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The therapeutic potential of mitochondrial channels in cancer, ischemia-reperfusion injury, and neurodegeneration

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Abstract

Mitochondria communicate with the rest of the cell through channels located in their inner and outer membranes. Most of the time, the message is encoded by the flow of anions and cations e.g., through VDAC and PTP, respectively. However, proteins are also both imported and exported across the mitochondrial membranes e.g., through TOM and MAC, respectively. Transport through mitochondrial channels is exquisitely regulated and controls a myriad of processes; from energy production to cell death. Here, we examine the role of some of the mitochondrial channels involved in neurodegeneration, ischemia-reperfusion injury and cancer in the context of their potential as therapeutic targets.

Keywords

mitochondrial channels; PTP (permeability transition pore); MAC (mitochondrial apoptosis-induced channel); VDAC (voltage dependent anion channel); cancer; degenerative diseases

1. Introduction

Ion channels are requisite to rapid communication within and between cells. Imbalances in these informational exchanges lead to dysfunctions that underlie multiple pathologies. Modulation of plasma membrane ion channels is a prime objective of many pharmacological agents and so it is not surprising mitochondrial channels present a new and exciting cache of potential therapeutic targets. Mitochondrial channels enable communication between the cytosol and the metabolic center of the cell, which also makes decisions about survival and death. Mitochondria are involved in many processes from generation of ATP, hemes and steroids to well-controlled apoptosis. While the number of ion channels in the outer and inner membranes of mitochondria continues to grow, here we will focus on a few identified that are now known to be linked to pathologies. As shown in figure 1, these channels include MAC (mitochondrial apoptosis-induced channel), mitochondrial porin or VDAC (voltage dependent anion-selective channel), the PTP (permeability transition pore), and the protein import translocases of the outer and inner membranes called TOM, TIM23 and TIM22. mCS, mRyR, CLIC and other mitochondrial channels are discussed in other articles within this special issue, e.g., potassium channels and calcium homeostasis, under the direction of our editors Drs. Adam Szewczyk and Jerzy Duszynski. In keeping with our experimental focus, this discussion will have an electrophysiological bent, as summarized in Table 1. It is

interesting to note that Bertil Hille defined selective permeability as an important property of ion channels, which generally limited their conductances to below 400 pS (Hille, 2001). However, this review focuses on MAC, VDAC, PTP, and the protein translocases, which are perhaps less classical, as their pores are such that their ion selectivity is low and conductance is high compared to most plasma membrane channels.

2. MAC unleashes cell death signals

While not detectable in healthy cells, opening of MAC corresponds to the mitochondrial outer membrane permeabilization that occurs during the commitment step of intrinsic apoptosis. MAC provides the aqueous pathway enabling the release of cytochrome c and other proapoptotic factors from the intermembrane space. Once in the cytosol, these signaling molecules facilitate the amplification of apoptosis, e.g. through apoptosome formation and caspase activation. MAC formation is tightly regulated by the Bcl-2 family of proteins, which contains pro- and anti-apoptotic members (Dejean et al., 2005; Guo et al., 2004; Martinez-Caballero et al., 2009; Pavlov et al., 2001). MAC is formed de novo by the oligomerization of Bax and/or Bak, which are pro-apoptotic Bcl-2 family members. MAC does not form and cytochrome c is not released in cells lacking both Bax and Bak or over expressing the anti-apoptotic members, Bcl-2 or Bcl-x_L (Martinez-Caballero et al., 2009). Furthermore, a recent report showed Bid, Bim, and PUMA are essential for direct activation of the Bax-and Bak-dependent cell death program (Kim et al., 2009; Ren et al., 2010).

MAC activity was first recorded in the native outer membrane by directly patch-clamping mitochondria isolated from apoptotic FL5.12 cells (Pavlov et al., 2001) and has since been recorded from many cell types in which apoptosis was induced by a variety of insults. MAC has multiple substates and a variable peak conductance. See Table 1 for details. Although transitions are observed indicating that MAC can spontaneously gate, current traces often reveal a stable open state of 3–4 nS. Gating is also seen in response to effectors as described below (Martinez-Caballero et al., 2004; Peixoto et al., 2009a). Polymer (dextran) exclusion experiments indicate that MAC with conductances between 1– 5 nS have pore diameters of several nm, which should be wide enough to allow the passage of ~3 nm cytochrome c (Chan et al., 1966; Dejean et al., 2006). For example, ~4 nS MAC allows passage of 10 kDa and 17 kDa dextrans but not 45 kDa or 71 kDa dextrans (Guo et al., 2004). We previously applied this sizing approach to the TIM23 protein translocase of mitochondria to show this pore had a double barrel (Martinez-Caballero et al., 2007). Like the effects of 10 kDa dextrans, conductances of 2–5 nS MACs are typically modified by cytochrome c as this protein partitions into the pore; much larger and much smaller conductance MAC are not affected by the 12.5 kDa cytochrome c (Guo et al., 2004). The dextran approach has also been used to ensure peak conductances reflect a single MAC and not multiple smaller pores in the current recordings. Unlike many channels, MAC is not voltage dependent and is only slightly cation-selective. These findings suggest that MAC is not a classical channel because of the low selective permeability and high conductance and that MAC may be modeled as a simple pore through the membrane (Martinez-Caballero et al., 2009). Nevertheless and as shown in figure 2, MAC formation can be visualized in real-time as step-wise increases in current flow after patch clamping mitochondria with micropipettes containing tBid; tBid triggers MAC formation and cytochrome c release in isolated mitochondria. Assuming the pore is formed by the two transmembrane α -helices of Bax or Bak, our modeling suggests mature MAC typically contain ~8–10 monomers and has diameters of 5–6 nm (Martinez-Caballero et al., 2009).

The possibility is being considered that MAC or its components participate in other cellular functions, e.g., regulation of mitochondrial morphology (for a review, see Suen et al., 2008). This is in part because mitochondria fragment during apoptosis and deletion of both Bax and

Bak leads to a decrease in mitochondrial size (Cleland et al., 2010; Karbowski et al., 2004; Karbowski et al., 2006). Interestingly, DDP1/Timm8a is an intermembrane space protein that is released presumably through MAC at the same time as cytochrome c; this protein can activate the fission protein Drp-1 and promote mitochondrial fragmentation (Arnoult et al., 2005). Furthermore, MAC and intrinsic apoptosis may play a role in generation of bystander effects, based on evidence that a death signal generated downstream of MAC and cytochrome c release can be propagated between different cells (Das et al., 2007; Peixoto et al., 2011; Peixoto et al., 2009b).

Several effectors of MAC have been identified (Table 2). As predicted, cytochrome c partitions into MACs pore to induce a reversible and dose dependent increase in noise and small decrease in conductance (4–50%) (Guo et al., 2004). We refer to this as a type 1 effect, which resembles the effects observed during the transit of ATP through VDAC (Rostovtseva and Colombini, 1996, 1997), DNA through α -hemolysin (Akeson et al., 1999), and maltodextrins through maltoporins (Kullman et al., 2002). Type 2 effects of cytochrome c correspond to a 50–90% reduction in conductance that is dose dependent and voltage independent. This response corresponds either to destabilization or plugging of the open state of MAC, which is not reversible (Guo et al., 2004). Similar effects are sometimes seen with 10 kDa dextran (unpublished results).

Early studies revealed that while the amphipathic cation dibucaine reversibly closed MAC, the inhibition by trifluoperazine and propranolol was not reversible (Table 2) (Martinez-Caballero et al., 2004). Specific Bax channel inhibitors (BCIs) were then developed (Bombrun et al., 2003; Hetz et al., 2005). More recently, several inhibitors of MAC (iMACs) were identified with nanomolar efficacy, some of which also blocked apoptosis (Peixoto et al., 2009a). This blockade is illustrated in figure 2C, where iMAC2 causes a stepwise decrease in current flow. The precise mechanism of inhibition is being investigated, but the current traces suggest the dibromo carbazole moieties of the iMACs irreversibly disassemble pre-formed MACs (unpublished results). This halts release of mitochondrial death signals such as cytochrome c and rescues the cell from apoptotic death. MAC is thus an exciting potential therapeutic target for many reasons, but especially for its central role in initiating intrinsic apoptosis.

3. VDAC is the food channel

VDAC is a β -barrel channel located in the mitochondrial outer membrane that provides a transport pathway for many solutes; dextrans up to 5 kDa are also permeable (most recently reviewed in Shoshan-Barmatz et al., 2010). This pore is responsible for the exchange of ATP and ADP between the cytosol and intermembrane space. Interestingly, VDAC interacts with many other proteins, e.g. hexokinase and creatine kinase, to convert newly generated ATP into high energy storage forms like glucose 6-phosphate and creatine phosphate, in brain and muscle, respectively. In keeping with a two way trafficking, VDAC enables the exit of newly formed hemes as well as the entrance of substrates for the electron transport chain, including pyruvate, malate, succinate, and NADH.

The single channel behavior of *Paramecium aurelia* VDAC was first reported in 1976 by Schein, Colombini & Finklestein in studies of purified proteins in planar bilayers (Schein et al., 1976). VDAC has a fully open state conductance of ~650 pS in 150 mM KCl (4 nS in 1 M KCl). See Table 1 for additional details. Open VDAC is typically slightly anion-selective and a cation-selectivity predominates when VDAC is half-open at ~300 pS. This shift to half-open and cation-selective is observed at both edges of VDAC's bell-shaped voltage dependence, i.e., at ± 25 to 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 (Rostovtseva and Colombini, 1996, 1997). VDAC structure has been deduced from NMR and crystallography studies (Bayrhuber et al., 2008; Hiller et al., 2008; Ujwal et al., 2008). These studies agree with earlier electron microscopy studies (Guo et al., 1995; Verschoor et al., 2001) which report a slightly elliptical $2.7\text{--}3.5 \times 2.4\text{--}3.1$ nm pore with 19 trans-membrane domains.

While the sequence homology is often below 30%, VDACs expressed in yeast and mammalian mitochondria have high functional homology as determined by various electrophysiological approaches. The fully open state of both rat and yeast VDAC is ~650 pS (150 mM KCl) and is generally anion-selective with similar voltage dependencies. Furthermore, both rat and yeast VDACs can assume a fully open or half open state with slight cation selectivity (Pavlov et al., 2005). There are two isoforms of VDAC in yeast and three in mammals, which are referred to as yVDAC1 and yVDAC2 for yeast and VDAC1, VDAC2, and VDAC3 in mammals. These isoforms have not yet been distinguished by electrophysiology approaches. While knocking out isoforms 1 and 2 in yeast is debilitating and causes large increases in the doubling time, knocking out isoforms 1, 2, and 3 in mouse is lethal. As discussed below, VDAC isoforms seem to play diverse roles in apoptosis and metabolism as well as allowing transport of substrates (Cheng et al., 2003; Lee et al., 1996; Shoshan-Barmatz et al., 2010). VDAC clearly fulfills essential roles, at least in higher organisms.

The single channel behavior of detergent-solubilized VDAC is not the same as that recorded from native membranes, suggesting modulators of this mitochondrial channel are lost during reconstitution. As shown in Table 2, several cytosolic factors decrease the open probability of VDAC reconstituted in planar bilayers. For example, NADH and Mg²⁺-NAD(P)H decrease ADP flux through VDAC (Zizi et al., 1994) and so does tubulin (Rostovtseva et al., 2008). Moreover, an unidentified intermembrane space protein (Holden and Colombini, 1993) and the BH3-only protein tBid (Rostovtseva et al., 2004) are putative endogenous VDAC inhibitors. VDAC can be phosphorylated and has binding sites for hexokinase I (Azoulay-Zohar et al., 2004) and hexokinase II (Vander Heiden et al., 2000). Paradoxically, hexokinase I closes VDAC, while hexokinase II seems to keep it open. The list of putative VDAC modulators is still growing – Roman and coworkers surveyed 55 novel interactions of VDAC with other proteins and small molecules (Roman et al., 2006). Furthermore, VDAC seems to mediate regulation of metabolism including calcium-dependent ATP uptake and a modification of conductance by physiological cytosolic calcium concentrations (Bathori et al., 2006).

Despite extensive research over the past 30 years, only a few inhibitors of VDAC with pharmacological potential or nanomolar efficacy have been described. König's polyanion strongly changes the voltage dependence of VDAC (Colombini et al., 1987). More recently, the Bcl-2 antisense oligonucleotide G3139 was found to close VDAC and induce caspase-dependent apoptosis (Lai et al., 2006). The metabolic effects of modulation of this channel bespeak the importance of controlling ion flux through the mitochondrial outer membrane to maintain cellular homeostasis. It also highlights VDAC as a potentially important therapeutic target. However, the functional configuration of VDAC is yet to be clarified. Is the channel intrinsically open in living cells as suggested by the low permeability barrier of the mitochondrial outer membrane or is it closed? Answering these questions is crucial to understanding VDACs function *in vivo* and how the modulators described above could be used in therapeutic interventions.

4. PTP is elusive and notorious but fills fundamental roles in mitochondrial signaling

The PTP is a yet unidentified pore in the inner membrane that is responsible for the mitochondrial permeability transition, a phenomenon first characterized in 1979 (Haworth and Hunter, 1979; Hunter and Haworth, 1979a, b). Opening of the PTP leads to a transition in permeability from low to freely permeable to solutes up to 1.5 kDa. The resulting swelling can be monitored by light scattering at wavelengths reflecting mitochondrial volume. The PTP can be opened in cells and metabolizing isolated mitochondria by numerous effectors but, most notably, calcium plus phosphate and reactive oxygen species (ROS) (See extensive review by Gunter and Pfeiffer, 1990) and more recent review (Halestrap, 2009)]. Removal of calcium with EGTA or the addition of ADP, magnesium, or cyclosporine A induces reversible PTP closure. Matrix calcium in the presence of phosphate typically triggers PTP opening, but the activating concentrations of both rely on several other factors. Higher calcium concentrations are needed for opening in the presence of inhibitors including high negative membrane potential, low matrix pH, ADP, magnesium and strontium. Conversely, lower calcium levels activate PTP if adenine nucleotides are depleted or mitochondria are depolarized by protonophores like carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP). Interestingly, Bcl-2 overexpression suppresses calcium activation of PTP in both swelling and patch-clamp experiments (Murphy et al., 1996; Murphy et al., 2001).

The single-channel behavior of PTP was characterized by directly patch-clamping mitoplasts, which are mitochondria treated to expose their inner membrane. PTP is voltage dependent with multiple conductance levels and a peak conductance of 1-1.5 nS (150 mM KCl). See Table 1 for details. In earlier electrophysiological studies, PTP was referred to as the multiple conductance channel and the mitochondrial megachannel. While the original studies identified this as a calcium-activated channel (Kinnally et al., 1989; Kinnally et al., 1996), the seminal work of Zoratti's group forged the link of this channel activity to PTP notably because it was closed by the specific inhibitor cyclosporin A and other PTP effectors (Szabo et al., 1992; Szabo and Zoratti, 1992).

Does PTP play a role in normal physiology? Clearly, opening PTP will result in matrix swelling that ruptures the outer membrane and spills pro-apoptotic factors into the cytosol to initiate cell death (Dejean et al., 2010; Kinnally et al., 2010; Peixoto et al., 2010; Ryu et al., 2010a). The role of PTP in various forms of cell death is further discussed below. Importantly, flickering of PTP putatively underlies a mitochondrial version of 'calcium-induced calcium release' between the cytosol and the mitochondrial matrix (Gunter and Sheu, 2009; Ichas et al., 1997; Jouaville et al., 1998). The rapid removal of calcium from the cytosol and its subsequent release from mitochondria through PTP flickering could prevent calcium-inactivation of plasma membrane channels essential to refilling calcium stores as well as allow for signaling in these microdomains. PTP might also play a role in ROS signaling. Brief openings of PTP cause transient depolarization and short bursts of ROS production (Wang et al., 2008). Mitochondria-generated ROS have putative roles in cell signaling as evidenced by the redox sensitivity of chaperones, kinases and even gene expression (Veal et al., 2007). Hence, PTP may impact many cellular processes that are not linked to cell death including muscle contraction and saliva secretion (Altschuld et al., 1992; Ryu et al., 2010b). Nevertheless, transient flickering of the PTP can result in production of sufficient ROS to cause sustained PTP opening and eventual cell death (De Giorgi et al., 2002; Forte and Bernardi, 2006).

It has been postulated that PTP was composed of VDAC in the outer membrane and the adenine nucleotide translocator in the inner membrane (Beutner et al., 1998). However,

knockout and knock down studies of these two proteins clearly established that the adenine nucleotide translocator (Kokoszka et al., 2004; Lee et al., 2009) and VDAC (Baines et al., 2007) are not essential components of PTP. Nevertheless, there is evidence that VDAC and the adenine nucleotide translocator may regulate this permeability transition. For example, VDAC1 regulates the transport of calcium, a PTP activator, across the outer membrane (Hajnóczky et al., 2002). Partial closure of VDAC1 increases its calcium permeability (Rostovtseva et al., 2005; Tan et al., 2007). Therefore, VDAC1 may under certain circumstances act as a PTP sensitizer even if it is not a component of the pore (Baines et al., 2007). Cyclophilin D has been confirmed to play a regulatory role in PTP using molecular approaches (Baines et al., 2005; Basso et al., 2005; Nakagawa et al., 2005). PTP is central to ischemia-reperfusion injury and necrosis and is further discussed below. Nevertheless, the molecular identity of the pore that underlies this well-studied phenomenon remains elusive more than thirty years after its initial characterization.

5. The protein import channels TOM, TIM23, and TIM22

Only a small fraction of mitochondrial proteins are encoded in the mitochondria; the vast majority must be translocated from the cytosol across one or two membranes to reach their final destination. These mitochondrial proteins are imported through water filled channels as unfolded precursors by the TOM, TIM22, and TIM23 complexes (for a comprehensive review, see Schmidt et al., 2010). The TIM23 pore was the first electrophysiological demonstration of the link between protein import and water-filled channels in mitochondria (Lohret et al., 1997). This was quickly followed by a report by Thieffry and Henry's group on the channel activity of TOM, the protein import complex of the outer membrane (Kunkele et al., 1998a). These two pores enable the coordinated passage of proteins across both membranes and into the matrix space.

TOM channels are the gatekeepers of protein import and recognize mitochondria-bound precursor proteins often by their N-terminal signal sequence (Andreoli et al., 2004). This signal can be a cysteine motif (Herrmann and Hell, 2005), a presequence (Roise and Schatz, 1988) or an internal targeting element (Brix et al., 1999). Like VDAC, TOM is a β -barrel channel located in the outer membrane. The main component of this complex is Tom40 but there are many accessory proteins and receptors including Tom22, Tom20 and Tom70.

The TIM23 complex works in a coordinated manner with the TOM40 complex to translocate matrix-bound proteins like isocitrate dehydrogenase. This complex is an intricate machine containing up to 10 subunits, depending on the final destination of the protein in route (Chacinska et al., 2009). The core components Tim17 and Tim23 (pore), and Tim50 (receptor) are sufficient for recognition of precursors and the initial, membrane-potential dependent translocation of the presequence across the inner membrane. The core interacts with other smaller complexes, e.g., PAM complex, depending on the protein's final destination (Chacinska et al., 2005; Mokranjac et al., 2005; Popov-Celeketić et al., 2008; van der Laan et al., 2005).

The TIM22 complex inserts multi-topic proteins like the phosphate carrier into the inner membrane in an energy independent manner (for a review, see Neupert and Herrmann, 2007). This 300 kDa complex consists of the three membrane proteins Tim22, Tim54, and Tim18 and three soluble intermembrane space proteins, Tim9, Tim10, and Tim12. Tim22 is the core of the complex and is related in amino acid sequence to Tim17 and Tim23 (Sirrenberg et al., 1996). Tim54 seems to function as a scaffold protein that holds the complex together and is essential for protein import (Kerscher et al., 1997; Kovermann et al., 2002). Tim18 is a distant homologue of subunit 3 of succinate dehydrogenase, Sdh3. The

precise functions of Tim54 and Tim18 in yeast are not known and, to date, their presence in mammalian mitochondria has yet to be confirmed.

The channel activities of TOM and TIM23 complexes recorded with electrophysiological approaches are indistinguishable without resorting to mutations in core elements or the use of other effectors (Muro et al., 2003). Comparisons of these activities with those of recombinant proteins Tim23 and Tom40 solidified the assignment of identities (Chich et al., 1991; Fevre et al., 1993, 1994; Henry et al., 1996; Juin et al., 1997; Kinnally et al., 2000; Kunkele et al., 1998b; Thieffry et al., 1992). TOM and TIM23 activities have a peak conductance of ~1 nS in yeast and ~750 pS in mammals (150 mM KCl). A half open state is frequently observed, especially at higher potentials. Modeling through single channel analysis suggests both TOM and TIM23 contain a double barrel pore (Martinez-Caballero et al., 2007; Muro et al., 2003). Both TOM and TIM23 activities are sensitive to short peptides whose sequences mimic those that target precursor proteins to the matrix; these peptides induce a concentration-dependent flickering of 200–500 transitions/sec in both channels.

While the electrophysiological behavior of yeast TIM22 is indistinguishable from that of TIM23 and TOM (Peixoto et al., 2007), mammalian TIM22 has yet to be characterized (Table 1). Furthermore, TIM22 channels are not modulated by the same signal sequences as TOM and TIM23, but rather by a peptide referred to as P2. The amino acid sequence of P2 peptide mimics the ‘stop’ translocation signal at the beginning of the fifth transmembrane segment of the phosphate carrier; this peptide was essential to the original detection of TIM22 activity.

Two human diseases have been described that are caused by defects in the mitochondrial protein import machinery: Mohr-Tranebjaerg syndrome and dilated cardiomyopathy with ataxia reviewed in Peixoto et al., 2010. However, we found no studies on therapeutic compounds targeted to these protein import machineries. Electrophysiological, biochemical and morphological characterization of these translocation complexes will reveal novel intricacies of protein import that may have therapeutic relevance to disease models.

6. Mitochondrial channels as therapeutic targets

Mitochondria compartmentalize numerous death-evoking signaling factors, which wreak havoc upon their release to the cytosol and eventually cause cell death through intrinsic apoptosis. Mitochondria are also central to programmed necrotic death that can be caused by ischemia-reperfusion injury and chemotherapeutic agents. Exciting new results have linked neurodegenerative diseases to mitochondria dysfunction and novel chemotherapies are targeting Bcl-2 family proteins located in mitochondria to restore the cell’s normal default ability to die. Here, we discuss details connecting these three disease processes with mitochondrial ion channels. For disease associated with other mitochondrial features like respiration, mitophagy, and dynamics some excellent reviews are available, including Campello and Scorrano, 2010; Chen and Chan, 2009; DiMauro and Schon, 2003; Schon et al., 2010.

6.1 Tumor formation and cancer

Because MAC formation and cytochrome c release are the last check points prior to the activation of downstream caspases, these events are considered the commitment steps in mitochondrial apoptosis. For that reason, MAC is an important target for novel cancer therapies. Importantly, the pore of MAC is formed from oligomers of Bax and/or Bak. MAC assembly is triggered by BH3-only protein members (Bid, PUMA, Bim) and inhibited by anti-apoptotic Bcl-2 and Bcl-x_L (Chipuk et al., 2010; Danial, 2007; Dejean et al., 2006; Kinnally et al., 2010; Kroemer et al., 2007; Leber et al., 2007; Wang and Youle, 2009).

Overexpression of BH3-only proteins can lead to MAC formation, cytochrome c release, and apoptotic death in multiple cancer cell lines (Brunelle and Letai, 2009). Interestingly, the sensitivity of cancer cells to overexpression of various BH3-only proteins, referred to as BH3 profiling, has developed since not all BH3-only proteins are effective in restoring apoptotic death in every tumor type. This profiling shows that BH3-only proteins can represent specific cancer markers that can then be selectively targeted for therapy (Brunelle and Letai, 2009; Certo et al., 2006; Letai, 2008).

Bcl-2 and Bcl-x_L are inhibitors of MAC-formation and were initially identified as proto-oncogenes (Egle et al., 2004; Meijerink et al., 2005; Swanson et al., 2004). In this respect, pro-lymphocytic cell lines overexpressing Bcl-2 exhibit both resistance to MAC formation and the ability to induce lymphoma upon injection in mice (Goping et al., 1998; Gross et al., 1998; Meijerink et al., 2005; Pavlov et al., 2001). Paradoxically, an over-abundance of Bcl-2 also causes a mitochondrial accumulation of Bax and Bim, two activators of MAC formation and activity (Certo et al., 2006; Letai, 2008; Teijido and Dejean, 2010). Interestingly, a physical interaction between the BH3 domains of Bcl-2 with Bax or Bim is required for this phenomenon to occur (Certo et al., 2006; Letai, 2008; Teijido and Dejean, 2010). This Bcl-2-induced sequestration of pro-apoptotic proteins in mitochondria has been coined 'priming for death', and this concept is currently being exploited to develop new cancer therapies (Certo et al., 2006; Letai, 2008). More particularly, BH3 mimetic compounds such as the ABT-737 and ABT-263 restore MAC formation and cell death by competitively inhibiting the interactions between Bcl-2 and the accumulated BH3-only proteins and/or Bax. They are promising chemotherapeutic agents against tumor cells that up-regulate Bcl-2 and/or Bcl-x_L and are currently in clinical trials (Brunelle and Letai, 2009; Letai, 2008; Oltersdorf et al., 2005; Teijido and Dejean, 2010).

Tumorigenesis and the accompanying resistance to apoptosis can also be initiated by down-regulation of pro-apoptotic Bax and Bak. In this case, the death program fails as MAC formation is prevented by a lack of building blocks (Reed, 2008). Chemotherapies in this latter case may involve the activation of Nur77 by the drug 3-Cl-AHPC; activation of Nur77 leads to the conversion of Bcl-2 into a core component of a new type of cytochrome c release channel (Han et al., 2006). Other outer membrane proteins are also being evaluated as potential therapeutic targets such as the TOM receptor Tom22, which was shown to stabilize Bax at the mitochondria (Cartron et al., 2008).

VDAC is the outer membrane gateway for adenine nucleotides and redox equivalents that are requisite to generate ATP through oxidative phosphorylation at the inner membrane. Interestingly, different VDAC isoforms may inhibit apoptosis and promote tumorigenesis through specific interactions with pro-apoptotic proteins, like VDAC2 with Bak (Cheng et al., 2003), or with enzymes favoring glycolysis, like VDAC1 with hexokinase II (Shoshan-Barmatz et al., 2010). VDAC is being examined as a cancer selective target since tumor cells have elevated glycolysis and increased expression of VDACS compared to normal cells. The anticancer agent furanonaphthoquinone (FNQs) induces caspase-dependent apoptosis via the production of NADH-dependent ROS, which is enhanced by VDAC1 overexpression (Simamura et al., 2008). Another drug, erastin, induces RAS-RAF-MEK-dependent non-apoptotic cell death via VDAC2 (Yagoda et al., 2007). Interestingly, VDAC activity is affected by G3139, an 18-mer phosphorothioate antisense oligonucleotide that also 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 (Lai et al., 2006). Finally, the plant hormone methyl jasmonate has selective anticancer activity in preclinical studies. The mechanism seems to involve disruption of the interaction between human hexokinase II and VDAC1, causing the inhibition of glycolysis and the induction of outer membrane permeabilization (Goldin et al., 2008). Intriguingly,

partial closure of the channel, but not its opening, increases the calcium permeability of VDAC1 (Rostovtseva et al., 2005; Tan and Colombini, 2007). Therefore, VDAC1 may, under certain circumstances, also act as a PTP sensitizer, and drugs inducing VDAC1 closure may be useful as a chemotherapeutic agent.

While also caused by unchecked proliferation, many cancer cells fail to die because of a breakdown in their apoptotic pathways (Tu et al., 2009). These cells are sometimes referred to as the undead. If apoptosis is not restored during severe insult, these undead cells tend to die by either autophagy, or perhaps more frequently, by necrosis. While not as useful for dissecting the mechanisms of tumorigenesis, a close examination of the undead could reveal additional chemotherapeutic targets.

6.2 Ischemia reperfusion injury

The central role of mitochondria in ischemia/reperfusion injury of the heart exemplifies the fundamental role of mitochondrial channels in pathophysiology (Halestrap, 2009). The end result of PTP opening is a relatively predictable necrotic cell death. The vast majority of the oxygen we breathe in is used by mitochondria during oxidative phosphorylation. During ischemia, the lack of an oxygen supply to tissues leads to a metabolic switch from oxidative phosphorylation to glycolysis as the electron transport chain is reduced and the pH of the cytosol begins to acidify. At the same time, reduced ATP levels dampen the activity of the Na^+/K^+ ATPase, which causes a general cellular swelling. Ribosomes detach from the endoplasmic reticulum as protein synthesis is stalled. These changes are reversible depending on extent, until the plasma membrane is damaged by excessive swelling or lysosomes release their enzymes. The cytosolic levels of ADP and Pi elevate, which may contribute to PTP opening. At an early stage, calcium accumulates in the matrix through the mitochondrial calcium uniporter (MCU), which again favors PTP formation. Once open, PTP disrupts the permeability barrier of the inner membrane and accumulated calcium is dumped into the cytosol, which triggers cell death. Paradoxically, reperfusion rapidly triggers oxidative stress, which again leads to opening of PTP and initiates programmed necrotic death. See Table 2.

Opening of the PTP upon reperfusion leads to a permeabilization of the inner membrane that is, if persistent, catastrophic for the integrity of these organelles. The high concentration of proteins in the matrix exerts a large colloidal osmotic pressure that causes the mitochondria to rapidly swell. The outer membrane ruptures, spilling pro-apoptotic factors into the cytosol. Depolarized mitochondria are a major liability for the cell as ATP is hydrolyzed in efforts to restore the mitochondrial membrane potential. In the best case scenario, these depolarized mitochondria will undergo autophagy. However, the collapse of the mitochondrial membrane potential and the leakage of NADH can lead to the generation of ROS via the direct transfer of electrons to molecular oxygen (Bernardi et al., 2006; Halestrap, 2009). ROS are also activators of PTP, which then self-amplifies to open more PTP in neighboring mitochondria. These reversible events set off a vicious cycle that may end in cell death.

Numerous experimental data support the involvement of PTP in ischemia/reperfusion injury. Cyclosporin A reduces the death of cardiomyocytes subjected to anoxia-reperfusion and is a high-affinity inhibitor of PTP that acts through a regulator cyclophilin D. Cyclosporin A is also effective in protecting these same cells against reperfusion-induced mitochondrial membrane potential collapse (Crompton, 1999). PTP opening further deregulates calcium homeostasis and inhibits the respiratory chain, resulting in further ROS production and additional PTP opening. Other examples of PTP involvement during necrosis are found in stroke models of primary brain cells (Jacobson and Duchen, 2002).

PTP plays a crucial role in necrosis and this idea is supported by observations in cyclophilin-D deficient mice (Baines et al., 2005; Basso et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005). Cyclophilin-D ablation increases the amount of calcium required for PTP opening and abolishes the sensitivity to cyclosporin A (Rasola et al., 2010). Cyclophilin-D knock out cells are resistant to necrotic stimuli such as A23187-induced calcium overload, and to the decrease of heart and brain injury following ischemia/reperfusion. However, cells isolated from these same animals still die in response to treatments with classical apoptotic inducers such as staurosporine or etoposide (Nakagawa et al., 2005).

While sustained opening of PTP will cause cell death, activation of the mitochondrial K^+ -ATP channels provides some cardio-protection against ischemia-reperfusion injury. See Table 2. Prior to PTP opening, reduced ATP-levels and/or moderate increases in ROS levels lead to opening of these K^+ channels, a slight depolarization and swelling of the matrix space, reduced calcium loading through the mitochondrial calcium uniporter (MCU), and reduction of ROS production. Each of these changes reduces the likelihood that PTP will open. Interestingly, inhibition of MCU facilitates post reperfusion recovery (Unitt et al., 1999) while activation of mitochondrial K^+ -ATP channels promotes cardioprotection (Lee et al., 2007).

6.3 Neurodegeneration

The common pathogenesis mechanisms for premature neuronal death seen in many neurodegenerative disorders are still largely unknown. These include Huntington's disease, Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, Friedreich ataxia, hereditary spastic paraplegia, dominant optic atrophy, Charcot-Marie-Tooth type 2A, neuropathy ataxia and retinitis pigmentosa, and Leber's hereditary optic atrophy. However, mitochondrial dysfunction seems to be a common denominator that leads to neuronal death; these defects impact the electron transport chain, ATP synthesis, calcium handling, and fusion and fission (Kwong et al., 2006). As discussed above, mitochondria orchestrate cell death through opening of ion channels and release of death signals. Neuronal cell death also involves mitochondrial ion permeability changes, release of apoptotic factors from mitochondria, e.g. cytochrome *c*, Smac/DIABLO, Htr2A/Omi or AIF, activation of caspases and, finally, condensation and degradation of nuclear DNA. An important regulatory step of this process occurs at mitochondrial membranes where pro- and anti-apoptotic members of the Bcl-2 family either promote or prevent membrane permeability transition (Polster and Fiskum, 2004). Thus, preventing formation of the mitochondrial killer channels MAC and PTP may be a definitive therapeutic approach in neurodegeneration. Since VDACs are constitutive channels required for oxidative phosphorylation, preventing their formation/function is not a viable alternative approach. Nevertheless, deciphering VDACs interaction partners could be revealing. For example, small molecules could be designed to modulate VDAC2 interaction with pro-apoptotic Bak.

As mentioned above, mitochondria play a pivotal role in calcium signaling that, when compromised, leads to dysfunction and cell death after ischemia reperfusion in the brain (Friberg and Wieloch, 2002; Norenberg and Rao, 2007; Starkov et al., 2004), as well as in diseases like Alzheimer's, Parkinson's, Huntington's and amyotrophic lateral sclerosis (ALS) (Bezprozvanny, 2009; Gibson et al., 2010). One study has shown that mutant huntintin reduces the calcium load needed to induce PTP opening (Choo et al., 2004). However, the possible involvement of PTP in neurodegeneration is still a matter of debate. While a significant body of evidence, both clinical and experimental, supports a role for PTP in ischemic injury (Baines et al., 2005; Friberg and Wieloch, 2002; Norenberg and Rao, 2007; Schinzel et al., 2005) and in neurodegenerative diseases (Bezprozvanny, 2009), uncertainty remains at least partly due to the inconsistent effects of cyclosporin A on neuronal mitochondria (Chinopoulos and Adam-Vizi, 2006; Nicholls, 2004, 2009).

Deciphering the molecular identity of PTP could help understand these discrepancies, as well as suggest novel therapeutic targets.

MAC inhibition is particularly attractive and relatively unexplored. Two small Bax inhibitors prevented cytochrome c release, mitochondrial depolarization, and apoptosis in an animal model of ischemic brain injury (Hetz et al., 2005). Stronger inhibitors of MAC (iMACs) are now available (Peixoto et al., 2009a; Peixoto et al., 2010), but their effects in neuronal cell death await investigation. Another promising study approaches MAC inhibition by targeting tBid (Becattini et al., 2004). Small 4-phenylsulfanyl-phenylamine derivatives were capable of occupying a deep hydrophobic crevice on the surface of Bid and inhibit tBid-induced Smac release, caspase 3 activation and cell death. In neuronal cell cultures, these novel inhibitors attenuated cell death induced by glutamate, glucose-oxygen deprivation, or β -amyloid in a dose-dependent manner (Culmsee et al., 2005). Finally, an interesting approach to MAC inhibition could be the targeting of anti-apoptotic Bcl-2 in neurons. Interestingly, a novel mechanism of apoptosis in Alzheimer's disease has been proposed, where presenelins destroy Bcl-2 (Wang et al., 2005). Thus, presenelin inhibition may protect Bcl-2 and prevent MAC formation in Alzheimer's disease.

The protein import channels TOM, TIM23 and TIM22 have also been implicated in disease models of neurodegeneration. The Mohr-Tranebjaerg syndrome (also called deafness dystonia syndrome) shows X-linked recessive inheritance and is characterized by progressive sensorineural hearing loss, dystonia, mental deficiency, and visual disability (Jin et al., 1996; Kokotas et al., 2007). It is caused by a mutation of the deafness/dystonia protein1 gene DDP1, also called Timm8A, which is part of the TIM23 complex. Finally, a study suggests that abnormal accumulation of amyloid precursor proteins across mitochondrial import channels is a hallmark of Alzheimer's. Mitochondria-associated amyloid precursor proteins formed stable complexes with Tom40 and Tim23 (Devi et al., 2006) and compromised mitochondrial function. Thus, regulating the mechanism of interaction between protein import complexes and amyloid precursor proteins is yet another possible therapeutic approach. However, no compounds have yet been identified that target these import complexes.

7. Closing remarks

Extensive research in the past decade has enormously increased the list of mitochondrial functions and elevated this organelle from being the power generator to one that orchestrates cell fate. But as the list of mitochondrial functions increases, so does the list of disease-related mitochondrial dysfunctions. In fact, mitochondrial dysfunction seems to be a common denominator of numerous pathological conditions like cancer, ischemic injury in heart and brain, and neurodegenerative diseases. When mitochondrial dysfunction leads to cell demise, it often does so by opening pores that release death signals into the cytoplasm. On the contrary, mitochondrial dysfunction in cancer cells abrogates the killing machinery. In either case, mitochondrial ion channels, especially MAC and PTP are rising stars as promising therapeutic targets; their modulation could help restore cell homeostasis. Electrophysiological studies will allow a direct evaluation of the effects of novel agents on specific channels; such studies can unambiguously identify the mechanisms by which channels are either opened or closed. However, one of the most compelling problems in this field is lack of information regarding the molecular basis and structure for many of these channels, in particular PTP and MAC. That identification will bring new insights into the roles mitochondrial channels fulfill in normal and pathological conditions. Finally, this identification may reveal interesting targets to be explored in combination with current therapies for cancer and degenerative diseases when mitochondrial dysfunction is the underlying cause.

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ABBREVIATIONS

BH	Bcl-2 homology
iMAC	inhibitor of MAC
MAC	mitochondrial apoptosis-induced channel
PTP	permeability transition pore
ROS	reactive oxygen species
TOM	translocase of the outer membrane
TIM	translocase of the inner membrane
VDAC	voltage dependent anion-selective channel

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Figure 1. Mitochondrial ion channels

The mitochondrial channels MAC, PTP, VDAC, TIM (TIM22 and TIM23), and TOM are represented. MAC and PTP are mainly associated with release of mitochondrial death signals and induction of apoptosis in numerous cell types. While MAC specifically forms and opens in the outer membrane to release death factors (red spheres), PTP opening in the inner membrane results in matrix swelling and disruption of the outer membrane, which spills these factors into the cytosol. Turning on or off either channel could induce or prevent cell death respectively in different therapeutic approaches.

Figure 2. MAC activity can be turned on or off to induce or prevent apoptosis

A. Phase contrast microscopic image illustrates patch clamping of a mitochondrion (arrowhead). tBid is typically included inside the patch pipette to activate MAC, whereas iMACs are perfused into the bath to inhibit MAC. Scale bar is 2 μ m. **B, C.** MAC activities were recorded in patches excised from mitochondria of mouse embryonic fibroblasts in presence of tBid (**B**) or from proteoliposomes containing mitochondrial outer membranes of apoptotic FL5.12 cells (**C**). Top current traces at +40 mV show inclusion of tBid in the patch pipette induced stepwise increase of MAC conductance. Bottom current traces at +20 mV show perfusion with buffer containing 25 nM iMAC2 induces stepwise blockade of MAC.

Diagrams above current traces represent progressive pore assembly or disassembly by changing the number of helices in the pores (gray circles) (Martinez-Caballero et al., 2009) in presence of tBid or iMAC2, respectively. Dashed lines are 0 pA at 0 mV.

Table 1

Comparison of the single channel behavior of mammalian megachannels

Property	PTP	MAC	VDAC	TOM	TIM22
Peak conductance (nS)	1.1 ± 0.1	1–5	0.7 ± 0.1	0.7 ± 0.1	1.1 ± 0.1
Transition size (nS)	0.3–1.0	0.3–2.0	0.36 ± 0.04	0.34 ± 0.03	0.50 ± 0.10
Ion selectivity	Sl. Cation	Sl. Cation	Sl. Anion	Sl. Cation	Sl. Cation
P _K /P _{Cl}	7	4.7 ± 1.3	0.7 ± 0.1	3.6 ± 0.8	8.4 ± 6.3
Voltage dependent	Yes	No	Yes	Yes	Yes
Pore diameter (nm)	2.8 ± 0.1 ^a	2.7–6.0 ^a	2.2 ± 0.1 ^a	1.5 ± 0.1 ^b	0.84 ± 0.3 ^b

Sl. – slightly.

^a calculated from peak conductance.

^b calculated from transition size. TIM23 activity is indistinguishable from data shown for TOM activity and so was not repeated. Yeast TIM22 is shown as mammalian is not yet available. Adapted from (Kinnally et al., 2010).

Table 2

Mitochondrial channels as therapeutic targets.

Location	Type	Potential role(s)	Modulators	Therapeutic potential
Outer membrane	MAC	MOMP, apoptosis	tBid ^a , cytochrome c, Bcl-2 ⁱ , Dibucaine ⁱ , trifluoperazine ⁱ , propranolol ⁱ , Bcl-2 ⁱ , iMACs ⁱ	Suppress apoptosis in degenerative disease: iMACs, Bcl1, Bcl2. Chemotherapy to induce apoptosis by BH3 mimetics: ABT-263 (Peixoto et al., 2009a; Schinzel et al., 2005; Trudel et al., 2007; Vogler et al., 2009)
	VDAC	Metabolic transport, apoptosis	NADH ⁱ , DIDS ⁱ , tubulin ⁱ , tBid ⁱ , hexokinase, Bak, Bcl-xL, Bcl-2, Ca ²⁺ , voltage	Chemotherapy to induce apoptosis: FNQs, erastin, G3139, and methyl jasmonate (Goldin et al., 2008; Lai et al., 2006; Simamura et al., 2008; Yagoda et al., 2007)
Inner membrane	PTP	Necrosis, apoptosis, ROS signaling, Ca ²⁺ homeostasis	cyclosporin A ⁱ , cyclophilin D, NIM811 ⁱ , bongkreikic acid ⁱ , atractyloside ^a , Ca ²⁺ , Pi, ADP ⁱ , voltage	Suppression of death in Ullrich and Bethlem syndromes, Alzheimer, cardioprotection from ischemia-reperfusion injury: cyclosporin A (Du et al., 2008; Hicks et al., 2009)
	MCU	Ca ²⁺ uptake, apoptosis	divalent cations ⁱ , ruthenium red ⁱ , Ru360 ⁱ	Inhibition facilitates post reperfusion recovery (Unitt et al., 1999)
	MitoK _{ATP}	Volume regulation, cardioprotection	Diazoxide ^a , glibenclamide ⁱ , 5-HD ⁱ , ATP ⁱ	Opening with BMS-180448 promotes cardioprotection (Lee et al., 2007)

^a activatorⁱ inhibitor