane, mice were fed cuprizone. The researchers observed a slightly selective anion channel, with voltage-dependent activity and a mean single-channel conductance of 107 pS (in 150 mM KCl); this channel was later named the centum-pS channel [40]. This channel was also found in the inner membrane of the liver and heart mitochondria of oxen and mice that were not treated with cuprizone [41]. The centum-pS channel responds to nanomolar concentrations of mitochondrial benzodiazepine receptor (mBzR) ligands. The channel activity is completely inhibited by protoporphyrin IX, PK11195 and Ro5-4864 (4-chlorodiazepam) with IC_{50} values in the nanomolar range. The high affinity of the benzodiazepine ligands to this channel suggests a putative association of the channel protein with the mBzR present in the outer membrane of mitochondria [40, 42]. Apart from the voltage-sensitive centum-pS channel [43, 44], Kinnally et al. also observed a 15-pS channel (low conductance channel, LCC) in the inner mitochondrial membrane patches; this channel was activated by alkaline pH and was inhibited by the presence of Mg^{2+} ions, and the authors suggested that this channel corresponds to IMAC [44]. With the increasing number of electrophysiological experiments on mitoplasts or isolated submitochondrial vesicles, the number of observed anion channel types has multiplied. There are actually several slightly differing anion channels that have been described, although one cannot conclude with certainty which of them could correspond to IMAC [44–49]. The anion channels were measured from mitochondrial membranes of different origin.

Anion channels in the inner membrane of brown adipose tissue mitochondria were characterized by a conductance of 108 pS in 150 mM KCl solution [45, 49]. These channels were measured by the patch-clamp technique in mitoplast-attached mode. Klitsch and Siemen [45] showed that this channel is inhibited by a low concentration of purine nucleotides; however, the channel is not identical to the uncoupling protein (UCP) [45, 50, 51]. UCP from brown adipose tissue mitochondria was also shown to behave as a chloride channel, with two steps of 75-pS conductance (in 100 mM KCl), to be sensitive to voltage and to be inhibited by nucleotides and DIDS. UCP is unaffected by pH changes and Mg^{2+} ions [50]. According to Borecky et al., the 108-pS channel may be a candidate for IMAC because of several properties: the most convincing ones are the pH dependence of the channel activity (quantified as the open probability of the channel) and the inhibition by Mg^{2+} ions [49]. Its activity was inhibited by propranolol [52], similar to the anion fluxes through IMAC [53].

In yeast mitochondria, a 45-pS channel (in 150 mM KCl) was detected; this channel was characterized by low activity, which was only slightly affected by voltage. The channel could be inhibited by ATP [47, 54].

A more thorough description of anion channels derived from sheep cardiac mitoplasts was given by Hayman et al. [46, 55]. Channels permeable to chloride were observed after the incorporation of vesicles into a planar lipid bilayer; one type of channel had a conductance of 100 pS in 150 mM KCl, was named intermediate conductance mitochondrial anion channel (INMAC), and had multiple subconductance states; the other type of channel had 50-pS conductance, had two distinct subconductance states, and was named small conductance mitochondrial anion channel (SMAC). Both channels were more selective for anions than for the potassium cation (P_{Cl}/P_K ~7–9). The responses of INMAC channels to pH changes were consistent with those of the brown adipose tissue 108-pS channel [49]; however, the response was not sensitive to ATP, Mg^{2+} ions or voltage [46].

Native chloride channel with a conductance of 129 pS in 250 mM KCl [56] and 97 pS in 150 mM KCl [57] were detected in purified rat cardiac mitochondria. These channels were

Name (citations)	Origin	Method	G [Ps] ([KCl])	P_{Cl}/P_K	Kinetic features	Substates
IMAC [34, 53]		Light scattering of swollen mitoplasts	Not defined for IMAC flux			
Centum pS [39, 40]	Mouse liver	Patch clamp	107 (150 mM)	~4.5	Bursts	
Centum pS [41]	Mouse heart and liver, ox heart	Patch clamp	~100 (150 mM)			
108 pS [45]	Brown adipose tissue	Patch clamp	108 (150 mM)			✓
108 pS [49]	Brown adipose tissue	Patch clamp	108 (150 mM)		Bursts	
UCP [50]	Brown adipose tissue	Patch clamp	2 × 75 (100 mM)	~17		
45 pS [47, 54]	Yeast	Patch clamp	45 (150 mM)	~3.2		
INMAC [46, 55]	Sheep heart	BLM	100 (150 mM)	~9		✓
SMAC [46, 55]	Sheep heart	BLM	50 (150 mM)	~7		✓
97-pS [56, 57]	Rat heart	BLM	97 (150 mM)	~3	Burst	✓
LCC [44, 52]	Rat liver	Patch clamp	15 (150 mM)			
CLIC5 [21, 83, 85]	Recombinant	BLM	26–400 (140 mM)	~0.5–9		✓

BLM is the method of ion channel reconstitution into a planar lipid bilayer. The conductance (G) and the selectivity for chloride over potassium ions (P_{Cl}/P_K) are summarized in columns 4 and 5, respectively. The concentration of KCl in which the conductance was determined is shown next to the value of conductance. Some channels possess a typical kinetic feature—bursts (column 6). The final column shows the presence of subconductance states (substates). The cells are empty when the corresponding information is missing.

Table 1. Origin, method of detection and biophysical parameters of different chloride channels from the inner mitochondrial membrane.

Name	Voltage	Mg^{2+}	pH	DIDS
IMAC [34, 53]		Inhibition	Acidic pH—inhibition	Partial inhibition
			Alkaline pH—activation	
Centum pS [39, 40]	Sensitive	(Mg^{2+} not present in the solutions)	Insensitive	
Centum pS [41]	Sensitive			Insensitive
108 pS [45]				
108 pS [49]	Sensitive	Inhibition	Acidic pH—inhibition	
			Alkaline pH—high activity	
UCP [50]	Sensitive	Insensitive	Insensitive	Inhibition
45 pS [47, 54]	Minimal	Insensitive		
INMAC [46, 55]	Slightly sensitive or insensitive	Insensitive	Acidic pH—inactivation	
			Alkaline pH—active channel	
SMAC [46, 55]		Decrease in amplitude	Insensitive	
97 pS [56, 57]	Sensitive	Activation	Acidic pH—slow ↑ G followed by inhibition	One-sided inhibition
			Alkaline pH—high activity, ↓ G	
LCC [44, 52]	Insensitive	Inhibition	Activation by alkaline pH	
			No activity at acidic pH	
CLIC5 [21, 83, 85]				

The table summarizes the effect of voltage, Mg^{2+} ions, pH and the nonspecific anion channel inhibitor DIDS on the activity of the chloride channels. Regulation by pH also affected the conductance of the channels (G); the arrows indicate the change in conductance. The cells are empty when the corresponding information is missing.

Table 2. Regulation of chloride channels from the inner mitochondrial membrane.

also permeable to large anions such as acetate. Concerning the selectivity of these channels, the channels were slightly selective for anions over potassium ions (P_{Cl}/P_K ~3) and practically nonselective among several tested types of anions. The channels responded to pH changes by changing their ionic conductance. An alkaline environment caused an immediate decrease in conductance but did not affect the activity. On the other hand, acidification induced a slow increase in conductance, as well as an abrupt inhibition after a delay of approximately 1 min [56]. A similar effect of pH on channel activity was seen in the study of the centum-pS channel from brown adipose tissue mitochondria [49], but no changes in conductance due to pH shifts were reported. The pH dependence of the conductance of the rat mitochondrial chloride channels was also measured with the gluconate anion, which was impermeant at 7.4 pH. Interestingly, gluconate conductance appeared at acidic pH and gradually increased with the acidity of the environment [56]. However, it seems

that in addition to the spatial dimensions of the anion size, the change in conductance can be a consequence of the change in hydration energy at different pH values and/or the change in the surface charge in the pore vestibule [58]. As mentioned before, DIDS is commonly used as a nonspecific inhibitor of anion channels, although not all of these channels are sensitive to DIDS. The chloride channels from rat cardiac mitochondria are inhibited by DIDS from one side only, with IC_{50} of ~12 μM. DIDS affected the complicated kinetics of these channels, which was previously described in detail [57]. In native mitochondrial chloride channels, the dependence of the activity on voltage has an approximately bell-shaped character [57], and these channels are more active in the presence of 1 mM Mg^{2+} ions than in Mg^{2+}-free solution [56]. Again, for these channels, some but not all properties correspond to IMAC.

The origin, methodical approach, biophysical properties and regulation of the inner mitochondrial membrane chloride channels are summarized in **Tables 1** and **2**. In these tables, the frequent value of ~100-pS conductance (in 150 mM KCl) and the similar selectivities suggest that the 108-pS channel, INMAC and the channel from purified rat cardiac mitochondria might all represent the centum-pS channel. Nevertheless, these studies did not lead to a definitive conclusion about the identity of IMAC and did not provide an ultimate match at a single-channel level. In addition, the potential role or roles of these channels are mostly unclear (rev. in [59]). In the first years of the third millennium, the interest in mitochondrial chloride channels began to slowly fade. At this time, the group of Brian O'Rourke published a set of studies on the oscillations of the mitochondrial membrane potential in whole cardiomyocytes, which revived interest in IMAC by indicating its importance in this phenomenon [60–62].

4. Role of mitochondrial chloride channels in mitochondrial membrane potential oscillations

Changes in the cardiac action potential (AP) duration lead to ventricular arrhythmias [42]. The pattern of AP is also determined by sarcolemmal ATP-sensitive potassium channels ($sarcK_{ATP}$) [63], whose activity responds to changes in ATP levels in the cytoplasm. Thus, the perturbation of mitochondrial bioenergetics can be one cause of AP heterogeneity because mitochondria produce the majority of cellular ATP [42]. A decrease in mitochondrial membrane potential ($\Delta\Psi_m$) is associated with a lowered ATP production, which affects the $sarcK_{ATP}$ that regulate the action potential duration [61, 64]. Several years ago, it was shown that metabolic stress can induce a collapse in $\Delta\Psi_m$ [60]; this collapse could be abolished by different inhibitors of ion channels of the inner mitochondrial membrane that are permeable to anions [37]. Arrhythmias can also arise as a consequence of the oxidative stress caused by ischemia/reperfusion [42]. A decrease, oscillations and even a collapse of $\Delta\Psi_m$ were observed in isolated cardiomyocytes exposed to oxidative stress [61]. Oxidative stress can be experimentally induced by exposing a small volume of the cell to a laser flash. The energy provided by the laser light induces a local increase in the production of reactive oxygen species (ROS) in the mitochondria in the flashed region. In 2000, Zorov et al. published a study showing that local ROS production can lead to a synchronous collapse of $\Delta\Psi_m$

in whole cardiomyocytes [65]. The increased ROS concentration in the mitochondria causes the release of excess ROS into the cytoplasm, in parallel with a fast depolarization of mitochondrial membrane potential. This process was named ROS-induced ROS release [65]. Before the ROS are scavenged by superoxide dismutase, the ROS cause depolarization of neighboring mitochondria. It is assumed that the prevailing form of the ROS responsible for this effect is the superoxide radical. Under physiological conditions, local $\Delta\Psi_m$ oscillations are characterized by a broad frequency range, and mitochondria act as oscillators that are weakly coupled by ROS. $\Delta\Psi_m$ oscillations, which are induced by oxidative stress or by insufficient concentrations of substrate, are characterized by one dominant frequency and a high amplitude (up to tens of millivolts) [61]. These oscillations spread into the whole cardiomyocyte [66]. Aon et al. showed that the ROS produced at complex III of the electron transport chain are from the main part responsible for synchronous $\Delta\Psi_m$ oscillations. The authors provided evidence that the superoxide radical is the ROS involved in the oscillations and their propagation throughout the cell. The role of IMAC in the process of $\Delta\Psi_m$ regulation was highlighted by the fact that inhibitors of anion channels (DIDS, 4′-chlorodiazepam) prevented the oscillations and collapse of $\Delta\Psi_m$ [61]. The presence of an anion channel inhibitor locked the ROS within the mitochondria exposed to a laser flash. These results led the authors to assume a double role of IMAC in both the dissipation of energy that causes $\Delta\Psi_m$ depolarization and the pathway that allows superoxide radicals to leave the matrix [61]. $\Delta\Psi_m$ changes are also accompanied by redox potential oscillations in the whole cell [61]. The redox pair reduced/oxidized glutathione (GSH/GSSG) is the major indicator of the cellular redox state [67]. It has been proved experimentally that the GSH/GSSG ratio determines the trend of $\Delta\Psi_m$ changes and their reversibility [68]. The GSH/GSSG ratio and the absolute concentration of these two compounds in cardiomyocytes affect the chloride fluxes in mitochondria under conditions of oxidative stress [68]. A computer model of a mitochondrial oscillator was based on oxidative phosphorylation, the cytoplasmic ROS-scavenging system, ROS and IMAC [62]. The results of this model are in good agreement with experimental data. IMAC, whose identity remains uncertain, was included in this model as an escape route for ROS. The conductance of IMAC was set as the conductance of the yeast 108-pS channel [49]. The model assumes that IMAC is activated in a positive feedback loop by the leaking of ROS from the matrix, where high concentrations of ROS accumulate under oxidative stress conditions. IMAC mediates the release of ROS from mitochondria if the ROS production in the matrix is increased to a critical value. The release of ROS provides communication within the mitochondrial network, which results in synchronous $\Delta\Psi_m$ oscillations throughout the whole cardiomyocyte [62, 69]. According to the model, the flux of anions through IMAC is responsible for the fast depolarization phase during the $\Delta\Psi_m$ oscillations [62, 66].

The role of IMAC in the process of mitochondrial membrane potential oscillations inspired a further search for evidence of the identity of the native chloride channels from highly purified rat cardiomyocyte mitochondria.

5. Kinetics, subconductance states and inferred channel structure

The basic biophysical properties and regulation of many of the described mitochondrial chloride channels are not consistently in agreement among themselves and with the accepted

properties of the IMAC pathway, but the gating and permeation through the channel pore seem to be more useful properties for comparison of these channels.

The 108-pS channel from brown adipose tissue mitochondria is characterized by bursts of fast flickering at negative voltages and long openings at positive voltages [49]. A similar behavior, but with inverse polarity, is visible in the traces of sheep chloride channels. These channels were reconstituted in a planar lipid bilayer; at an applied voltage of −50 mV, the openings were long, and the transitions to the closed state were infrequent. By contrast, the channel under +50 mV applied potential also exhibited bursts of fast events (see Figures 2 and 3 in [55]). The different polarities of this effect can be caused by different orientations of the channel in the measuring system. The bursting behavior was also reported for the chloride channels of rat cardiac mitochondria [57]. A report that the gating kinetics was affected by voltage was also found for the 45-pS yeast mitochondria channel [47]. It is unfortunate that the gating kinetics was not thoroughly described for many of the reported mitochondrial chloride channels; this information might have been helpful for comparison of these channels, which differ in pharmacological regulation in many cases.

In general, ion channels can have not only a main conducting state but also states with lower than maximal conductance, which are called subconductance states (or simply substates). The presence of substates was described and analyzed in native chloride channels from rat cardiac mitochondria [70], which are similar to the chloride channels from sheep cardiac mitochondria [55]. Three distinct substates were detected in the chloride channels of rat origin, and their conductances corresponded to 29, 50 and 74% of the maximal conductance. The occupancy of states with a lower conductance is small and less than 2% for each state. SMACs from sheep mitochondria displayed substates with 25 and 50% of the maximal conductance. The occupancy of the 75% substate was rare and below 1% [55]. Hayman proposed that the channel is formed by four subunits. INMACs exhibited several substates, but these states were not described in more detail [46].

An unusual observation of the decomposition of the chloride channel of rat cardiac mitochondria into substates was described by Tomasek et al. [70]. This channel is characterized by the absence of a maximal conducting state and an increased gating frequency between substates, leading to complete loss of the channel activity. The behavior of the channels was in line with the suggested model of four conducting subunits that cooperate as one channel. The gating of the four units is synchronized, and it seems that the subunits are unstable when they do not work in the cooperating complex.

As the occupancy of substates that were analyzed is low, it is not surprising that the occupancy has not been described or even mentioned in other studies of chloride channels from the inner mitochondrial membranes. On the other hand, the presence of substates was described for some chloride intracellular channels called CLICs [71].

6. CLIC localization

Thirty years ago, chloride intracellular channel (CLIC) proteins were isolated from bovine kidney cortex membrane vesicles for the first time [15]. The protein was isolated using a high-affinity ligand of chloride channels, indanyloxyacetic acid (IAA-94), and the transport through

the channel was detected by the uptake of the $^{36}Cl^-$ isotope. In 1989, CLIC proteins were purified from bovine kidney cortexes and the apical membranes of bovine trachea and reconstituted into lipid vesicles; the single-channel properties of CLIC were measured in a planar lipid bilayer system [21]. Since then, the family of CLIC proteins has grown, and it contains six vertebrate members, three invertebrate members and at least four plant members [71]. These proteins can adopt two forms—soluble and integral membrane [72]. The soluble form of CLIC1 was crystallized, and the obtained structure is a structural homologue of the glutathione-S-transferase superfamily [72, 73]. The CLIC1 isoform was also crystallized in the presence of glutathione (GSH), for which these proteins possess a binding site [72]. CLIC1 has been thoroughly studied at the single-channel level, but an extremely broad range of channel conductances from 17 to 160 pS has been reported for this isoform [71, 72, 74–78]. The channel activity was not affected by the presence of 1 mM Ca^{2+} or 1 mM Mg^{2+}, although the activity was blocked by both the oxidized and reduced forms of glutathione [74], which is consistent with the presence of a glutathione-binding site. This channel is localized in intracellular membranes, as are other CLIC family members, and in plasma membranes [71]. The conductance of expressed CLIC4 channels was measured in several studies, and the conductance spanned a broad range of values from 1 [79] up to 43 pS [80, 81]. Although the conductance varies among these studies, there is an agreement concerning the selectivity of the channel, which is poorly selective for anions over cations ($P_{Cl}/P_{choline}$ ~3) [79, 80]. Littler et al. resolved the structure of CLIC4 at 1.8 Å resolution [81], indicating that CLIC4 is a monomeric protein, and it was structurally similar to CLIC1 with a difference in the domain of CLIC1 that undergoes structural changes upon oxidation. It was shown, however, that CLIC4 also responds to redox agents. Incubation with H_2O_2 favored the fusion of CLIC4 into the planar lipid bilayer; on the other hand, pretreatment with DTT resulted in no channel activity [81]. Intriguingly, the CLIC4 expressed in HEK293 cells was not affected by the nonspecific chloride channel inhibitor DIDS [82]. The absence of DIDS inhibition was also found for the centum-pS channel [41], and a one-sided effect was reported for anion channels derived from rat cardiac mitochondrial membranes [57].

The recombinant CLIC5 isoform was also studied at the single-channel level [83]. Similar to other CLIC isoforms, CLIC5 is poorly selective for monovalent ions (either negatively or positively charged), and several values of conductance ranging between 26 and 400 pS were reported [21, 84, 85]. Different substates were observed in single-channel current recordings of CLIC1 and CLIC5 proteins incorporated into planar lipid bilayers [77, 83]. The observed broad range of conductance values was interpreted to indicate a cluster of functioning chloride channel pores [71, 86].

CLIC proteins possess two peculiar properties. One already mentioned property is that CLIC proteins can exist in two different forms: a soluble protein form and an integral membrane protein form, which is unusual as the structural characteristics of integral proteins differ from those of soluble proteins. This double structure is deduced from the fact that CLIC proteins were found in both the aqueous phase and the membrane fraction during the process of protein isolation [87]. However, the crystallographic structure of the integral form has not yet been determined. The second peculiarity is that these proteins lack the conserved mitochondrial targeting sequence [88–90]; nevertheless, in 2016, the research group of H. Singh reported that some CLIC isoforms are localized in the mitochondria of cardiomyocytes [89, 90].

Indeed, this finding was a breakthrough after three decades of searching for the identity of chloride channel proteins in the inner mitochondrial membrane. The CLIC4 and CLIC5 isoforms were detected on mitochondrial membranes using immunofluorescence imaging and colocalization of fluorescent markers for CLIC isoforms 1, 4 and 5 and MitoTracker dye via a super-resolution STED microscope. The presence of the corresponding proteins was detected by Western blot analysis of the purified mitochondrial fraction. Interestingly, the CLIC5 and CLIC4 proteins do not have the same distribution in the mitochondrial membrane. The CLIC4 isoform has a cluster distribution similar to that of a voltage-dependent anion channel of the outer mitochondrial membrane, VDAC1 [91]. Higher levels of the CLIC4 isoform were found in the outer mitochondrial membrane, while CLIC5 had a uniform distribution mainly concentrated in the inner mitochondrial membrane.

7. Discussion of the identity of the mitochondrial chloride channel

The spectrum of chloride channels found in the native inner mitochondrial membranes seems to be broad, and there are many experimental inconsistencies among these channels. Some properties are nevertheless notable when one is searching for the identity of the channel. The discussed channels were all measured either from highly purified mitochondrial membrane fractions fused to planar lipid bilayers or from patched mitoplasts. The most frequently reported channel has ~100-pS conductance [39, 41, 46, 49, 57]. Several of the channels share a bursting pattern of single-channel activity, voltage-dependent activity and gating kinetics. In addition, some of these channels maintain the pH regulation described for the anion flux through IMAC [49, 53, 56]. An anion channel having a 100-pS conductance was successfully incorporated in a model of the process of ROS-induced ROS release and synchronized mitochondrial membrane potential oscillations [62]. This model describes well the measured experimental data.

The only chloride channel protein detected in the inner mitochondrial membrane is the CLIC5 channel. Naturally, the question arises as to whether the CLIC5 channel is identical to the channel with the measured single-channel currents described in several studies. To answer this question now, we can consider only the available (and not very abundant) experimental data from recombinant CLIC5 single-channel measurements [21, 84, 85]. The inhibitory effect of IAA-94, which was studied with native mitochondrial chloride channels of the rat heart [56], favors a connection between these channels and CLICs. The hints of the structural features of native channels of both rat [70] and sheep [55] origin are also in good agreement with the structural model suggested for CLICs [71]. Rat mitochondrial chloride channels, which are similar in many ways to the centum-pS channel, suggest that these channels correspond to IMACs, and the rat mitochondrial chloride channels were reported to be redox-sensitive [70]. Unfortunately, the redox regulation has not been thoroughly described, but the behavior of the channels in reduced and oxidized environments seems to be consistent with the CLIC redox sensitivity. Both CLICs and (the many) mitochondrial chloride channels are poorly selective, and the conductance of all the discussed chloride channels is within the broad range reported for CLIC channels [71].

8. Conclusion and future prospects

There is one crucial task ahead of us to reach the ultimate convergence point: the connection between the native inner mitochondrial membrane chloride channels and the CLIC protein must be proved unambiguously. If this connection was proven, the experimentation on the mitochondrial membrane potential can progress to a completely new level. This progression might provide important knowledge concerning the process of cardiac arrhythmias caused by $\Delta\Psi_m$ oscillations, with a potential clinical impact in the future. The use of molecular genetic techniques, such as silencing or conditional knock-out, and studies on the structure-function relationship will be feasible. Once the gene for the mitochondrial chloride channel is known, many options for experimentation will become available. The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway can provide benefits against ischemia/reperfusion injury via changes in the expression or activity of some proteins, including the proteins of the respiratory chain [92]. In the context of the ROS-induced $\Delta\Psi_m$ oscillations that occur during mitochondrial stress, it seems to be causally important to minimize ROS formation by improving mitochondrial function. STATs, which were also identified in cardiomyocyte mitochondria, can be useful for this task. In addition to being involved in mitochondrial respiratory function [93], STAT3 may regulate the mitochondrial permeability transition pore [94, 95]. It would be of interest to know whether the inner membrane mitochondrial chloride channel is also affected by STAT3 or the STAT3 activation pathway under conditions of ischemia/reperfusion.

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Conflict of interest

There is no conflict of interest.

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