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Review

Mitochondrial ion channels as therapeutic targets

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ABSTRACT

The study of mitochondrial ion channels changed our perception of these double-wrapped organelles from being just the power house of a cell to the guardian of a cell's fate. Mitochondria communicate with the cell through these special channels. Most of the time, the message is encoded by ion flow across the mitochondrial outer and inner membranes. Potassium, sodium, calcium, protons, nucleotides, and proteins traverse the mitochondrial membranes in an exquisitely regulated manner to control a myriad of processes, from respiration and mitochondrial morphology to cell proliferation and cell death. This review is an update on both well established and putative mitochondrial channels regarding their composition, function, regulation, and therapeutic potential.

1. Introduction

Ion channels are central to communication within the body; they are responsible for your thought processes and keep your heart beating. Ion channels are the target of most pharmaceutical products. Hence, it is not surprising that mitochondria rely heavily on a battery of ion channels to interface the communication between the cytosol and the site of energy production, which also holds the key to life and death decisions. Here, we will describe the multiple channels of the outer and inner membranes of mitochondria that orchestrate a myriad of cellular processes from ATP, steroid, and heme synthesis to protein import and apoptosis. As we are electrophysiologists, we will use the single channel behavior of these channels as our central theme.

During the 70s and 80s the notion that mitochondria might have channels was typically dismissed because the consensus was that mitochondria could not possibly maintain the membrane potential and high resistance essential to oxidative phosphorylation if they had channels; opening channels would cause uncoupling. Historically, VDAC (voltage dependent anion-selective channel), or mitochondrial porin, was the first channel identified in mitochondria. Isolated from paramecium mitochondria, VDAC's activity was characterized in planar bilayers in a report in 1976 by Schein et al. [1]. Hence, the molecular identity and function of VDAC has been known for many years. The first characterization of the permeability transition pore or PTP quickly followed that of VDAC in 1979, in a series of articles by Hunter and Haworth [2]; these were not electrophysiological, but rather based on swelling assays using a photometric approach. At that time, few appreciated the impact of this channel, which we now realize is central to ischemia-reperfusion injury and necrosis. Our group and that of Zoratti independently characterized PTP single channel behavior 10 years later by patch clamping mitoplasts, which are mitochondria stripped to reveal the inner membrane [3,4]. Intriguingly, 30 years after the initial reports, the molecular identity and function of PTP remain a matter of speculation and open argument. Nevertheless, the persistent application of patch clamping techniques to mitoplasts led to the discovery of many other intriguing channels. Sorgato first reported the existence of a novel anion-selective channel, mCS (mitochondrial Centum-pico-Siemen channel) in mitoplasts; the molecular identity and function of this channel remains elusive [5]. Not much later, our lab found the TIM23 channel, which was the first electrophysiological demonstration of the link between protein import and water-filled channels in mitochondria [6]. This was quickly followed by a report from Juin et al. on the channel activity of TOM, the protein import complex in the outer membrane [7] (Fig. 1). And, there are numerous other channels like AAA, ACA, TIM22, and various K^+ channels, which were discovered by applying these techniques to native membranes. Here, we will provide you with an update on where

Abbreviations: AAA, alkaline-induced anion-selective activity; ACA, alkaline-induced cation-selective activity; ANT, adenosine nucleotide translocator; CLIC, chloride intracellular channel; IMAC, inner membrane anion channel; MAC, mitochondrial apoptosis-induced channel; mCS, mitochondrial Centum-pico-Siemen channel; MCU, mitochondrial calcium uniporter; PTP, permeability transition pore; TIM, translocase of the inner membrane; TOM, translocase of the outer membrane; VDAC, voltage dependent anion-selective channel

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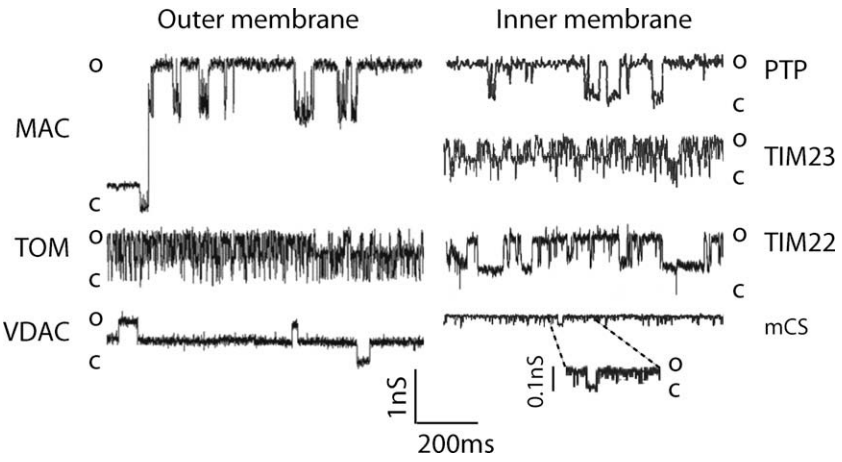

Fig. 1. The main channel activities of mitochondrial membranes. Current traces were recorded from patches excised from either reconstituted or native membranes under symmetrical 150 mM KCl (see original articles). Pipette voltages vary from +5 to + 40 mV, but current traces are represented in the same scale for comparison. All the traces show open channels (o) with downward transitions to sub open and closed (c) states. Traces were adapted from [100] (mouse MAC, TOM and VDAC), [101] (human TIM23 and mCS), [60] (yeast TIM22), and [102] (mouse PTP).

we stand in identifying the molecular basis and function of mitochondrial ion channels.

2. The food channel, VDAC

A decade before we trained our micropipettes to chase tiny mitochondria in suspension, VDAC conductance was reconstituted in phospholipid bilayers [1]. A great deal of information has been collected about this voltage dependent anion-selective channel over the past 30 years using electrophysiological, biochemical and functional approaches [8]. VDAC is the most abundant protein in the outer membrane of mitochondria and it was the first mitochondrial channel to be identified. While the sequence homology is often below 30%, multiple VDAC isoforms are expressed in paramecium, yeast, and all higher mammalian mitochondria with surprisingly strict functional homology. While knocking out both isoforms of VDAC in yeast is debilitating, knocking out all three VDAC isoforms in mouse is lethal. Hence, VDAC fulfills an essential role, at least in higher organisms. Importantly and as a testament to their functional homology, expression of human VDAC in yeast rescues the defects observed in the double knockout (reviewed in [9]).

VDAC provides the aqueous pathway across the outer membrane for the transfer of the food from the cytosol to the inner membrane. Hence, it is dubbed the 'food channel'. That is, the substrates, including pyruvate, malate, succinate, NADH as well as ADP and phosphate that are used by mitochondria to generate ATP through oxidative phosphorylation, pass through VDAC on their way to the electron transport chain in the inner membrane. In keeping with two way trafficking, the ATP and hemes generated by mitochondria traverse VDAC to reach the cytosol. Interestingly, VDAC interacts with many other proteins, e.g., hexokinase and creatine kinase, to trap the newly generated ATP in high energy storage forms like glucose 6-phosphate and creatine phosphate, in brain and muscle, respectively [9,10].

Bacterial porin has striking similarities to VDAC. Both channels have β-barrel pores formed from anti-parallel β-pleated sheets. Both channels are voltage dependent and have quite similar functions in delivering foodstuffs. These similarities underlie the often used other name for VDAC, which is mitochondrial porin. There are however differences. For example, VDAC has a single pore and normally exists as a monomer while bacterial porins can form trimers and adopt different configurations (reviewed in [11]).

For the most part, there is a consensus that VDAC has a single pore 2.5–3 nm wide when fully open. In its fully open state (~650 pS in 150 mM KCl) (Fig. 1), VDAC's pore has a slight anion-selectivity that shifts to a cation selective state when it partially closes [12]. This shift to a half open and cation-selective state is observed at both edges of VDAC's bell-shaped voltage dependence, i.e., at ±25–50 mV. In agreement with its dynamic gating properties, fully open VDAC allows the flux of metabolites like ATP, ADP, and Pi, along with Ca^{2+}, K^+, and Na^+ across the outer membrane [13]. When partially closed, the pore is ~1.8 nm wide and cation-selective. While a cationic fully open VDAC has been reported, its permeability to ATP awaits further examination. There are three mammalian isoforms of VDAC and similar single channel behaviors are recorded using electrophysiological approaches regardless of species. That is both rat and yeast have a fully open state of ~650 pS that are generally anion-selective with similar voltage dependencies. In addition, both species of VDAC can assume a fully open or half open state with slight cation selectivity.

VDAC structure has been deduced from NMR and crystallography studies [14,15]. These studies agree with earlier electron microscopy studies which report a slightly elliptical 2.7–3.5 × 2.4–3.1 nm pore [16]. In the 3D models, this pore straightens in the middle into 1.5–2.7 × 1–1.7 nm. These dimensions are likely to represent those of a fully open VDAC pore. Hence, the channel would be composed of up to 19 β-strands plus one α-helix. A consensus has yet to be achieved about the role and location of this positively charged α-helix. While the 3D models locate this N-terminal helix next to the barrel's wall, facing the c-terminus, others suggest that this helix may be part of the barrel's wall or perhaps located outside in the cytoplasm. In fact, current recordings of VDAC lacking the α-helix show smaller conductances. In a recent opinion article, Colombini suggests that these and other discrepancies regarding 3D and "functionally-derived" structures are inherent to the refolding and reconstitution procedures used to obtain the 3D structures [17]. Interestingly, and in contrast to AFM images, the 3D structures lack superficial protrusions. These protrusions could be formed by the α-helix, by a β-strand, or possibly by a putative accessory protein.

Hundreds of papers have been published on VDAC, a few hundred of which show channel recordings in reconstituted systems. However, not a single paper reports VDAC gating in the outer membrane of isolated mitochondria that corresponds to the activity seen after reconstitution in planar bilayers or proteoliposomes. Thus, a putative modulator that controls VDAC gating in isolated

mitochondria could possibly be lost in reconstituted systems. In agreement, several cytosolic factors decreased the open probability of VDAC reconstituted in planar bilayers. For example, NADH and Mg^{2+}-NAD(P)H decreases ADP flux through VDAC. Moreover, an intermembrane space (IMS) protein and tBid (a cleaved form of Bid) has been reported as a putative endogenous inhibitor of VDAC. Finally, VDAC can be phosphorylated and has binding sites for hexokinase I and hexokinase II. Paradoxically, while hexokinase I seems to close the channel, hexokinase II seems to keep it open. The list of putative VDAC modulators is still growing – Roman and co-workers surveyed 55 novel interactions [18]. While it is reasonable that the most abundant protein in the mitochondrial outer membrane might be the main pathway of communication with the cytosol, putative modulators are based on biochemical and electrophysiological data and also on functional studies.

Determinations of VDAC conductance and modulation of metabolite flux have just begun in intact cells. In an elegant variation of the patch clamp techniques, Jonas and co-workers showed that a large outer membrane channel with voltage dependence typical of VDAC can be activated in intact neurons by a train of synaptic action potentials or by a cleaved form of Bcl-xL. These channels are inhibited by NADH or hypoxia [19]. Other studies include an ethanol induced shift of cytosolic NADH/NAD+ redox potential to a reduced state which may inhibit ADP flux across the outer membrane and a report that the conductance of VDAC is Ca^{2+} dependent at physiological cytosolic Ca^{2+} concentrations [20]. Finally, Ca^{2+} pretreatment of permeabilized cells enhanced subsequent ATP uptake, implying a VDAC-mediated regulation of metabolism. All these studies show that the best studied mitochondrial channel VDAC has many faces that are just now beginning to be revealed.

3. Apoptosis channels

While generating the energy currency of the cell, ATP, the many functions that mitochondria perform are just now being discovered. Mitochondria play a central role in apoptosis. These organelles compartmentalize a myriad of death-evoking signaling proteins, like cytochrome *c* and AIF, which upon their release to the cytosol wreak havoc and eventually induce cell death through apoptosome formation and caspase activation. There are at least two ways to break down the permeability barrier of the outer membrane (Fig. 2). Activation of the inner membrane channel PTP causes matrix swelling and ruptures the outer membrane. This event results in a spilling of IMS contents into the cytosol. Alternatively, de novo formation of a gigantic channel called MAC (mitochondrial apoptosis-induced channel) permits cytochrome *c* and other pro-apoptotic factors to freely diffuse from the IMS to the cytosol. Here, we discuss the channels MAC and PTP, and the possibility these channels act synergistically in the suicide of the cell.

3.1. MAC

During apoptosis, pro-apoptotic proteins assemble into a large channel, MAC, which makes the mitochondrial outer membrane permeable to proteins normally constrained within the IMS. Once in the cytoplasm, these proteins trigger a chain of events that lead to the destruction of the cell (Fig. 2). MAC formation and mitochondria outer membrane permeabilization (MOMP) have been consistently reported in a variety of cell lines during at least two different apoptotic insults, IL-3 deprivation [21] and kinase inhibition [22]. After induction of apoptosis, a time dependent increase in membrane permeability resulted from formation of MACs with conductances of 3–5 nS (in 150 mM KCl) (Fig. 1). Regardless of its heterogeneous peak conductance, >1.5 nS MAC is voltage independent and slightly cation-selective, consistent with its proposed function as a cytochrome *c* release channel (cytochrome *c* is cationic). MAC with a conductance of 4 nS is permeable to up to 17 kDa dextran. This polymer exclusion method indicates that MAC with conductances between 1.5 and 5 nS have pore sizes of 2.9–7.6 nm, which should be large enough to allow the passage of 3 nm cytochrome *c*. Cytochrome *c* in fact changes MAC conductance in an interesting way. A reversible and dose dependent increase in noise and decrease in conductance (4–50%) is observed, suggesting cytochrome *c* is partitioning into the pore. This effect was named type 1 and resembles that seen with other channels, e.g., ATP on VDAC [13,23], DNA on α-hemolysin [24], maltodextrins on maltoporins [25], and ampicilin on OmpF [26]. The Type 2 effects of cytochrome *c* correspond to a more robust reduction in conductance (50–90%) that is dose dependent, irreversible, and voltage independent. This response corresponds either to a destabilization or a plugging of the open state of MAC. Possibly, binding sites for cytochrome *c* may exist in the structure of 2–4 nS MAC, which could block the passageway and may be important in synchronization of apoptotic events.

Unlike the mitochondrial protein import complexes (see below), MAC allows release of soluble proteins from the IMS into the cytosol. And, although MAC pore is at least twice as big as those from the protein import complexes, its function is in contrast to that of the import complexes, as this channel allows for the export of folded proteins from mitochondria. Consistent with its function, MAC conductance decreases in presence of cytochrome *c* [27], as discussed above. Induction of MAC formation by several means, such as staurosporine treatment, IL-3 withdrawal, exogenous expression of Bax and tBid lead to release of cytochrome *c* in different cell lines. For example, staurosporine treatment induced increased expression and mitochondrial relocation of green fluorescent Bax. When mitochondria containing green fluorescent Bax where isolated and patch clamped, they showed an increase in conductance of ~6 nS, consistent with the presence of MAC. Immunocytochemistry analysis showed cytochrome *c* leaked to the cytoplasm under these conditions [22]. Such release can in turn be prevented by knocking out Bax and Bak, overexpressing Bcl-2 or Bcl-xL, or using MAC inhibitors [28]. The result of knocking down MAC's main components or its pharmacological inhibition is prevention of cytochrome *c* release and protection against apoptosis.

Whether MAC or its components participate in other cellular functions is of increasing interest. Some pieces of evidence exist of a possible interaction of Bcl-2 family proteins, e.g., Bax and Bak with the mitochondrial morphology machinery (for a review, see [29]). Recent studies implicate Bax and Bak with maintenance of the mitochondrial network in the absence of apoptosis, since double knockout cells had smaller mitochondria than control cells. While there are alternative interpretations, DDP/Timm8a, an IMS protein that is released presumably through MAC and at the same time as cytochrome *c*, can activate the fission protein Drp-1 and promote mitochondrial fragmentation [30]. These findings support the hypothesis that fission of mitochondria occurs after MAC formation. Yet another role has been recently proposed for MAC in generation of a so called bystander effect, based on evidence that a death signal generated downstream of MAC and cytochrome *c* release seems to propagate through gap junctions to kill adjacent cells [31].

The molecular identity of MAC, or at least most of it, has been determined using functional knockouts, immunoprecipitations, and pharmacological inhibitors. VDAC1 and VDAC3 have been excluded as MAC components, since MAC formation, conductance and function were unaffected by either knockouts [32]. Nevertheless, two pro-apoptotic Bcl-2 members, Bax and Bak have been established as functionally and structurally redundant core com-

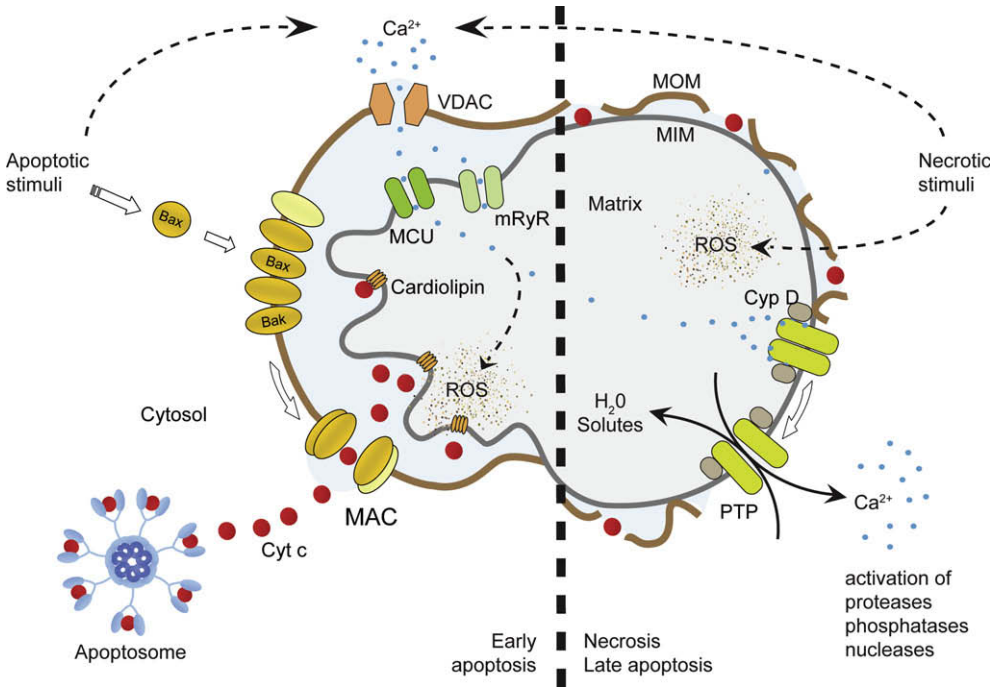

Fig. 2. Mitochondrial ion channels in apoptosis. *Left*, Apoptotic stimuli induce relocation of Bax from the cytosol into the mitochondrial outer membrane (MOM). Bax, Bak and possibly other unidentified protein(s) oligomerize and form MAC. An increase in ROS production, possibly due to Ca^{2+} entry through VDAC, MCU and mRyR, detaches cytochrome *c* from cardiolipin in the inner membrane (MIM). Cytochrome *c* spills into the cytoplasm and binds Apaf-1 to form apoptosomes and amplify the apoptotic cascade. *Right*, Necrotic stimuli lead to exacerbated Ca^{2+} uptake and ROS generation by mitochondria. High levels of Ca^{2+} and ROS induce a cyclophilin D (Cyp D)-sensitive opening of PTP that leads to swelling of the matrix and release of Ca^{2+}. Swelling disrupts the outer membrane while released Ca^{2+} activates proteases, phosphatases and nucleases that lead to necrotic degradation.

ponents of MAC. That is, either Bax or Bak knockouts are equally capable of attaining MAC conductance and releasing cytochrome *c* upon apoptotic stimulation. Hence, one can envision that MAC exists in three populations: two of them formed by Bax or Bak homo-oligomers and one by Bax–Bak hetero-oligomers. However, Mikhailov et al. showed that, although Bax and Bak in the oligomeric state are able to co-immunoprecipitate, they fail to crosslink [33]. Hence, although Bax and Bak are unquestionable components, their structural relationship has yet to be clarified, as well as the possible existence of additional components, for which an extensive proteomic approach might be necessary.

Much of MAC structure has been deduced from functional, biochemical, genetic and patch clamp studies. The pore of functional MAC is thought to be minimally a hexamer of Bax and/or Bak assuming each monomer contributes two transmembrane helices. In fact, α-helices 5 and 6 of Bax are amphipatic, which makes them good candidates for pore components [34]. Moreover, Bax lacking helices 5 and 6 do not release cytochrome *c* [35,36]. Given the structural similarities between Bax and Bak, the same helices could underlie pore formation in MAC by either protein. Single channel analysis of tBid-induced MAC formation in freshly isolated mitochondria suggested that MAC formation can be explained by sequential incorporation of additional Bax or Bak monomers to an existing pore [32]. However, other studies with outer membrane preparations suggest a lipidic component to the pore [37].

3.2. PTP

The mitochondrial permeability transition pore (PTP) was originally described in swelling experiments on isolated mitochondria 30 years ago [2]. Opening of the PTP leads to a transition in permeability of the inner membrane from extremely low to freely permeable to solutes up to 1.5 kDa. These changes were typically monitored by light scattering at wavelengths known to reflect mitochondrial volume. The PTP can be reversibly closed by removal of Ca^{2+} or by the addition of cyclosporine A, magnesium or ADP. A myriad of effectors including Ca^{2+} plus Pi and reactive oxygen species have been reported [38]. This channel has been implicated in the apoptotic cascade as a means of releasing cytochrome *c* from mitochondria. However, the role of PTP in early stages of apoptosis is unclear, since its opening suspends oxidative phosphorylation and leads to depletion of ATP reserves required for apoptosome formation. PTP opening is frequently observed downstream of MAC formation and outer membrane permeabilization, but it is not clear if PTP has a role in induction of apoptosis. Some models, however, advocate for a PTP dependent apoptosis prior to MAC formation [39]. Persistent PTP opening results in loss of mitochondrial membrane potential and massive swelling of mitochondria likely involved in necrotic cell death (Fig. 2).

The connection between the PTP and channel activities recorded directly from the inner membrane by patch clamping mammalian mitoplasts was made over a few years mostly by Zoratti's group [40]. In earlier studies, the PTP activity was referred to as MCC (mitochondrial multiple conductance channel) or MMC (mitochondrial megachannel). Although much smaller than MAC and Bax channels, PTP has a high conductance of ~1.2 nS with multiple conductance levels (Fig. 1). Transitions between sublevels are typically 0.3–1 nS. Unlike MAC, this slightly cation-selective channel is voltage dependent, closing with matrix positive potentials. Interestingly, while overexpression of Bcl-2 had no detectable effect on the channel activity, this protein suppressed calcium-activation of PTP in both swelling and electrophysiological experiments [41,42]. Recently, Zoratti's group described a new channel activity in mitoplasts that resembles plasmalemmal maxi-anion channels, but differs pharmacologically and was observed more frequently at high Ca^{2+} concentrations, known to induce PTP [43]. The channel conductance, gating and selectivity rather seems closer to VDAC than PTP, although its occurrence

was unaffected in VDAC-1$^{-/-}$/VDAC-3$^{-/-}$ double knockouts. Because the detection of this channel was inversely proportional to that of PTP in five different cell lines, the authors coined this new activity HP (Half PTP). Interestingly, HP was not affected by cyclosporine A.

The molecular identity of PTP remains unresolved despite extensive efforts. ANT and VDAC were thought to be pore components of PTP based on the inhibitory effects of ANT inhibitors, and on co-immunoprecipitation experiments of VDAC with ANT. These candidates were later discarded because both ANT and VDAC knockouts expressed PTP activity [44,45]. Cyclophilin D knockouts, however, modified the permeability transition and reduced injury after reperfusion. Knockout experiments demonstrated that cyclophilin D represents the target for PTP inhibition by cyclosporin A, and that it modulates the sensitivity of the PTP to Ca^{2+} [46]. Recently, the mitochondrial phosphate carrier was proposed as a new candidate based on its co-immunoprecipitation with cyclophilin D [47]. This interaction was prevented by cyclosporin A, a classical PTP inhibitor, and was promoted by oxidative stress, a classical PTP inducer. However, cyclophilin D has many partners in mitochondria. Furthermore, an essential role for the phosphate carrier in PTP formation awaits a demonstration that knockdowns fail to undergo a permeability transition. While the phosphate carrier has reported channel activity [48], assignment as the PTP would require that the channel activity be sensitive to cyclophilin D and cyclosporine A. Finally, as a non-protein candidate, polyphosphate has also proposed as the molecular basis of the PTP [49].

Studies propose alternative structural candidates in the inner membrane, but what about the outer membrane counterpart(s)? Most of the studies that advocated for or against VDAC participation on PTP used matrix swelling assays. This argument may be over-interpreted because PTP is probably not the sole mediator of matrix swelling. In fact, PTP inhibitors reduce, but do not completely abolish swelling. But the question remains. What, if one exists, is the outer membrane counterpart of PTP? If PTP really spans both the inner and outer membranes, its conductance must be detectable in the outer membrane of intact mitochondria. Virtually all current traces attributed to PTP were recorded from mitoplasts, or reconstituted systems, both devoid of the outer membrane. The only large conductances reported to date in isolated mitochondria are attributed to MAC and depleted in Bax/Bak double knockouts [22,32]. In summary, VDAC function (or dysfunction) can affect ROS production and onset of PTP, but knockout studies argue against a physical interaction and no other outer membrane protein has been associated with the permeability transition of the inner membrane. In addition, the outer membrane is thought to not represent an osmotic barrier, thus swelling is probably reliant exclusively on yet unidentified inner membrane components.

4. Protein import channels

4.1. TOM

Only 13 of the ~1500 proteins found in human mitochondria are encoded in its genome. That means mitochondria import almost all of their proteins. Regardless of their final destination, imported proteins must pass through the gatekeeper, TOM, located in the outer membrane (Fig. 3). In general, precursor proteins carry a signal sequence in their N-terminus signal that targets them to mitochondria. This signal can be a cysteine motif, a presequence or an internal targeting element [50]. All these targeting sequences are recognized by the receptor proteins Tom70 and Tom20, which then deliver the precursor protein to Tom22. Finally, the precursor is handed off to the translocation pore of the TOM complex. Tom40 forms the protein-conducting pore, while the small accessory proteins Tom5, Tom6 and Tom7 regulate stability of the complex [50]. From this point, the general import pathway diverges into several machines that finish the sorting and send the precursor proteins to their final destination. Two of these machines, TIM23 and TIM22, have inner membrane channels that will be discussed later.

The channel activity of TOM was first discovered in native outer membrane preparations as a peptide sensitive channel, named then PSC [7]. Later studies showed recombinant Tom40 was capable of forming a channel with similar characteristics in bilayer systems. Finally, comparative studies confirmed those two channel activities reflected the same entity, and the name PSC fell into disuse [51]. TOM activity has a high conductance of ~1 nS in yeast and ~750 pS in mammals (in 150 mM KCl) (Fig. 1). A half open state is frequently observed, especially at higher potentials. TOM activity is sensitive to peptides whose sequences mimic that used to target

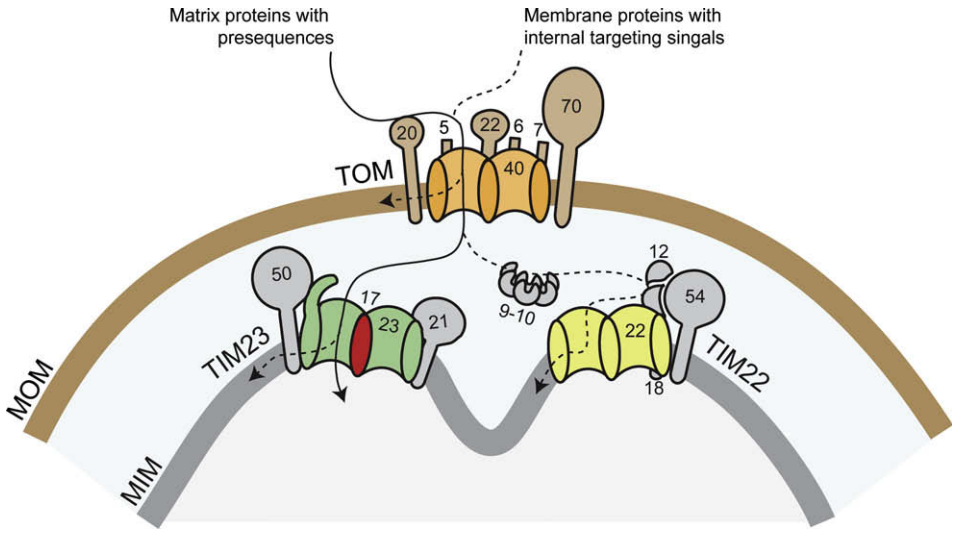

Fig. 3. Mitochondrial ion channels in protein import. The three major protein import complexes TOM, TIM23, and TIM22 are represented. Numbers indicate molecular weights of subunits and arrows indicate sorting pathways of mitochondrial proteins according to their targeting sequence. The detailed pathway, additional routes and components are reviewed in [54]. The three complexes rely on ion channels for protein import into mitochondria. In the outer membrane (MOM), Tom40 forms the channel of TOM. In the inner membrane (MIM), Tim23 and Tim17 form the channel of TIM23, while Tim22 forms the channel of the TIM22 complex.

precursor proteins; these peptides cause a rapid flickering of up to 500 transitions/s.

Many structure-function studies support the channel properties observed in patch clamp and bilayer studies. For instance, transmission micrographs of negatively stained TOM show 2 or 3 pores, consistent with the channel's substrates. Channel flickering in presence of presequences is thought to reflect transient pore occlusions during protein transport. Sizing with dextrans indicates that this channel indeed has a double barrel pore. Finally, the cation selectivity could be important for transport of positively charged presequences. However, recent analysis of TOM conductance levels reports up to 6 different states and suggests the double pore structure may be an oversimplified view of the channel [52].

Although TOM is considered the general import pathway to mitochondria, its participation in transport of some outer membrane proteins is still a matter of debate. For example, the insertion of peripheral benzodiazepine receptor seems to rely exclusively on Tom70. A similar role of Tom70 was suggested for the docking of peripheral outer membrane proteins Mfb1 and Mcl1 to the mitochondrial surface. Moreover, although an involvement of the TOM complex has been suggested for docking of pro-apoptotic Bax, some studies suggest the opposite. Finally, the assembly of the peripheral outer membrane protein Sam37 into the SAM complex was found to occur independent of any TOM component [53]. Hence, the processes underlying the insertion of proteins containing membrane-spanning α-helices into, or association of peripherally attached proteins with the outer membrane need further investigation.

4.2. TIM23

Most of the nuclear-encoded proteins that cross TOM have the matrix as their final destination. These proteins carry a presequence that allows their passage through TIM23, one of the protein translocases of the inner membrane. The TIM23 complex is an intricate machine that could contain up to 10 subunits, depending on the final destination of the protein in route [54]. The core components are Tim17 and Tim23 (pore), and Tim50 (receptor), which are sufficient for recognition of precursors and the initial, membrane-potential-dependent translocation of the preprotein across the inner membrane. Tim21 reversibly interacts with the core of TIM23 and regulates the lateral release of proteins into the inner membrane. In absence of Tim21, the core rather interacts with the PAM complex to allow translocation of proteins into the matrix. PAM is an ATP-consuming motor formed by Pam16–18, Pam44, Ssc1 (yeast homologue is Hsp70) and Mge1. While the mechanism of lateral release needs more investigation, translocation into the matrix is currently better characterized. Through ATP cleavage cycles, Mge1 assists Ssc1 to grab and pull the precursor protein into the matrix. When translocation is complete, the presequence is excised by a mitochondrial peptidase and matrix chaperones assist in protein refolding.

Patch clamp studies in isolated mitochondria from yeast and rat liver identified a channel whose high conductance in mitoplasts (Fig. 1) was activated by signal peptides [6,55]. Recombinant Tim23 showed a similar activity in bilayer systems, but signal peptides were not needed for activation. Interestingly, reconstitution of isolated inner membranes into liposomes had a similar effect, suggesting a regulating factor present in native preparations was lost after reconstitution. Because yeast MCC activity was depleted or altered by Tim23 antibodies or by Tim23 mutation [6], it was established that yeast MCC was linked to Tim23, much like PSC and TOM and then, like PSC, the name MCC also fell in disuse. Note, early studies of mammalian MCC actually corresponded to recordings of two distinct channel activities (TIM23 and PTP), which were later resolved by pharmacology. For example, cyclosporine A blocks PTP, but not TIM23 channels in mammalian mitoplasts. Furthermore, PTP has not yet been identified in yeast.

TIM23 activity is essentially identical to that of TOM and reflects the cooperative and presequence-sensitive gating of twin pores. However, recombinant Tim23 has a smaller conductance than TIM23, suggesting additional components comprise the pore. Indeed, mutation or knockdown of Tim17 diminish conductance. In the case of Tim17 knock down, polymer exclusion experiments showed the double pore was replaced by a single, presequence-insensitive pore, incapable of protein import. Hence, Tim17 was proposed as a component of the pore of TIM23 [56].

Although TIM23 is classically known to transport proteins into the matrix, some studies show it could insert proteins into the inner membrane by two different ways [54]. One group of inner membrane proteins are transported into mitochondria by a conservative sorting pathway that involves N-terminal presequence-driven translocation into the matrix via TIM23 and subsequent insertion into the inner membrane from the matrix side. Another group uses a stop-transfer mechanism for mitochondrial import and sorting. A presequence signals translocation into the matrix but a hydrophobic stop-transfer sequence stalls translocation, leading to lateral release at the level of the TIM23 complex. A third group of inner membrane proteins that use the TIM23 complex have an internal targeting signal that is composed of a transmembrane domain directly followed by a presequence-like element. Whether switching between these different transport modes involves conformational changes of the complex or assembly and disassembly processes is currently under debate.

Most of the structural and functional features currently attributed to TIM23 were obtained from yeast models, although electrophysiological recordings were also performed in mammalian mitoplasts. In a pioneering effort, Ahting and co-workers generated the first mouse knockout model of the Tim23 protein [57]. While homozygous Tim23 mice were not viable, heterozygous F1 mutants showed a 50% reduction of Tim23 protein in Western blot, a neurological phenotype and a markedly reduced life span, underlying the critical role of the mitochondrial import machinery for maintaining mitochondrial function in mammals. Electrophysiological, biochemical and morphological characterization of mitochondria from this knockout model may reveal novel intricacies of protein import, mitochondrial dynamics, and other essential functions.

4.3. TIM22

Insertion of multi-topic proteins like the phosphate carrier into the inner membrane is accomplished by the TIM22 translocase in an energy independent manner (for a review, see [50]). This 300 kDa complex consists of the three membrane proteins Tim22, Tim54, and Tim18 and three soluble IMS proteins, Tim9, Tim10, and Tim12. Tim22 is the core of the complex and is related in amino acid sequence to Tim17 and Tim23. Tim54 seems to function as a scaffold protein that would hold the complex together and is essential for protein import [58]. Tim18 is a distant homologue of subunit 3 of the succinate dehydrogenase, Sdh3. The precise functions of Tim54 and Tim18 are not known and, to this date, their presence in mammalian mitochondria has yet to be confirmed. A complex consisting of the small Tim subunits Tim9, Tim10, and Tim12 is permanently tethered to the IMS side of the TIM22 complex. Tim54 might contribute to the binding of this Tim9–10–12 complex because its association with Tim22 was destabilized in Tim54 deletion mutants [59]. Tim12 is an essential protein, and the Tim9–10–12 complex may play a vital role for substrate recognition by the TIM22 complex.

While the channel activity of TIM23 in both artificial and native systems was identified almost concurrently, the channel activity of

yeast TIM22 in native mitochondrial membranes was only recently described [60]. TIM22 is a quiescent channel that requires signal sequence interaction for its activation in both reconstituted and native mitochondrial inner membranes. Once open, TIM22 channel has activity remarkably similar to TIM23 and TOM, in agreement with their purportedly analogous function at early stages of protein translocation. In reconstituted inner membrane vesicles, the channel behavior is that of twin cooperative pores with a fully open state of ~1 nS and a 500 pS half open state (Fig. 1), much like TIM23 and TOM. However, smaller substates are also observed in mitoplasts.

5. Mitochondrial Ca^{2+} channels

Mitochondrial Ca^{2+} channels participate in many intracellular signaling pathways in physiological and pathological conditions [61]. By allowing Ca^{2+} flux in and out of mitochondria, these channels help regulate cellular bioenergetics. Importantly, inhibition of mitochondrial Ca^{2+} uptake affects ADP-induced state 3 respiration [62]. Thus, mitochondrial Ca^{2+} channels may allow mitochondria to sense and coordinate changes in cytosolic energy needs. In pathological conditions, however, mitochondrial Ca^{2+} overload contributes to the generation of reactive oxygen species [63,64], opening of PTP [65,66], and cell death [67]. Also, dysregulation of mitochondrial Ca^{2+} handling is associated with contractile dysfunction during heart failure [68].

Mitochondrial Ca^{2+} uptake has been recognized for more than 50 years – yet, its mediator(s) remains unidentified. It is often referred to as the mitochondrial calcium uniporter or MCU. Electrophysiological and biochemical approaches indeed have yielded many channel activities associated with the functions described above and they seem to belong to at least six seemingly different entities, including MCU, MiCa, mCa1, mCa2, RaM and the mitochondrial ryanodine receptor mRyR. However, only mRyR has been molecularly identified and much debate exists around the idea that the remaining five activities represent different kinetic states of MCU. These channels and the mentioned debate are thoroughly addressed in a review on mitochondrial Ca^{2+} channels by Ryu et al., in this same issue. In short, the hallmark common feature among these Ca^{2+} channels is their sensitivity to nM Ru360, although mCa2 only partially closes at ~10 μM [69,70]. MCU and MiCa also share a similar permeability to some divalent cations ($Ca^{2+} \approx Sr^{2+} \gg Mn^{2+} \approx Ba^{2+}$) and impermeability to Mg^{2+}. However, MiCa has a much higher Ca^{2+} flux rate than previously reported for MCU [69]. mCa1 and mCa2 have inward rectification Ca^{2+} currents at negative potentials like MiCa does, but mCa1 has a smaller overall open probability and ~10-fold higher conductance, while mCa2 has a smaller conductance [70]. Finally, RaM is currently described as a rapid kinetic mode of mitochondrial Ca^{2+} uptake that is inactive at μM concentrations and less sensitive to Ru360. At this moment, it is not known whether RaM is mediated by the same molecular complex as that of MCU. While it is possible that all these channel activities represent different molecular entities, differences could also be due to experimental conditions and cell types used.

Collective efforts have been made to identify MCU over the years. Ca^{2+}-binding glycoproteins and ruthenium red labeled proteins were isolated from mitochondria, but they remain unidentified. More recently, mutagenesis and knockdown studies with the uncoupling proteins 2 and 3 (UCP2/3) suggest they are fundamental for mitochondrial Ca^{2+} uptake [71]. However, it is not clear yet whether or not the UCP2/3 actually form the Ca^{2+} conducting pore(s) responsible for the MCU. Brookes et al. disputed this idea by showing that neither UCP inhibitors, GDP and genipin, nor UCP2/3 knockouts were able to alter Ca^{2+} uptake in normal mitochondria [72]. Further studies on Ca^{2+} currents in mitoplasts from both normal and UCP2/3 knockouts along with point mutations on the putative Ca^{2+} conducting pore regions could be helpful to address this issue.

6. Mitochondrial K^+ channels

Electrogenic mechanisms for K^+ entry into mitochondria (K^+ channel) have been reported for 40 years, and are thought to play a key role in the regulation of matrix volume [73,74]. The existence and properties of the K^+ channels in mitochondria have been inferred from their functional characterization. A number of channel activities associated with K^+ flux through the inner membrane were reported, but the molecular identities remain a matter of investigation. The first strong evidence of a channel activity was obtained from patch clamp studies on fused giant mitoplasts from rat liver [75]. Channel properties resembled those of the plasma membrane cardiac KATP channel, except that the blocker HMR1098 had a lower effect on this putative mitochondrial counterpart. Another large conductance (~300 pS) Ca^{2+} activated K^+ channels found in heart and brain mitoplasts of which biophysical characteristics are similar to plasma membrane BK channels showing its inhibition by charybdotoxin and activation by NS1619 [76,77]. Finally, a margatoxin-sensitive voltage gated Kv1.3 channel was identified in T lymphocyte mitoplasts and immunodetected in mitochondria. Like other mitochondrial potassium channels, Kv1.3 is similar in mitochondrial and plasma membranes, but the mitochondrial membranes used lacked plasmalemmal and endoplasmic reticulum markers. This topic is further discussed in the review in this same issue by Adam Szewczyk.

7. Mitochondrial anion channels

7.1. mCS

The mitochondrial Centum pico-Siemens channel, mCS, was the first to be identified by direct patch clamping of mitoplasts [5]. This activity has been recorded in mitoplasts from different mammalian tissues and species, such as mice liver, heart, brain, pancreas and adrenal, rat liver, heart and brown adipose tissue, ox heart or mitoplasts from human tissue culture cells, but has not been reported in yeast. The channel was slightly anion-selective ($PCl^-/PK^+ = 4.5$) and showed a strong voltage dependence. At least two closed and two open states of the same conductance were proposed: a 50% sub-conductance level, as well as infrequent channel openings 1/3 and 2/3 of the most frequent conductance level of 107 pS (Fig. 1). Additional peak conductances >140 pS in the presence amiodarone have also been reported [78]. All these conductances had the same voltage dependence, and at least the 50 pS substate showed the same anionic selectivity. mCS was largely unaffected by variations of pH from 6 to 9, on the matrix or cytoplasmic side of the membrane [5]. While Sorgato et al. recorded the channel activity without any specific activation procedure, we found the channel was normally quiescent but could be activated if calcium was chelated from the cytoplasmic side of mitoplasts during isolation procedures.

Pharmacology has enabled the elimination of a variety of proteins that might underlie mCS activity. ANT was discarded because mCS was not affected by carboxyatractylate and brongkrekate (see below). Similarly, mCS was not associated with the F_0 region of the ATP-synthase as its activity was insensitive to oligomycin and DCCD. Klitsch and Siemen reported that mCS was inhibited by submillimolar levels of di- and trinucleoside phosphates, as well as GMP, when added to the outside of patched mitoplasts [79]. These results indicated that mCS was not related to the uncoupling protein, thermogenin, which was insensitive to GMP. Furthermore,

Inoue et al. [75] reported that mCS was not affected when millimolar Mg^{2+} or ATP and micromolar ADP was applied on the matrix side of excised patches. Finally, the mitochondrial inner membrane anion channel (IMAC) is a channel inferred from light scattering (matrix swelling) studies, which had broad anion-selectivity and conducted mono-, di-, and trivalent anions [80]. This channel is believed to be an important component of the volume homeostatic mechanism of mitochondria, and was maintained closed or inactive by matrix Mg^{2+} and H^+. The lack of effect of Mg^{2+} [81], DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid), quinine or DCCD [82], all of them well known blockers of IMAC, indicated this putative channel is not related to mCS.

7.2. AAA and ACA

Two other pH-sensitive channel activities were reported in liver mitoplasts. Both of them displayed greater open probability upon alkalinization of the matrix side of the membrane, and were activated by depletion of Mg^{2+} [78]. They were referred as ACA (alkaline-induced cation-selective Activity, 15 pS) and AAA (alkaline-induced anion-selective activity, 45 pS), respectively. AAA was initially thought to correspond to IMAC because of their similar induction by alkaline pH or Mg^{2+} depletion. However, IMAC is ~100-fold more selective than AAA for Cl^- over glucouronate. The conductance and voltage dependence of ACA were similar to those of the ATP-sensitive K^+ channel (see above). However ACA was insensitive to 4-aminopyridine and glibenclamide plus ATP. The selectivity and inhibition by Mg^{2+} suggested that ACA channel activity may correspond to one of the cation uniporters implicated in volume homeostasis, whose existence was inferred from suspension studies [83,84].

7.3. CLICs

Chloride intracellular channels (CLICs) represent a new class of intracellular anion channels that have been identified by their homology to the p64 protein. A mitochondrial homolog, mtCLIC, has been identified from differential display analysis of differentiating mouse keratinocytes from $p53^{+/+}$ and $p53^{-/-}$ mice. MtCLIC colocalized with cytochrome oxidase in keratinocyte mitochondria but also was detected in the cytoplasmic compartment. This p53-regulated putative channel has been associated with apoptosis and may exist as either a soluble or a transmembrane form that may translocate to the nucleus in response to cell stress [85].

8. Putative mitochondrial channels

Aside from mitochondrial channel activities with unresolved identities, some studies have rather proposed putative channel activities with known identities. Osmotic experiments in absence and presence of Hg^{2+} suggested aquaporin 8 forms water channels in mitochondria that might regulate mitochondrial shape [86]. In this case, a channel activity has yet to be demonstrated. In other cases, purified recombinant proteins like Bcl-2, Bcl-xL and Omp85, a close relative of Sam50 in mammals, were capable of forming a channel after reconstitution in lipid bilayers [87–89]. However, it is not clear if these channel activities occur in native systems. Finally, protein free mitochondrial membrane extracts manifested a channel activity in lipid bilayers that is attributed to polyphosphate channels [49].

9. Mitochondrial channels as therapeutic targets

Mitochondrial dysfunction is at the epicenter of many devastating diseases that increasingly affect the human population. In the United States alone, 1000–4000 children per year are born with a type of mitochondrial disease. Also, many diseases of aging are now being attributed at least to a great degree to mitochondrial dysfunction. These include but are not limited to: Type 2 diabetes, Parkinson's disease, atherosclerotic heart disease, stroke, Alzheimer's disease, and cancer. Thus, mitochondria have emerged as therapeutic targets and so have the mitochondrial ion channels, since they are direct transducers of mitochondrial function, or dysfunction, to the rest of the cell. This section summarizes recent studies that raise interest to some mitochondrial ion channels as potential therapeutic targets (Table 1).

Based on the fact that VDACs are expressed more highly in cancer than normal cells, some studies propose VDAC-dependent cytotoxic agents can have cancer-selectivity. For example, furanonaphthoquinones (FNQs) induce caspase-dependent apoptosis via the production of NADH-dependent reactive oxygen species (ROS) by VDAC1. The ROS production and the anti-cancer activity of FNQs were increased by VDAC1 overexpression [90]. Another drug, erastin, induces RAS-RAF-MEK-dependent non-apoptotic cell death via VDAC2 [91]. VDAC is also a pharmacological target for G3139, an 18-mer phosphorothioate antisense oligonucleotide that targets the initiation codon region of the Bcl-2 mRNA and down-regulates the expression of Bcl-2. G3139 interacts with and closes VDAC, inducing caspase-dependent apoptosis [92]. Finally, the plant hormone methyl jasmonate has been shown to have selective anti-cancer activity in preclinical studies and the mechanism seems to be through disruption of the interaction between human hexoquinase and VDAC, causing the inhibition of glycolysis and the induction of MOMP [93].

Because MAC formation and cytochrome *c* release are the last check points typically prior the activation of the caspase cascade, these events are considered the point of no return in mitochondrial apoptosis. For that reason, MAC is a potential target for novel therapies, as the use of agonists or antagonists of this channel could induce or prevent cell death, respectively. For example, agonists of MAC could restore cytochrome *c* release and cell death in lymphomas. Some BH3 mimetic compounds like ABT-737 and its orally active analog, ABT-263, are currently in clinical trials and could potentially induce MAC in B-cell malignancies and myelomas [94]. Alternatively, antagonists of MAC potentially could protect transplanted neuronal precursor cells from apoptosis, as well as prevent HIV-1 induced lymphocyte depletion, severe congenital neutropenia, and other pathologies associated with Bax-induced cytochrome *c* release.

Cyclosporin A, a classical PTP inhibitor has been orally administered in a clinical study to treat Ullrich congenital muscular dystrophy and Bethlem myopathy patients. The mechanism of action of this drug may be due to its binding to cyclophilin D and inhibition of peptidyl–prolyl cis–trans isomerase activity. After a month of treatment, muscle biopsies showed decreased apoptosis markers and improved mitochondrial function [95]. In another disease model, Alzheimer, an accumulation of β-amyloid in mitochondria and its direct interaction with cyclophilin D promotes calcium-induced PTP opening, suggesting this channel could also be a therapeutic target in this neurodegenerative disease [96].

Although disease models are available for defects in the protein import machineries, we found no studies on therapeutic compounds targeted to these machineries. Two human diseases have been described that are caused by defects of the mitochondrial protein import machinery: the Mohr-Tranebjaerg syndrome and the syndrome of dilated cardiomyopathy with ataxia (DCMA). DCMA is a type of 3-methylglutaconic aciduria, which is clinically characterized by severe, early onset dilated cardiomyopathy, growth retardation, ataxia and optic atrophy. It is caused by a splice mutation in the DNAJC19 gene, which putatively corresponds to the human homolog of yeast Tim14. The Mohr-Tranebj-

Table 1
Mitochondrial ion channels as therapeutic targets.

Location	Type	Modulators	Potential role(s)	Therapeutic potential
Outer membrane	VDAC	Bax, Bak, Bcl-xL, Bcl-2, Tom20, Ca^{2+}, pH, ΔV, NADH, DIDS	Metabolic transport, apoptosis	FNQ, erastin, G3139, and methyl jasmonate induce apoptosis in cancer cells through VDAC [68–71]
	MAC	Bax, Bak, tBid, Bcl-2, Dibucaine, TFP, propranolol, Bci1, Bci2, iMACs	MOMP apoptosis	iMACs, Bci1, Bci2 and BH3 mimetics modulate apoptosis through MAC [22,72,81,82]
Inner membrane	TIM23	Presequences, Ca^{2+}, pH, ΔV, CsA	Protein import	Mohr-Tranebjaerg and DCMA syndromes [83–86]
	PTP	CsA, NIM811, bongkrekic acid, Ca^{2+}, pH, ΔV, Thiols, Bax, cyclophilin D	Necrosis, apoptosis	CsA on Ullrich and Bethlem syndromes, Alzheimer, cardioprotection [74,87]
	MCU	Divalent cations, nucleotides, RuRed, Ru360, ryanodine	Ca^{2+} uptake, apoptosis	Inhibition facilitates post reperfusion recovery [76]
	MitoKATP	ATP, GTP, palmitoyl CoA, Mg^{2+}, Ca^{2+}	Volume regulation protection against ischemic injury	Opening with BMS-180448 promotes cardioprotection [75]
	UCP	Fatty acids	Thermogenesis	Overexpression protects against oxidative stress [77]

See text for details and abbreviations.

aerg syndrome (MTS, also called deafness dystonia syndrome, DDS) shows X-linked recessive inheritance and is characterized by progressive sensorineural hearing loss, dystonia, mental deficiency, and visual disability. It is caused by a mutation of the deafness/dystonia protein1 gene (DDP1), also called translocase of the inner membrane 8a (TIM8A).

A prototype mitoKATP channel opener, BMS-180448 [97], has cardioprotective effects and is currently in clinical trials. However, it also opens plasmalemmal KATP channels. The role of mitochondrial potassium channels in cardioprotection is a matter of debate because of the lack of molecular identity and the ambiguous or unspecific effects of chemical agents thought to induce or prevent ischemia-reperfusion injuries.

Mitochondrial calcium influx is an attractive target for the treatment of reperfusion injury, based on in vitro studies with ruthenium red. However, this compound is unsuitable for therapeutic use and further studies are needed to develop novel compounds. One study proposed the use of dinuclear Cobalt complexes 10 years ago, but there have been no further developments [98].

Finally, mitochondrial dysfunction is a prominent feature of excitotoxic insult in neurons. In vitro studies have demonstrated these events are dependent on mitochondrial Ca^{2+} cycling and that a reduction in membrane potential is sufficient to reduce excitotoxic cell death. This concept has gained additional support from experiments demonstrating that the overexpression of endogenous mitochondrial uncoupling proteins (UCP), which decrease the mitochondrial membrane potential, decreases cell death following oxidative stress. Thus, upregulation of UCP activity can reduce excitotoxic-mediated ROS production and cell death whereas a reduction in UCP levels increases susceptibility to neuronal injury. These findings raise the possibility that mitochondrial uncoupling could be a potential novel treatment for acute CNS injuries [99].

10. Future perspectives

Mitochondria express a myriad of channel activities and the function of many of these channels is not well understood. One of the most compelling problems in this field is lack of information regarding the molecular basis for many of these channels. That identification will bring new insights into the roles channels fulfill in normal and pathological conditions. Mitochondria present technical challenges for monitoring channel activities in native membranes which we have overcome. However, monitoring mitochondrial channels inside cells, e.g., using the approach of the Jonas lab [19], or even in vivo presents additional technical challenges especially when one considers that the outer membrane shields the inner membrane from our electrodes. Nevertheless, mitochondrial channels are emerging as promising therapeutic targets for many diseases like cancer, aging, neurodegenerative diseases, stroke and infarct.

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