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The mitochondrial permeability transition pore: an evolving concept critical for cell life and death

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ABSTRACT

In this review, we summarize current knowledge of perhaps one of the most intriguing phenomena in cell biology: the mitochondrial permeability transition pore (mPTP). This phenomenon, which was initially observed as a sudden loss of inner mitochondrial membrane impermeability caused by excessive calcium, has been studied for almost 50 years, and still no definitive answer has been provided regarding its mechanisms. From its initial consideration as an *in vitro* artifact to the current notion that the mPTP is a phenomenon with physiological and pathological implications, a long road has been travelled. We here summarize the role of mitochondria in cytosolic calcium control and the evolving concepts regarding the mitochondrial permeability transition (mPT) and the mPTP. We show how the evolving mPTP models and mechanisms, which involve many proposed mitochondrial protein components, have arisen from methodological advances and more complex biological models. We describe how scientific progress and methodological advances have allowed milestone discoveries on mPTP regulation and composition and its recognition as a valid target for drug development and a critical component of mitochondrial biology.

Key words: mitochondria, mitochondrial permeability transition pore, calcium, cell death

CONTENTS

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I. INTRODUCTION

The mitochondrial permeability transition (mPT) is a pathophysiological state of the inner mitochondrial membrane (IMM). Under favourable conditions, including calcium (Ca^{2+}) overload, oxidative stress, increased phosphate concentration, and decreased adenine nucleotide availability, the IMM becomes highly permeable to solutes with a molecular weight of up to 1500 Da (Halestrap, 2009; Morciano *et al.*, 2015). Since biological and evolutionary features generally make the IMM relatively impermeable, the mPT leads initially to a considerable and unselective influx of solutes and to an abrupt loss of mitochondrial metabolites, causing mitochondrial homeostasis perturbation that can result in cell death (Izzo *et al.*, 2016). The mPT is evolutionarily highly conserved, with critical features present in yeast, mammals, and plants; only the crustacean *Artemia franciscana* seems to lack a regulated mPT, which may contribute to its long-lasting hypoxia tolerance (Menze *et al.*, 2005). The mPT is caused by the opening of proteinaceous channels at the juxtaposition of the IMM and outer mitochondrial membrane (OMM) rather than changes to the phospholipid bilayer composition (Crompton, Costi & Hayat, 1987). The opening of mPT pores (mPTPs) opening can be monitored using fluorescent dyes or absorbance assays in living cells, isolated mitochondria and tissues. Pore opening results from relatively severe perturbations of the mitochondrial matrix and the consequences of mPT are dictated by several factors such as the pore open time, the number of channels present, and the number of mitochondria affected by this pathophysiological event. A long-lasting mPT can lead to irreversible consequences, including matrix swelling, dissipation of mitochondrial potential, ATP hydrolysis into ADP and free phosphate ions, and uncoupling of oxidative phosphorylation, causing cell death. The modalities by which mPT leads to cell death are still debated; based on recent reports, apoptosis or necroptosis may occur depending on ATP concentration and availability (Brenner & Moulin, 2012).

mPTP opening, considered by some authors as a cellular catastrophe (Briston *et al.*, 2019), has been considered a critical mechanism in the development of several pathologies and organ damage caused by toxicity. For nearly two decades, the mPTP has been associated with cardiac dysfunction observed after ischemia/reperfusion of the heart (Borutaite *et al.*, 2003). In early work, Steenbergen *et al.* (1987) demonstrated an increase in cytosolic Ca^{2+} concentration after an ischemic episode. Cytosolic Ca^{2+} overload induces the opening of the mPTP and, consequently the release of cytochrome c from mitochondria to the cytosol and activation of the mitochondria-dependent apoptotic pathway (Whittington *et al.*, 2018). During ischemia, the mPTP remains closed because of cytosolic acidification since protons are mPTP inhibitors (Ong *et al.*, 2015). By contrast, opening of the mPTP is strongly potentiated during reperfusion after an ischemic episode. During reperfusion, the reintroduction of oxygen has a deleterious effect, resulting in a burst of reactive oxygen species (ROS) production (Di Lisa *et al.*, 2001; Hausenloy, Duchen & Yellon, 2003; Gonzalez-Montero *et al.*, 2018). ROS potentiate mPTP opening, and this can result in cardiac cell death and cardiac dysfunction. mPTP opening after episodes of ischemia/reperfusion was also observed in the brain (Halestrap, 2006). Inhibition of mPTP opening has been suggested as a neuroprotective strategy to prevent cerebral ischemia–reperfusion injuries (Leger *et al.*, 2011; Rekuviene *et al.*, 2017; Matsumoto *et al.*, 2018). The mPTP is also associated with metabolic diseases, including insulin-resistance or diabetes: Taddeo *et al.* (2014) demonstrated that mPTP opening is required for induction of insulin resistance in skeletal muscle. Work performed in animal models demonstrated how the mPT is induced in diabetic conditions, which may contribute to complications including diabetic cardiomyopathy or hyperglycemia (Oliveira *et al.*, 2003; Lumini-Oliveira *et al.*, 2011; Taddeo *et al.*, 2014; Riojas-Hernandez *et al.*, 2015). As another example, accumulation of hydrophobic bile acids in hepatic cells during cholestasis induces apoptosis of hepatic cells through mPTP opening (Yerushalmi *et al.*, 2001; Rolo, Palmeira & Wallace, 2003).

The opening of the mPTP may also be observed during drug-induced toxicity. For example, high doses of caffeine enhance the Ca^{2+}-dependent cardiac mPT in isolated mitochondrial fractions (Sardao, Oliveira & Moreno, 2002), causing concern over the excessive consumption of high-energy drinks and dietary supplements with high doses of caffeine. Doxorubicin, a potent chemotherapeutic drug but with associated cardiotoxicity, decreases the threshold for mPTP opening in cardiac cells, impairing the contraction performance of the heart (Montaigne *et al.*, 2011), as demonstrated

in rodent models (Oliveira *et al.*, 2004, 2005; Pereira *et al.*, 2011). The role of the mPTP in cell toxicity induced by xenobiotic compounds that cause oxidative stress and in different diseases has been extensively explored (Bernardo *et al.*, 2013; Carvalho *et al.*, 2018; Teixeira *et al.*, 2018). Due to the importance of the mPTP for mitochondrial function and, consequently, cellular performance, the dynamics and regulation of the mPT have been the subject of intensive research.

Although mPTP opening is often associated with disease conditions, there is evidence for a critical physiological role in its flickering or transient opening mode, especially in the heart (during cardiac development and in damage protection) (Hausenloy *et al.*, 2004; Korge *et al.*, 2011), brain (putative or indirect involvement in synaptic efficacy and plasticity) (Mnatsakanyan *et al.*, 2017), and in metabolic functions (Hom *et al.*, 2011).

II. THE mPTP AND MITOCHONDRIAL Ca^{2+} UPTAKE OR OVERLOAD

The cellular concentration of free Ca^{2+} regulates an array of biochemical reactions and is crucial for signal transduction (Rizzuto, Duchen & Pozzan, 2004; Elustondo *et al.*, 2017; Herst *et al.*, 2017; Krebs, 2017; Santulli, 2017; Giorgi *et al.*, 2018*a*; Giorgi, Marchi & Pinton, 2018*b*; Del Re *et al.*, 2019; Glaser *et al.*, 2019; Hausenloy *et al.*, 2020). Mitochondria are fundamental to cellular energy metabolism, supplying energy in the form of ATP and affecting cell physiology through the regulation of Ca^{2+} homeostasis (Picard, Wallace & Burelle, 2016; Krebs, 2017). Mitochondria have a large capacity to accumulate Ca^{2+} and can transiently store it, thus contributing to cell calcium homeostasis. Their ability to accumulate calcium for later release makes mitochondria essential cytosolic stores or buffers for Ca^{2+} in the context of cell physiology and pathophysiology (Dedkova & Blatter, 2008; Elustondo *et al.*, 2017; Ludtmann & Abramov, 2018; Delierneux *et al.*, 2020). Intramitochondrial free calcium plays a significant part in Ca^{2+} homeostasis in cells, also being important in cell survival and death (Bhosale *et al.*, 2015; Picard *et al.*, 2016; Santulli, 2017; Ludtmann & Abramov, 2018; Del Re *et al.*, 2019). It has been demonstrated that a basal level of Ca^{2+} in the mitochondrial matrix is needed for correct mitochondrial functioning, while the pathophysiological role of Ca^{2+} overload, which occurs in a wide range of pathologies, still remains to be clarified (Burgoyne *et al.*, 2012; Bertero & Maack, 2018; Ludtmann & Abramov, 2018).

(1) Mitochondrial calcium influx

Calcium accumulation in mitochondria was first described in the 1960s (Deluca & Engstrom, 1961). Since then, the role of Ca^{2+} in the regulation of mitochondrial bioenergetics and diverse cellular functions has been well established. Calcium homeostasis in mitochondria is regulated by a complex system of mitochondrial Ca^{2+} influx and efflux mechanisms. This Ca^{2+} transport system in mitochondria comprises specific transporters in the IMM and OMM. To access the mitochondrial matrix, Ca^{2+} must first pass through the OMM. This membrane is permeable to ions, in particular to Ca^{2+}, and to small proteins, due to the presence of a large conductance channel – the voltage-dependent anion channel (VDAC) – which allows the exchange of molecules of molecular weight up to 1500 Da (Schein, Colombini & Finkelstein, 1976; Colombini & Mannella, 2012; Krebs, 2017; Becker & Wagner, 2018; Magri, Reina & de Pinto, 2018). The VDAC is responsible for Ca^{2+} transport from the cytoplasm into mitochondria, with its permeability controlled by ATP and other regulatory factors. Three different VDAC isoforms have been identified: VDAC1, VDAC2 and VDAC3 (Mertins, Psakis & Essen, 2014; Krebs, 2017; Ponnalagu & Singh, 2017). Although these isoforms share some structural and functional properties, they appear to perform different physiological roles (Cheng *et al.*, 2003; De Pinto *et al.*, 2010; Magri *et al.*, 2018; Rostovtseva *et al.*, 2020). While limited information is available regarding the functions of VDAC2 and VDAC3 in the Ca^{2+}-influx mechanism (De Pinto *et al.*, 2010; Lemasters *et al.*, 2012; Magri *et al.*, 2018), VDAC1 has been the subject of detailed research. VDAC1 is highly expressed in most cells (Shoshan-Barmatz & Golan, 2012) and seems to be the most prevalent isoform involved in Ca^{2+} transport into the intermembrane mitochondrial space (Krebs, 2017).

Ca^{2+} transport through the IMM is regulated *via* several transporters. At present, three main mechanisms of Ca^{2+} influx through the IMM are proposed (Fig. 1): (*i*) an electrogenic mitochondrial Ca^{2+} uniporter (MCU), (*ii*) the rapid mode of Ca^{2+} uptake (RaM), and (*iii*) the mitochondrial ryanodine receptor (mRyR). Three main mechanisms of Ca^{2+} efflux through the IMM are also known: (*i*) a Na^{+}/Ca^{2+} exchanger (NCXm), (*ii*) a H^{+}/Ca^{2+} exchanger (HCXm), and (*iii*) the mPTP. The leucine zipper- EF-hand containing transmembrane protein (LETM1) was also proposed as a Ca^{2+}-transport system. However, its role in mitochondrial Ca^{2+} influx and efflux through the IMM is still under discussion. Ca^{2+} transport across the IMM was initially thought to involve a single mechanism that was demonstrated to be highly sensitive to ruthenium red and lanthanides (Gunter & Pfeiffer, 1990). The molecular identity of this ruthenium- and lanthanide-sensitive Ca^{2+} transport was unclear for several decades until this transporter was identified as the MCU complex, following identification of the gene encoding the pore-forming subunit of the MCU (Baughman *et al.*, 2011; De Stefani *et al.*, 2011).

Currently, Ca^{2+} influx through the MCU multi-protein complex is the best-characterized pathway for mitochondrial Ca^{2+} uptake. It is driven by the large electrochemical gradient (mitochondrial membrane potential ~180 mV) for Ca^{2+} across the IMM (Elustondo *et al.*, 2017; Mishra *et al.*, 2017; Mammucari *et al.*, 2018; Belosludtsev *et al.*, 2019). It is now considered that this multi-protein MCU complex adapts to

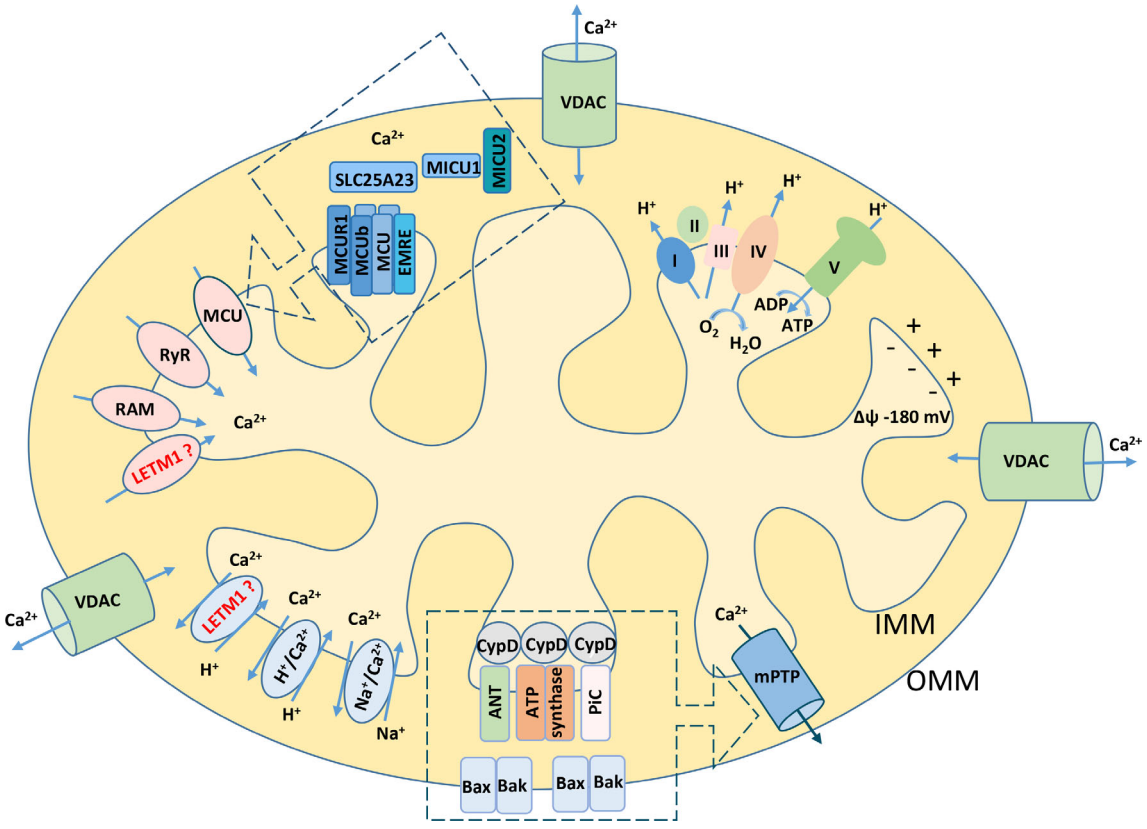

Fig 1. Mitochondrial Ca^{2+} transport system. Ca^{2+} transport into mitochondria takes place through the outer mitochondrial membrane (OMM) *via* a large-conductance channel, the voltage-dependent anion channel (VDAC). For Ca^{2+} influx through the inner mitochondrial membrane (IMM), three main mechanisms are known: the mitochondrial Ca^{2+} uniporter (MCU), rapid mode of Ca^{2+} uptake (RaM), and the mitochondrial ryanodine receptor (mRyR). The MCU is a macromolecular complex composed of pore-forming and regulatory subunits: mitochondrial Ca^{2+} uniporter (MCU), MCU dominant negative beta subunit (MCUb), essential MCU regulator (EMRE), the family of mitochondrial Ca^{2+} uptake proteins (MICU1–3), mitochondrial Ca^{2+} uniporter regulator 1 (MCUR1), and SLC25A23. Ca^{2+} influx through the MCU multi-protein complex is driven by the large electrochemical gradient (mitochondrial membrane potential ∼−180 mV) for Ca^{2+} across the IMM, generated by proton pumps (Complexes I, III and IV) of the electron transport chain. For Ca^{2+} efflux through the IMM, three main mechanisms are available: the Na^+/Ca^{2+} exchanger (NCXm), H^+/Ca^{2+} exchanger (HCXm) and the mitochondrial permeability transition pore (mPTP). The current proposal about the mPTP is based on the formation of ATP synthase dimers, in which the adenine nucleotide translocator (ANT) and Cyclophilin D (CypD) are likely regulators of the mPTP complex. The phosphate carrier (PiC) may also be a pore regulator, while the pro-apoptotic proteins Bax/Bak function in the outer membrane to contribute to regulation of mPTP opening and release of pro-apoptotic factors from mitochondria. The leucine zipper- EF-hand containing transmembrane protein (LETM1) was proposed as another Ca^{2+}-transport system. However, the role of LETM1 in mitochondrial Ca^{2+} influx and efflux through the IMM is still under discussion.

multiple states and is composed of several subunits, including transmembrane core components and membrane-associated regulatory subunits in the intermembrane space. Three proteins have been identified as core components of the MCU complex: the mitochondrial Ca^{2+} uniporter (MCU), the MCU dominant negative beta subunit (MCUb), and the essential MCU regulator (EMRE) (Raffaello *et al.*, 2013; Sancak *et al.*, 2013; Mishra *et al.*, 2017; Mammucari *et al.*, 2018; Cui *et al.*, 2019; Wang, Baradaran & Long, 2020*a*). The MCU gene (previously known as *CCDC109a*; 40 kDa protein) was identified through bioinformatics screening of the MitoCarta database, a compendium of mitochondrial proteins identified by mass spectrometry analyses on mitochondrial preparations from different mouse tissues (Baughman *et al.*, 2011; De Stefani *et al.*, 2011; Mammucari *et al.*, 2018). The MCUb gene (*CCDC109b*; 33 kDa protein) was identified through an MCU sequence homology screening (Raffaello *et al.*, 2013), and the incorporation of MCUb into the MCU complex has also been demonstrated by proteomic experiments (Sancak *et al.*, 2013). EMRE (*C22ORF32*; 10 kDa protein) was the last identified component of the MCU pore complex (Sancak *et al.*, 2013) and is essential for MCU activity, as demonstrated by experiments in EMRE knockout cells (Patron *et al.*, 2014). EMRE has been proposed to play a fundamental role in interactions between the pore core subunits and the regulatory subunits (Sancak *et al.*, 2013; Mammucari *et al.*, 2018).

The family of mitochondrial Ca^{2+} uptake proteins (MICU 1–3), mitochondrial Ca^{2+} uniporter regulator 1 (MCUR1), and SLC25A23 are now considered the main membrane-associated regulatory subunits of the MCU multi-protein complex (Hoffman *et al.*, 2014; Patron *et al.*, 2014; Wang *et al.*, 2014; Mishra *et al.*, 2017; Marchi *et al.*, 2019; Vais *et al.*, 2020). MICU1 (*CBARA1/EFHA3*; 54 kDa protein) is a soluble (or membrane-associated) protein in the intermembrane space. It is proposed to be pivotal in both gatekeeping and cooperative activation of MCU; keeping the channel closed under resting conditions (Csordas *et al.*, 2013; Patron *et al.*, 2014; Wang *et al.*, 2014; Foskett, 2020). Other isoforms of the MICU family of proteins – MICU2 (known as *EFHA1*) and MICU3 (known as *EFHA2*) – are also identified as regulatory subunits of the MCU multi-protein complex, and display EF-hand domains in their protein structure, but share only 25% sequence identity with MICU1 (Plovanich *et al.*, 2013). However, the location of MICU proteins and the nature of MICU-dependent regulation are still controversial (Vais *et al.*, 2020; Wu *et al.*, 2020; Wang *et al.*, 2020*b*). The diverse functions of MICU family proteins maintain normal mitochondrial Ca^{2+} levels under resting conditions and enable prompt activation of the MCU to mediate rapid mitochondrial Ca^{2+} uptake (Cui *et al.*, 2019).

MCUR1 (*CCDC90A*; 40 kDa protein), which consists of two transmembrane domains and one coiled-coil region, was also demonstrated to be a regulatory component of the MCU complex (Mallilankaraman *et al.*, 2012). More recently, it was shown that MCUR1 binds to the MCU pore and EMRE through their coiled-coil domains that stabilize the MCU complex (Tomar *et al.*, 2016).

SLC25A23, which belongs to a family of Mg-ATP/Pi solute carriers, was also proposed as an essential component of the MCU complex (Bassi *et al.*, 2005; Hoffman *et al.*, 2014; Krebs, 2017). A mutation of the EF-hand domain of SLC25A23 reduces mitochondrial Ca^{2+} accumulation, but whether this depends on direct MCU activity regulation or whether it affects mitochondrial bioenergetics or mitochondrial Ca^{2+} buffering capacity is still debated (Bassi *et al.*, 2005; Rueda *et al.*, 2015; Mammucari *et al.*, 2018). In addition to the MCU, there are other mitochondrial Ca^{2+} influx mechanisms, including the mRyR, RaM, and LETM1, which all have unique biophysical properties that differ from those of the MCU (Gunter & Gunter, 2001; Beutner *et al.*, 2005; Jiang, Zhao & Clapham, 2009; Elustondo *et al.*, 2017; Krebs, 2017; Mammucari *et al.*, 2018).

mRyR is the largest known ion channel (about >2 MDa), localized in the IMM, and can function as an alternative mechanism for mitochondrial Ca^{2+} uptake, especially in the mitochondria of cardiac and neuronal cells (Beutner *et al.*, 2001, 2005; Jakob *et al.*, 2014). Three different subtypes of RyR isoforms (RyR1, RyR2, and RyR3) with different pharmacological properties and tissue-specific expression have been described. RyR1, the primary isoform in skeletal muscle, was identified in the IMM of isolated heart mitochondria through [^{3}H]ryanodine binding, immunogold labelling and Western blot techniques. It is thought to mediate ryanodine-sensitive, rapid mitochondrial Ca^{2+} transport and is believed to play a central role in mitochondrial Ca^{2+} uptake (Beutner *et al.*, 2001, 2005). RyR2 is mostly present in cardiac muscle cells (Bhat *et al.*, 1999), while RyR3 is widely expressed in the endoplasmic reticulum (ER) of different vertebrate tissues (Giannini *et al.*, 1995) and may be co-expressed with RyR1 and RyR2. There are suggestions that under certain situations (e.g. mitochondrial Ca^{2+} overload), mRyR channels may also mediate Ca^{2+} efflux (Ryu *et al.*, 2010).

In isolated heart mitochondria, RaM has been described as an additional mechanism for Ca^{2+} transport, capable of sequestering significant amounts of Ca^{2+} hundreds of times faster than the MCU (Gunter & Gunter, 2001). RaM is activated only transiently, facilitates rapid sequestration of Ca^{2+} by mitochondria at the beginning of each cytosolic Ca^{2+} pulse, and rapidly recovers between pulses, allowing mitochondria to respond to repeated Ca^{2+} transients (Sparagna *et al.*, 1995). Compared with the MCU, this transporter is activated at much lower Ca^{2+} concentrations (−50 to 100 nM *versus* >500 nM) (Sparagna *et al.*, 1995). However, a protein responsible for this rapid mode of Ca^{2+} uptake has not been identified, although it has been speculated that the RaM comprises a substate of MCU operation since both are inhibited by ruthenium red and RaM activity does not appear to be present in MCU-knockout mitochondria (Baughman *et al.*, 2011; De Stefani *et al.*, 2011).

LETM1 was initially identified as a K^+/H^+ exchanger; however, it was later reported as a Ca^{2+}/H^+ antiporter, localized to the IMM (Jiang *et al.*, 2009). LETM1 transports Ca^{2+} bidirectionally across the inner membrane, depending on the pH gradient, and is inhibited by ruthenium red (Jiang *et al.*, 2009). However, further studies are needed to characterize its role in Ca^{2+} transport, as well as its sensitivity to ruthenium red, given recent demonstrations in which LETM1 protein was reconstituted in liposomes and was demonstrated to be a ruthenium red-insensitive electroneutral $Ca^{2+}/2H^+$ antiporter (Tsai *et al.*, 2014). Other studies have reinforced the role of LETM1 in K^+ homeostasis and suggested that it functions as an electroneutral H^+/K^+ exchanger (Nowikovsky & Bernardi, 2014). This hypothesis was supported by results showing that LETM1 was not responsible for efflux of Ca^{2+} from the mitochondria (De Marchi *et al.*, 2014*b*). There are some suggestions that the role of LETM1 could change according to specific conditions (O-Uchi, Pan & Sheu, 2012; Austin & Nowikovsky, 2019).

(2) Mitochondrial calcium efflux

While the biochemical characteristics and physiological functions of the mitochondrial systems for Ca^{2+} influx have been widely studied, understanding the molecular nature and properties of mitochondrial Ca^{2+} efflux systems has just begun, although functional characterization of this system began in the 1970s (Carafoli *et al.*, 1974). Currently, two separate mechanisms have been proposed to account for Ca^{2+}

extrusion from the mitochondrial matrix: Na^+-dependent (NCXm) and Na^+-independent (HCXm).

In most cells, the main mechanism of Ca^{2+} extrusion from mitochondria is the NCXm. Although Na^+-dependent Ca^{2+} efflux from mitochondria was first discovered in isolated rat heart mitochondria and was described as the mitochondrial Na^+/Ca^{2+} exchanger years ago (Carafoli *et al.*, 1974), its molecular identity was also only recently resolved (Palty *et al.*, 2010). It appears to extrude Ca^{2+} from the mitochondrial matrix to the intermembrane space and, more specifically, to constitute a $Na^+/Ca^{2+}/Li^+$ exchanger in the IMM (Palty *et al.*, 2010; Palty, Hershfinkel & Sekler, 2012). This Na^+-dependent Ca^{2+} exchange activity is benzodiazepine and CGP-37157 sensitive and is found in a wide variety of tissues. Although it is dominant in the heart, brain, skeletal muscle, parotid gland, adrenal cortex, and brown fat (Gunter *et al.*, 2004; Takeuchi, Kim & Matsuoka, 2015), it is also present in liver, kidney, and lung mitochondria, although its activity there is lower (Haworth, Hunter & Berkoff, 1980). The NCXm is active primarily in excitable cells and, in contrast to the plasma membrane Na^+/Ca^{2+} exchanger, it uniquely has the ability additionally to transport Li^+ ions (Carafoli *et al.*, 1974; Palty *et al.*, 2004). The stoichiometry (ion-exchange ratio) and the electrogenicity of the NCXm were controversial, but it was believed to be electroneutral (Affolter & Carafoli, 1980; Wingrove & Gunter, 1986*b*). The use of permeabilized rat ventricular myocytes demonstrated that the NCXm is voltage-dependent and electrogenic, suggesting a stoichiometry higher than $3Na^+$ for one Ca^{2+} (Kim & Matsuoka, 2008). This stoichiometry and the electrogenic nature of the NCXm were proved recently using the whole-mitoplast patch-clamp technique (Islam, Takeuchi & Matsuoka, 2020). Detailed mechanisms of the regulation of NCXm activity and sensitivity to effectors have not yet been clarified, but some studies demonstrate its regulation by a stomatin-like protein 2 (Da Cruz *et al.*, 2010) and the mitochondrial phosphatase and tensin homolog deleted on chromosome 10 (PTEN)-induced kinase 1 (PINK1) (Gandhi *et al.*, 2009).

In the tissues with low NCXm activity (e.g. the liver, kidney, lung, and smooth muscle; Takeuchi *et al.*, 2015), the HCXm has a dominant effect on the release of Ca^{2+} from mitochondria (Gunter & Pfeiffer, 1990). The molecular identity of the HCXm is still debated, but most likely its activity is electroneutral with a stoichiometry $2H^+$ for one Ca^{2+} (Gunter, Zuscik & Gunter, 1991). Studies of HCXm activity in rat isolated liver mitochondria demonstrated that the rate of efflux *via* the HCXm decreases with increasing pH gradient and suggest that this mechanism is an active rather than passive Ca^{2+} for $2H^+$ exchanger (Gunter *et al.*, 1991).

LETM1 is considered an additional and/or alternative mechanism of Ca^{2+} efflux from mitochondria (Nowikovsky *et al.*, 2012; Takeuchi *et al.*, 2015; Krebs, 2017; Austin & Nowikovsky, 2019). Ca^{2+} transport through the IMM could be mediated by LETM1, since this protein functions as a Ca^{2+}/H^+ antiporter under certain conditions (Mailloux & Harper, 2011). Studies using digitonin-permeabilized S2 or 293 cells expressing the mitochondrial Ca^{2+}-sensor protein pericam, and purified protein reconstituted in liposomes, showed that LETM1 mediates H^+/Ca^{2+} exchange (Jiang *et al.*, 2009). Later work using reconstituted proteoliposomes reported that LETM1 mediates electroneutral $2H^+/Ca^{2+}$ antiport, which is insensitive to ruthenium red (Tsai *et al.*, 2014). Further studies focused on intracellular-store-dependent Ca^{2+} dynamics provided evidence that LETM1 acts as a $2H^+/Ca^{2+}$ exchanger (Huang *et al.*, 2017). Combined with a theoretical analysis (Nowikovsky *et al.*, 2012), this lends credibility to the role of LETM1 as an important Ca^{2+} efflux mechanism.

Another important mechanism for Ca^{2+} release from mitochondria is suggested as the transiently open form of the mPTP. Under pathophysiological conditions, in which this high-conductance non-specific pore opens, it may function as a Ca^{2+}-efflux system (Takeuchi *et al.*, 2015; Biasutto *et al.*, 2016; Hurst, Hoek & Sheu, 2017; Britti *et al.*, 2018; Briston *et al.*, 2019). Opening of the mPTP is directly regulated by the concentration of free calcium, is triggered by Ca^{2+} overload, and enables rapid Ca^{2+} efflux (Hurst *et al.*, 2017). Detailed information regarding the function of the mPTP in Ca^{2+} transport is provided in Section VII.

It is important to note that the localization of mitochondria within the cell is a crucial factor for mitochondrial Ca^{2+} uptake. It is now well accepted that the location of mitochondria in proximity to the plasma membrane or the ER/sarcoplasmic reticulum (SR) is essential in modulating a variety of cellular functions, as well as in Ca^{2+} transport to subcellular structures, in particular to mitochondria (Rowland & Voeltz, 2012; van Vliet, Verfaillie & Agostinis, 2014; Stefan, 2018; Silva *et al.*, 2020). These interactions between mitochondria and the ER/SR have been described as physiologically and pathophysiologically significant for Ca^{2+} crosstalk between mitochondria and other cellular and subcellular structures (Giacomello *et al.*, 2010; Rowland & Voeltz, 2012; van Vliet *et al.*, 2014; Takeuchi *et al.*, 2015; Rieusset, 2018; Yousuf *et al.*, 2020). During Ca^{2+} movement through the plasma membrane or its release from the ER/SR, these interactions promote Ca^{2+} influx to neighbouring mitochondria, determining the particular properties of Ca^{2+} transport into these organelles (Lawrie, Zolle & Simpson, 1997; Park *et al.*, 2001; Giacomello *et al.*, 2010). There is some evidence of Ca^{2+} channelling through plasma membrane Ca^{2+} channels to nearby mitochondria, as well as in the opposite direction (Korzeniowski *et al.*, 2009). Mitochondria–ER/SR communication is also reported as an essential regulatory factor in a variety of cellular processes, including Ca^{2+} signalling, lipid biosynthesis, and mitochondrial division (Friedman *et al.*, 2011; Rieusset, 2018; Granatiero *et al.*, 2019; Namgaladze, Khodzhaeva & Brune, 2019). It is now clear that bidirectional Ca^{2+} crosstalk between both mitochondria and the plasma membrane and mitochondria and the ER/SR is crucial for regulating a wide range of cellular functions.

(3) Ca^{2+} induces mitochondrial swelling

Hunter, Haworth & Southard (1976) first introduced the concept of Ca^{2+}-induced mitochondrial swelling. In a series of seminal experiments performed on isolated beef heart mitochondria, the authors observed that when low levels of Ca^{2+} were added, mitochondria underwent a configurational transition from a shrunken matrix with large intracristal spaces to a swollen matrix with decreased intracristal spaces (Fig. 2). Mitochondria were described to transition from an aggregated to an orthodox configuration in this process. Using electron microscopy, it was concluded that for each time point post-Ca^{2+} addition, the mitochondrial population was heterogeneous with only the two different configurations present. The authors argued that the transition sequence for each mitochondrion consisted of a lag phase in the aggregated form followed by a sudden transformation to the orthodox form.

To assess the correlation between this configurational change and membrane permeability, Hunter *et al.* (1976) measured the permeability of mitochondrial membranes of isolated beef heart mitochondria to [^{14}C]sucrose in the presence of Ca^{2+}. In samples taken at the same time points as those used for electron microscopy imaging, it was observed that permeability to [^{14}C]sucrose increased simultaneously with the decrease in the number of aggregated mitochondria. This important result made it clear that Ca^{2+}increased the permeability of mitochondria to [^{14}C]sucrose. Importantly, the swelling that follows the addition of Ca^{2+} was caused by mitochondrial osmotic water influx that accompanies sucrose entry into the matrix space (Hunter *et al.*, 1976).

Further experiments addressed the effect of IMM permeability on mitochondrial respiratory activity. The uncoupler respiratory control index (i.e. the magnitude of the respiration increase) was compared with configurational changes at several time points after the addition of Ca^{2+}. This analysis showed that coupling in an individual mitochondrion follows an all-or-nothing law, precisely as for the configurational transition. However, experiments using hypotonically swollen mitochondria led to the conclusion that the changes in

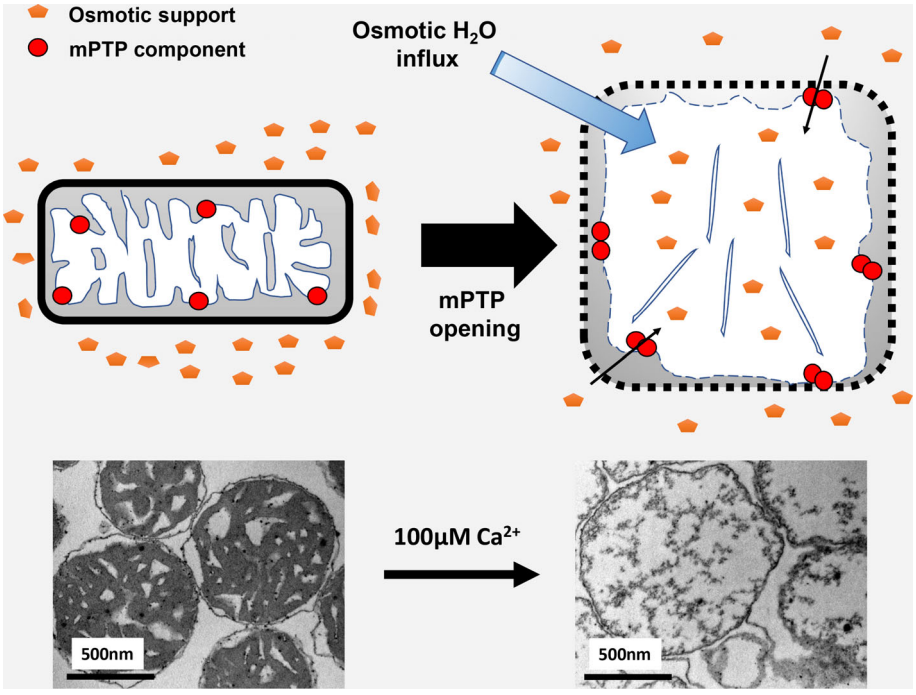

Fig 2. Mitochondrial swelling as a classical hallmark of the mitochondrial permeability transition pore (mPTP) *in vitro*. The upper part of the figure shows mitochondrial swelling associated with mPTP when induced *in vitro*. Isolated mitochondrial fractions (e.g. from liver or heart) are normally suspended in a reaction buffer with sucrose or KCl as osmotic support (~200 mOsm), represented in the figure as orange hexagons. Mitochondrial membranes are particularly impermeable to sucrose and show regulated permeability to K^+ and Cl^- ions. Upon opening of the mPTP (e.g. using a pro-oxidant agent in the presence of excess calcium), conformational alterations in inner mitochondrial membrane (IMM) proteins (in red) leads to the formation of the mPTP complex through which molecules under 1450 Da flow (including osmotic support molecules). Water follows by osmosis, leading to swelling of IMM and rupture of the outer mitochondrial membrane (OMM) due to the smaller surface area of the latter. The intra-cristal space also decreases. This effect leads to uncoupled mitochondrial respiration and mitochondrial depolarization, leading to calcium-induced calcium release. Mitochondrial swelling can be observed by following the pseudo-absorbance of the mitochondrial suspension at ~540 nm, the isosbestic point for mitochondrial cytochromes (the wavelength at which the total absorbance of the different cytochromes in mitochondria does not change during the event). The lower panel shows electron microscopy images of liver mitochondria before (left) and after (right) undergoing calcium-induced mPT. Images kindly provided by Drs. Sabine Schmitt and Hans Zischka, Helmholtz Center Munich, Germany.

coupling are caused by changes in the permeability of the IMM, rather than changes in configurational transition (Hunter *et al.*, 1976). This clarified previous misconceptions (Harman & Feigelson, 1952; Lehninger, 1962) that mitochondrial swelling by itself did not determine mitochondrial functioning. Hunter *et al.* (1976) also observed that the transition was reversible and that arsenate, phosphate, and fatty acids induced the phenomenon.

In later studies, light scattering was used to measure mitochondrial swelling, based on changes in pseudo-absorbance of a suspension of beef heart mitochondria (Hunter & Haworth, 1979*a*). This methodology, still used today for isolated mitochondrial fractions, enabled the authors to show that mitochondrial metabolites and small molecules and ions can counteract the Ca^{2+}-induced membrane transition: endogenous NADH, ADP, and Mg^{2+} all prevented the mPT. The same effect could be achieved with mitochondrial energization (Hunter *et al.*, 1976). Following these pioneer discoveries, Haworth & Hunter (1979) then explored the mechanisms by which Ca^{2+} leads to mPT, and how its inhibitors could function.

One important observation was that mitochondria which had been previously subjected to Ca^{2+}-induced mPT lost their endogenous protection. Also, the concentration of Ca^{2+} in the incubation medium was able to regulate the permeability of the IMM in a dose-dependent manner, as seen by measuring light scattering in suspensions titrated with different concentrations of Ca^{2+}. Together with the fact that the membrane transition needed no energy source (Hunter & Haworth, 1979*a*), this led to the conclusion that the physical binding of Ca^{2+} to units in the IMM could cause the mPT. An important observation was that each preventive agent showed a specific mode of action by which it counteracted the membrane transition. For example, Mg^{2+} was observed to inhibit the transition competitively. Hunter *et al.* (1976) had already proposed that Mg^{2+} could bind to 'trigger sites', but Ca^{2+}, with an apparently higher affinity for those sites, could exclude $Mg^{2+}$and induce the transition. The effects of ADP and NADH were investigated in more detail later (Haworth *et al.*, 1980). ADP and NADH have synergistic effects on inhibition of the Ca^{2+}-induced transition in isolated mitochondria. ADP was thought to have an inner membrane binding site which, when bound to ADP, could significantly inhibit binding of Ca^{2+} to the trigger site and therefore inhibit the transition. The inhibition by NADH was observed to be reduced by NADPH (Haworth *et al.*, 1980). Several cations were identified as competitive inhibitors of the modulating effects of Ca^{2+} (Hunter & Haworth, 1979*a*), including divalent cations, such as Sr^{2+} and Mn^{2+}, and the monovalent cations, K^+, Na^+, and tetramethylammonium (TMA). The trivalent metal inhibitor La^{3+} ion exhibited competitive inhibition more potent than these mono and divalent cations. Protons (H^+) were also a competive inhibitor, an observation that formed the basis for studies that eventually led to inhibition of the mPT by ischemia-induced cytosolic acidification (Qian *et al.*, 1997). These observations suggested that the trigger site could be involved in high-affinity Ca^{2+} uptake. However, Ca^{2+}-induced transition progression was found to be ruthenium red insensitive. Because high-affinity Ca^{2+} uptake is sensitive to ruthenium red (Moore, 1971), Hunter & Haworth (1979*a*) assumed that another mitochondrial site, independent from those involved in high-affinity Ca^{2+} uptake, must be involved in the transition.

Hunter & Haworth (1979*b*) further investigated pathways for the release of Ca^{2+} that occurred during the transition. They concluded that Ca^{2+} release was due to the Ca^{2+}-induced transition because: (*i*) agents that blocked the transition, such as Mg^{2+}, ADP and bovine serum albumin (BSA), inhibited Ca^{2+} release under steady-state respiration; (*ii*) mitochondria that accumulated Sr^{2+}, did not release it subsequently, indicating that the spontaneous release mechanism was selective for Ca^{2+}, similar to the transition itself; (*iii*) the use of light-scattering and electron microscopy to assess the configurational change during Ca^{2+} release showed that the transition to the orthodox state took place at the same time as Ca^{2+} release. Additional observations showed that the kinetics of Ca^{2+} release was similar to that of the transition: measurement of $^{45}Ca^{2+}$ fluxes indicated that mitochondria release their entire pool of Ca^{2+} at the same time, following a lag phase without any release. The authors also investigated the release of Ca^{2+} induced by the addition of an uncoupler molecule that dissipated the transmembrane electric potential ($\Delta\Psi$) (Hunter & Haworth, 1979*b*), observing that as Ca^{2+} accumulated in mitochondria, so did the amount of Ca^{2+} released by addition of the uncoupler trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP). Addition of FCCP also provoked a configurational change from the aggregated to the orthodox form, i.e. a permeability transition was observed. The percentage of mitochondria that underwent the transition was higher in populations with higher levels of Ca^{2+} accumulation. Again the pattern of uncoupler-induced Ca^{2+}-dependent Ca^{2+} release was sudden and total and followed a lag phase of no release. In conditions where the NADH protective mechanism was lost, mitochondria were observed to transition to an orthodox conformation without the requirement for additional Ca^{2+}, and to release Ca^{2+} much faster than mitochondria that still possessed this protective mechanism, thus implying that Ca^{2+}release depended on the permeability transition.

In addition, Sr^{2+} release was dependent on Ca^{2+} accumulation and was ruthenium red insensitive, both characteristics of the transition. Supported by this evidence, the uncoupler-induced release of Ca^{2+} was concluded to be another consequence of the Ca^{2+}-induced transition, but dependent on the level of Ca^{2+} accumulated.

Hunter & Haworth (1979*b*) also explored whether the release of Ca^{2+} caused by Na^+ was mediated by the Ca^{2+}-induced transition. Because no mitochondrial configurational changes upon addition of Na^+ and consequent Ca^{2+} release were observed, it was concluded that not all release of Ca^{2+} from mitochondria depends on the mPT. Na^+-induced Ca^{2+} release was later shown to originate from a protein membrane exchanger (Wacquier *et al.*, 2017).

III. FROM THE INITIAL OBSERVATIONS TO THE COMPOSITION AND REGULATION OF THE PERMEABILITY TRANSITION: AN EVOLVING MODEL

Over the past three decades, our understanding of the structure of the mPTP has evolved; while we now know some of the modulators involved, precise details of the pore-forming parts, including their dynamics, remain elusive. Advances in technology have enabled a better understanding of the mPTP pore complex composition and assembly, and its regulation in intact cells and tissues. Early work (Raaflaub, 1953; Lehninger & Remmert, 1959; Lehninger, 1962) used gravimetric and optical methods to show that isolated liver mitochondria can take up water and thereby increase in volume. Later studies were based on measuring either light transmittance (transparency) or light scattering (opacity, turbidity) of mitochondrial suspensions. Interestingly, Lehninger (1959) also observed the reverse process, which he termed 'contraction', consisting of the extrusion of water from mitochondria, resulting in a decrease in mitochondrial volume manifested by increased turbidity (light scattering) of mitochondrial suspensions. The standard method used for isolated mitochondria is still the mitochondrial swelling assay, which records a decrease in absorbance at 520/540 nm that accompanies mitochondrial swelling, with Ca^{2+} administered to preparations at pH 7.4.

This mitochondrial swelling, subsequently studied by Azzi & Azzone (1965*a*), was termed 'large-amplitude swelling' as it eventually led to complete rupture of the OMM and the formation of IMM 'ghosts' of low light absorbance (Fig. 2). By contrast, 'low-amplitude swelling', first observed by Chance & Packer (1958) and studied by Azzi & Azzone (1965*b*), was fully reversible and exhibited variation in the energy and metabolic state of mitochondria. These low-amplitude changes in mitochondrial volume and structure could also be observed inside living cells (Hackenbrock *et al.*, 1971). The term 'permeability transition' of the IMM was probably first used by Wingrove & Gunter (1986*a*). Large-amplitude and (most likely) low-amplitude mitochondrial swelling could be interpreted as resulting from an unspecific increase in permeability of the IMM to low molecular weight solutes. This increased permeability allowed the concentrations of low molecular weight compounds to equilibrate inside and outside the mitochondria, whereas concentrations of high molecular weight compounds, in particular, soluble intramitochondrial proteins, remained unchanged. This resulted in a higher osmotic pressure (so-called colloidal or oncotic pressure) inside mitochondria, resulting in the influx of water.

In contrast to mitochondrial swelling, mitochondrial contraction was assumed to be an active process (Lehninger, 1959). While the release of non-esterified fatty acids accompanied mitochondrial swelling, their contraction involved the re-incorporation of these fatty acids into mitochondrial phospholipids as demonstrated using isotopically labelled [^{14}C]oleate and glycerol 3-[^{32}P]phosphate (Wojtczak, Wlodawer & Zborowski, 1963). These studies suggested that contraction was enabled by the removal of accumulated free fatty acids and the restoration of some lipid compounds indispensable for the normal low permeability of the inner membrane.

A systematic study using electron microscopy revealed that the swelling-related increase in mitochondrial matrix volume is accompanied by the rupture of the OMM (Wlodawer *et al.*, 1966) (Fig. 2). By contrast ATP-induced contraction was reflected by matrix condensation, although this never led to the restoration of the original structure of the intact mitochondrion. Impermeability of the OMM to cytochrome c (Wojtczak & Zaluska, 1969; Wojtczak & Sottocasa, 1972) led to the development of an assay for its intactness in preparations of isolated mitochondria based on oxidation of externally added reduced cytochrome c (Wojtczak *et al.*, 1972). The same protocol can be used to detect the release of cytochrome c from mitochondria that might occur during mitochondrial swelling. In living cells, release of cytochrome c to the cytosol accompanied by the rupture of the outer membrane (Vander Heiden *et al.*, 1997; Petit *et al.*, 1998) can be investigated with the use of antibodies against cytochrome c; however, this requires isolation of cytosolic fractions. It is also important to note that there are other possible ways in which cytochrome c can be released from mitochondria into the cytosol without swelling and rupture of the OMM (Wieckowski *et al.*, 2001).

Experiments on isolated mitochondria were carried out in the late 1970s (Haworth & Hunter, 1979; Hunter & Haworth, 1979*a*,*b*) (see Section II.3) in research into the molecular mechanisms underlying the mPTP, its regulation, and the biological function of this transition that employed the 'controlled' environment offered by isolated and de-energized organelles. Despite being artificially devoid of endogenous substrates, de-energized mitochondria preserve a permeability transition, allowing its analysis in a relatively variable-free environment. Methods applied to isolated mitochondria partly replaced previous methodologies using enzymatic reactions performed, for instance, on ashed preparations from rat heart that were often inaccurate and technically difficult (Slater & Cleland, 1953). Although the earlier methods allowed Ca^{2+}-dependent mitochondrial swelling to be observed (Slater & Cleland, 1953), studies on isolated mitochondria enabled the demonstration of the sudden opening of a reversible permeability state of the IMM, termed the 'Ca^{2+}-induced transition' (Hunter *et al.*, 1976), and enhancement of that effect by increased concentrations of phosphates, arsenate and fatty acids (Hunter & Haworth, 1979*a*). The ability to add different substrates to mitochondrial preparations to identify their regulatory properties allowed exploration of the protective mechanisms against mPTP opening, such as Mg^{2+} which competes with Ca^{2+} for a shared binding site inside mitochondria. Thus, Ca^{2+} was established as the central positive modulator of the mPT (Azzi & Azzone, 1966; Hunter *et al.*, 1976). Other protective mechanisms included the reduction status of mitochondrial NAD^+, bongkrekic acid, and the energetic status of mitochondria (Hunter *et al.*, 1976). This evidence was confirmed more recently when it was demonstrated that Ca^{2+}-

dependent swelling was efficiently inhibited by bongkrekic acid administration, an ADP-based medium and by the immunosuppressant Cyclosporin A (Halestrap & Davidson, 1990). This work highlighted the usefulness of mitochondrial swelling to study this unselective pore's opening and highlighted a regulatory role for the adenine nucleotide translocator (ANT) and cyclophilin D (CypD), the molecular target of Cyclosporin A.

Critical to modern studies was the description of two ways by which a non-specific pore opens: one caused by low physiological Ca^{2+} concentrations involving a peptidylprolyl isomerase (PPi)-dependent mechanism that is insensitive to Cyclosporin A, and a second in response to higher Ca^{2+} concentrations and that is Cyclosporin A-sensitive (Davidson & Halestrap, 1987). These observations led to the proposal of a model of pore opening, based on the CypD–ANT protein interaction in the presence of Ca^{2+} overload (Halestrap & Davidson, 1990). In this model, by binding CypD, Cyclosporin A would promote its dissociation from the translocator and block the pore. The ability of ADP, ATP, and bongkrekic acid strongly to inhibit mPTP opening suggested that they also may bind the ANT carrier, reversing its conformation caused by Ca^{2+} addition. A combination of isolated mitochondrial preparations and patch-clamp techniques allowed monitoring of mPTP properties in response to ANT oligomers in artificial membranes (Brustovetsky & Klingenberg, 1996).

It was also suggested that the mPTP might result from structural and functional 'cooperation' between the IMM and OMM (Kottke *et al.*, 1988; McEnery *et al.*, 1992; Kinnally *et al.*, 1993). This view identified the contact sites between the two membranes as possible loci regulating not only the permeability but also the metabolic and energetic functions of mitochondria (Nicolay *et al.*, 1990; Bucheler, Adams & Brdiczka, 1991; Wieckowski, Brdiczka & Wojtczak, 2000). Arguments that the mPTP is located at the junction between the two membranes were based on observations that proteins supposedly participating in this pore came from both the OMM and IMM.

Early studies on isolated mitochondria identified putative proteinaceous channels between the IMM and OMM with ANT and VDAC as the core components of the mPTP (Fig. 3A), and a plethora of regulators, including the OMM 18-kDa peripheral benzodiazepine receptor (TSPO), glycogen synthase kinase 3β (GSK3β), hexokinase II (HKII) and creatine kinase (CK) (Ong *et al.*, 2014; Morciano *et al.*, 2015; Tanaka *et al.*, 2018). Although the VDAC enables the transport of most solutes across the IMM, the new era of genetic studies challenged this proposed pore structure (Baines *et al.*, 2007), demonstrating that mitochondria isolated from VDAC-knockout (KO) mice still exhibited a Ca^{2+}-dependent mPT very similar to that found in wild-type mitochondria. Similarly, cell death was unaltered in VDAC-KO cells. Gene inactivation studies using KO animal models confirmed the presence of a functional mPTP also in ANT-KO mice, thus excluding it as a pore-forming component of the mPTP (Kokoszka *et al.*, 2004) and conferring to CypD a more important modulatory role (Schinzel *et al.*, 2005; Hurst *et al.*, 2020). The recent innovative generation of a triple KO for ANT1, ANT2, and ANT4 has revisited the contribution of this translocator to the mPTP cascade; the progressive deletion of these isoforms led to equal decreases in sensitivity of the pore to opening (Karch *et al.*, 2019), implying that ANT, under favourable biochemical conditions and in a given tissue, may constitute an alternative to ATP synthase during mPTP formation.

CypD then remained the only protein whose involvement in the mPTP was uncontested: experiments performed with transgenic mice lacking the peptidylprolyl isomerase f (*Ppif*) gene confirmed that this protein is a key element of the mPTP that is responsible for its sensitivity to Cyclosporin A (Basso *et al.*, 2005; Valasani *et al.*, 2014, 2016; Lindblom *et al.*, 2020; Torpey *et al.*, 2020; Panel *et al.*, 2021). However, it should be stressed that CypD plays a regulatory role and is not involved in the pore itself (Fayaz, Raj & Krishnamurthy, 2015). The elucidation of the regulatory role of CypD in mPTP opening was facilitated by discovering that Cycloporin A (the gold-standard inhibitor of the mPTP) targeted this protein. To date, multiple investigations have shown that CypD inactivation (using genetic or pharmacological approaches) inhibits mPTP induction and cell death in several *in vitro* and *in vivo* models. Signalling events also can target CypD to regulate mPTP opening.

McEnery *et al.* (1992) investigated the interaction of TSPO with the ANT and VDAC proteins. At that time, studies on IMM–OMM contact sites were the focus of interest, and ligands of TSPO with nanomolar affinity and joined to VDAC/ANT proteins were shown to have mPTP-like channel activities, as recorded using the patch-clamp technique (Kinnally *et al.*, 1993). However, biochemical attempts to identify the mPTP core complex by conditional deletion assays led researchers to conclude that TSPO was dispensable in terms of mPTP regulation and hence does not participate in mPTP-dependent cell death (Sileikyte *et al.*, 2014).

It was proposed independently by two research teams (Alcala *et al.*, 2008; Leung, Varanyuwatana & Halestrap, 2008) that the phosphate carrier (PiC) could be a good candidate to form the core of the mPTP (Fig. 3B). The observed concentration-dependent inhibitory effect of n-ethylmaleimide (NEM), ubiquinone 0 (UQ0), and (spiro [cyclopentane-1,5′-[5H]dibenzo[a,d]cyclohep-ten]-2-one,10′,11′-dihydro-3-methylene (Ro 68-3400) on both the mPTP and PiC suggested that the PiC could act as a pore-forming component. This was reinforced by the fact that phosphates greatly enhanced mPTP opening, so it was believed that an additional protein of the IMM (such as the PiC) could represent the putative pore-forming part. Varanyuwatana & Halestrap (2012) subsequently questioned this concept by showing that a 70% or more decrease in expression of the PiC in HeLa cells did not affect mPTP opening. This was confirmed by other authors using KO models (Gutierrez-Aguilar *et al.*, 2014). Interestingly, the involvement of the PiC in the PTP could not be fully excluded because complete genetic deletion of this carrier in mouse

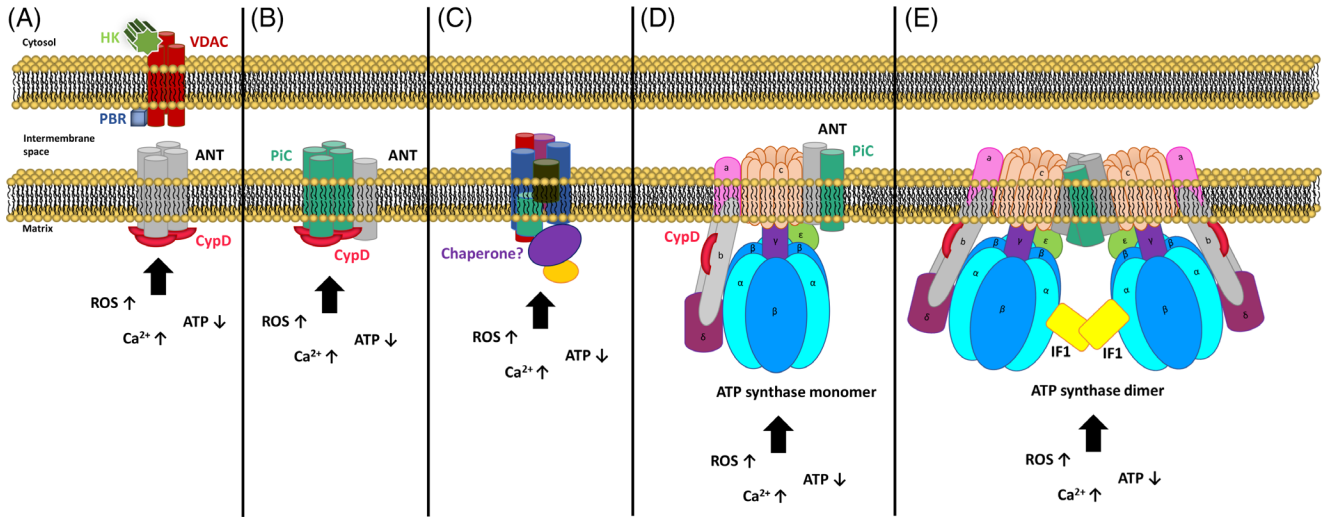

Fig 3. Different models for the composition and structure of the mitochondrial permeability transition pore (mPTP). (A) The initial model, in which the adenine nucleotide translocator (ANT) in the inner mitochondrial membrane was proposed to have a structural role, together with the outer mitochondrial membrane voltage-dependent anion channel (VDAC). The mPTP structure and opening is regulated by the matrix chaperone cyclophilin D (CypD), while hexokinase (HK) and the peripheral benzodiazepine receptor (PBR) also have regulatory roles through their interaction with the VDAC. (B) Knock-out experiments revealed that neither the VDAC or the ANT are indispensable for the mPTP complex. Instead, the phosphate transporter (PiC) was proposed as the mPTP main structural component, whose opening is regulated by the ANT and CyD. (C) Other proposals suggested that the mPTP is composed of, not one, but several possible entities comprising distinct proteins and which are converted into the mPTP through the binding of matrix chaperones. (D, E) The most recent proposal suggests that the structural component of the mPTP is ATP synthase, with two alternative theories: the first proposing that the ATP monomer forms the mPTP (D), while the second suggests that ATP synthase dimers are the core of the mPTP complex (E). In both these models, the ANT and PiC, both components of the mitochondrial phosphorylative system, have a regulatory role. Regardless of the model proposed, there are common features, including a contribution of increased levels of reactive oxygen species (ROS) and increased [Ca^{2+}] and of decreased [ATP] contributing to pore opening.

cardiac mitochondria desensitized the mPTP (Kwong *et al.*, 2014). The PiC was thus instead suggested to be a regulatory rather than a core component of the mPTP (Kwong *et al.*, 2014).

In another perspective, Karch *et al.* (2013) provided evidence for B-cell lymphoma-2 (Bcl-2)-associated X (Bax) and Bcl-2-antagonist/killer 1 (Bak) members of the Bcl-2 family as mPTP components; using transgenic animal models, they demonstrated that the absence of both proteins promoted resistance to mitochondrial swelling and resulted in the lack of channel activity, as evidenced by patch-clamp studies on mitoplasts (Karch *et al.*, 2013). Thus, even though Bax and Bak play a structural role in the OMM part of the mPTP complex, an increase in solutes in the mitochondrial matrix also requires permeability of the IMM. This pore model suggested a subdivision between IMM- and OMM-pore formation, returning to previous concepts of IMM–OMM contact sites in mPTP assembly (Beutner *et al.*, 1998). Despite solid data supporting the contact site model of the mPTP, another model of mPTP formation has been proposed (Kowaltowski, Castilho & Vercesi, 2001; He & Lemasters, 2002). This model suggests that the pore could be formed by misfolded mitochondrial proteins modified, among other agents, by oxidative damage and unconnected with the presence of a pre-existing inner membrane pore. It has been proposed that opening of such unregulated pores occurs when the number of amphipathic protein clusters exceeds the number of chaperones available to block their conductance (Fig. 3C).

Although Ca^{2+} overload is widely recognized as the primary inducer of the mPT, other positive regulators have been reported. Halestrap (1991) demonstrated that pH can act as an additional and precise regulator of the mPT in rat heart and liver mitochondria. mPTP opening is thought to be inhibited in an acidic matrix pH because of the displacement of Ca^{2+} at the trigger site by H^+ ions (Halestrap, 1991). Atractyloside, a natural toxic glycoside, increases mPTP opening by modifying the conformational state of ANT, locking it in a cytoplasmic-side open conformation. ROS production and low mitochondrial membrane potential (MMP) are two other known inducers. Mitochondria are an important source of ROS (Giorgi *et al.*, 2018*c*), and conditions that lead to increased oxidative stress in cells, such as reoxygenation following hypoxia, strongly sensitize mPTP opening and consequent cell demise (Assaly *et al.*, 2012). Thus, it is a classical notion that the mPT is related to the redox state of mitochondria, including that of coenzyme Q (Kowaltowski, Castilho & Vercesi, 1995), and NADPH (Bernardes *et al.*, 1994), with mitochondrial oxidative stress playing an important role

even in de-energized mitochondria (Kowaltowski, Castilho & Vercesi, 1996; Vercesi *et al.*, 2018).

A unique role is also played by thiol groups of proteins localized in the IMM. Connern & Halestrap (1994) showed that exogenous administration of thiol oxidizing reagents to purified mitochondria preparations promoted key modifications in Cys56, Cys159 and Cys256 residues of the ADP/ATP translocator. In particular, Cys56 oxidation potentiated the binding of mitochondrial CypD with the IMM leading to pore opening (Connern & Halestrap, 1994). Cys56 and Cys159 are thought to be involved in antagonizing the inhibitory properties of ADP *via* the nucleotide-binding site on the ANT protein, as shown by adding phenylarsine oxide (PhAsO) to de-energized mitochondria, with its effect independent of CypD binding (Halestrap, Woodfield & Connern, 1997). All these effects were reversed in the presence of antioxidants (Kowaltowski *et al.*, 2001; Vercesi *et al.*, 2018). Further, evidence for mPTP inhibition in the presence of rotenone and the sensitivity of the mPT to inorganic phosphate (Pi) prompted the suggestion that NADH:ubiquinone oxidoreductase (Complex I) could also be implicated in the negative modulation of the mPTP, establishing a novel level of regulation. This was proposed to occur *via* conformational changes of Complex I affecting its interaction with the mPTP, in a manner that depends on the availability of Pi and CypD expression (Li *et al.*, 2012).

The open conformations of the mPTP may assume a low or a high conductance. The ability to switch from a low to a high conductance was investigated by Ichas & Mazat (1998) who also reported that this transition became irreversible upon reaching the high-conductance state. Historically, physiological roles have been attributed to the low-conductance open conformation; indeed, mitochondrial functions are preserved while in the so-called 'flickering mode' that regulates cellular Ca^{2+} homeostasis (Gunter & Pfeiffer, 1990; Altschuld *et al.*, 1992), with very limited diffusion of solutes through the IMM (cutoff <300 Da) and precise regulation by matrix pH changes and mitochondrial Ca^{2+} uptake (Ichas, Jouaville & Mazat, 1997). Conversely, when the mPTP switches to the high-conductance state, the consequences that are not compatible with cell life, including increased permeability of the IMM to solutes of 1500 Da, rupture of the OMM, and activation of the apoptotic cascade.

Our understanding of the processes leading to the association of ATP synthase (Complex V) with the mPTP (Fig. 3D, E) began with the discovery of regulatory analogies between these two complexes. Two mPTP inhibitors (ADP and Mg^{2+}) interact to block the hydrolytic activity of Complex V (Feniouk, Suzuki & Yoshida, 2006), while phosphates, known to be positive regulators of mPTP opening, reversed this state. In addition, the mPTP subunits ANT and PiC form a so-called ATP synthasome by interacting with ATP synthase (Ko *et al.*, 2003). Similarly, mitochondrial CypD, the target of the mPTP inhibitor Cyclosporin A, has been demonstrated to interact with the peripheral stalk of ATP synthase, the oligomycin-sensitivity-conferring protein (OSCP) (Giorgio *et al.*, 2009). This protein is able to modulate ATP synthase activity, causing it to decrease when mitochondrial CypD is anchored and to regulate mPTP opening (and cell death) *via* its C141 and H112 residues. These residues respectively affect the sensitivity of the pore to oxidation and its dependence on H^+ binding (Antoniel *et al.*, 2018; Carraro *et al.*, 2020).

In recent years, much effort has been made to identify putative component(s) of the IMM with channel properties. The c-subunit of Fo-ATP synthase, an IMM-resident protein, was identified as a key mPTP component with voltage-sensitive channel properties in its monomeric form and in the presence of both CypD binding and high Ca^{2+} concentrations (Alavian *et al.*, 2014). Studies from three independent research groups confirm the knowledge that this c-subunit plays a pivotal role in mPTP opening and link physio-pathological effects to its intracellular expression (Bonora *et al.*, 2013) and phosphorylation status (Azarashvili *et al.*, 2014). How the mPTP complex takes shape and which other proteins contribute to the structure of the pore-forming region remains elusive. Biochemical studies (Pavlov *et al.*, 2005; Elustondo *et al.*, 2016) reported the presence of higher numbers of c-subunits in mitochondria following induction of the mPT. This suggested the possibility that the c-subunit modifies interaction within F_1/F_O-ATP synthase dimers. They described an essential role of complexes formed by the c-subunit–inorganic polyphosphate (polyP)-hydroxybutyrates (PHB) axis in the formation of a non-specific pore channel during Ca^{2+}-induced stress. These results suggest that the c-subunit may form water-filled pores with polyP possibly serving as a hydrophilic coating of the pore, despite the F_1/F_O-ATP being a hydrophobic protein. Further confirmation of the importance of the c-subunit in mPTP activity has been published recently (Neginskaya *et al.*, 2019).

Two separate hypotheses justify a contribution of ATP synthase to mPTP assembly and activity (Fig. 3D, E): first, dimerization of ATP synthase is essential for the generation of the pore opening (Giorgio *et al.*, 2013) (Fig. 3E); second, ATP synthase monomers with a proper c-ring (the arrangement of c-subunits in the IMM) conformation constitutes the pore-forming part (Fig. 3D) (Bonora *et al.*, 2017). However, currently both models present certain critical issues that remain to be addressed (Bauer & Murphy, 2020). Concerning the first model (Giorgio *et al.*, 2013), there is irrefutable proof of the involvement of ATP synthase dimers (and oligomers) in beneficial bioenergetic functions of mitochondria, partially due to their role in ensuring the correct curvature of the IMM (Daum *et al.*, 2013), thus dimers of ATP synthase are unlikely also to be a mediator of Ca^{2+}-dependent cell death. For the second hypothesis, a structural model by which a non-specific current occurs in the c-ring is still needed. The controversial nature of the mPTP was highlighted by two recent publications in which KO experiments showed that the c-subunit and the peripheral stalk subunit of ATP synthase are not required for mPT opening (He *et al.*, 2017*a*,*b*). However, electrophysiological analysis

of isolated mitoplast KOs for the c-subunit demonstrated that, in their absence, the current recorded differed from that expected for mPTP. Indeed, the c-subunit-KO cells did exhibit a Ca^{2+}-inducible current, which could be inhibited by Cyclosporin A, but this was much lower than in wild-type cells (0.3 nS *versus* 1.3 nS). Moreover, the data suggest that, in c-subunit-KO cells, a second current could be generated by ANT (Neginskaya *et al.*, 2019). These findings thus confirm the key role of the c-subunit in mPTP activity. In another study, fully reconstituted active ATP synthase in liposomes was responsive to Ca^{2+}, converting dimers, but not monomers, into a channel. Interestingly, the activity was sensitive to adenine nucleotides, but not to ligands of ANT or VDAC (Urbani *et al.*, 2019). Recent decades of research have led to the discovery of a large number of inhibitors (Morciano *et al.*, 2018) and inducers of the mPT (see Fig. 4).

IV. UNDERSTANDING THE mPTP WITH THE USE OF ELECTROPHYSIOLOGICAL STUDIES

Important information about the mPTP structure and functioning has been obtained using the patch-clamp technique. To record channel activity of the IMM using this technique, it is necessary to remove the OMM and to create single-membrane mitoplasts. Two methods can be used to achieve this: the French press (Decker & Greenawalt, 1977) or osmotic swelling (Gupte *et al.*, 1984).

The first patch-clamp recordings of mitochondrial channel activity from the IMM (Sorgato, Keller & Stuhmer, 1987) led to the description of a single, slightly anion-selective channel of 107 pS conductance in 150 mM KCl. A 350 pS channel attributed to the OMM was also observed (Sorgato *et al.*, 1987). Subsequently, a study on mouse liver mitochondria (Kinnally, Campo & Tedeschi, 1989) reported a variety of conductances of 10–20, 45, 80, 120–150, 200, 350, and 1000 pS, with the latter uncharacterized due to its rarity. A parallel study on rat liver mitochondria (Petronilli, Szabo & Zoratti, 1989) observed a similarly large-conductance activity of about 1.3 nS. This activity exhibited a flickering nature, and its main subconductance state was around 0.63 nS at +20 mV. However, a range of other subconductance levels was observed at different voltages, for instance, at +30 mV subconductances were 450, 350, 860 pS while at −40 mV they were 650, 450, 1000 pS. This study was carried out in 150 mM KCl with 0.1 mM $CaCl_2$, while Kinnally *et al.* (1989) employed no addition of $CaCl_2$. A follow-up study (Szabo & Zoratti, 1991) found that this large-conductance activity characterized by multiple subconductance states, for which they coined the term 'mitochondrial megachannel' (MMC), was inhibited by 100–200 nM Cyclosporin A, a known inhibitor of the mPTP (Broekemeier, Dempsey & Pfeiffer, 1989). The kinetic features of the single-channel events supported the idea that the MMC is composed of cooperating subunits. Further properties of the MMC were characterized in a subsequent study, showing that the MMC was non-selective, activated by Ca^{2+}, and inhibited by Mg^{2+}, Cyclosporin A, and ADP, probably acting at matrix-facing sites (Szabo & Zoratti, 1992). It then was shown that Mg^{2+}, Mn^{2+}, Ba^{2+}, and Sr^{2+} and Cyclosporin A act as competitive inhibitors for Ca^{2+} (Szabo, Bernardi & Zoratti, 1992), and that the MMC is regulated by pH in the physiological range. Lower pH values caused MMC closure in a Ca^{2+}-reversible manner. For example, MMC activity that was blocked by pH 6.5 in the presence of 0.5 mM Ca^{2+} could be reinstated by 1.2 mM Ca^{2+}. Patch-clamp experiments were carried out using a so-called 'inside-out' configuration in which the matrix side of the membrane patch is exposed to the medium. Together, these patch-clamp results led to the conclusion that the modulating sites involved in these effects are located on the matrix side of the IMM. Bernardi *et al.* (1992) provided evidence that the Ca^{2+}-induced mPT is affected by the above agents, supporting the identification of the MMC as responsible for mPT. This pharmacological characterization of the MMC/PTP was set aside in subsequent studies in which the voltage dependence of the megachannel was re-investigated (Szabo & Zoratti, 1993). It was noted that the closed state(s) of the MMC was favoured at negative (physiological) transmembrane potentials. MMC conductance was 4.35 nS in symmetrical 0.5 M KCl with gating events involving a flickering half-size conductance (2.2 nS), which corresponded to that of the fully open VDAC in these conditions (Szabo, de Pinto & Zoratti, 1993). This led to the proposal that the MMC consisted of two cooperating porin (VDAC) molecules. Beutner *et al.* (1996) then characterized high molecular weight complexes isolated by Triton X-100 extraction from rat brain homogenate. These complexes were tested with specific antibodies and contained hexokinase, creatine kinase, VDAC, and ANT. After incorporating artificial bilayers, channel activity of 6 nS in 1 M KCl with an asymmetrical voltage dependence was recorded.

A claim that ANT was responsible for the mPTP was made a couple of years earlier (Tikhonova *et al.*, 1994). In their studies, purified ANT was incorporated into liposomes and fused into black lipid membranes (BLM) in the presence of 800 mM urea. Channel activity was induced by mersalyl. They observed a range of conductances from 200 pS to 2.2 nS in 180 Na_2SO_4, 20 mM 2-(N-morpholino)ethanesulfonic acid (MES)-NaOH, pH 7.0, in the presence of 3 mM Mg^{2+}. Involvement of ANT in MMC activity was also proposed in other studies (Brustovetsky & Klingenberg, 1996; Brustovetsky *et al.*, 2002). Brustovetsky & Klingenberg (1996) used bovine heart ANT, purified and reconstituted into giant membrane vesicles. Large conductance channels of 300–600 pS [in buffer containing 100 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 4 mM potassium gluconate, 5 mM MES, and 5 mM trisaminomethane (Tris) at pH 7.2] were observed. These channels exhibited low cation selectivity ($PK^+/PCl^- = 4.3 \pm 0.6$), were activated by Ca^{2+} (1 mM), inhibited by protons (pH 5.2), and by a combination of bongkrekate and ADP. Channel closing was induced at

Fig 4. Mitochondrial permeability pore direct and indirect regulators (inducers and inhibitors). ABT-737, 4-[4-[[2-(4-chlorophenyl) phenyl]methyl]piperazin-1-yl]-N-[4-[[(2R)-4-(dimethylamino)-1-phenylsulfanylbutan-2-yl]amino]-3-nitrophenyl] sulfonylbenzamide; AntiOXBen 3, 5–(6-(3,4,5-trihydroxybenzamido)hexylamino) carbonylpentyl]triphenylphosphonium bromide; AUL-12, AuIIIBr2(ethylsarcosinedithiocarbamate); B4G2, 23-hydroxybetulinic acid derivative; CO donors, e.g. transition metal carbonyls; CsA, Cyclosporin A; DIDS 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; EM20-25, 5-(6-chloro-2,4-dioxo-1,3,4,10-tetrahydro-2H-9-oxa-1,3-diaza-anthracen-10-yl)-pyrimidine-2,4,6-trione; Ginsenoside Rd, 2-O-β-D-glucopyranosyl-(3β,12β)-20-(β-D-glucopyranosyloxy)-12-hydroxydammara-24-en-3-yl-β-D-glucopyranoside; GNX-4728, substituted cinnamic
(Figure legend continues on next page.)

extreme voltages. In a follow-up study, recombinant ADP/ATP carrier (rAAC) from *Neurospora crassa* was expressed in *Escherichia coli* (Brustovetsky *et al.*, 2002). Purified rAAC was reconstituted and its activity recorded by patch-clamp. Its behaviour was similar to that observed for ANT from bovine heart. In addition, it was shown that cyclophilin isolated from *Neurospora crassa* suppressed channel gating, thus increasing channel open probability, while Cyclosporin A abolished the cyclophilin effect. When ADP was applied to cyclophilin-activated channels it induced flickering of the channel, effectively decreasing channel open probability. By contrast, channel gating was diminished by the pro-oxidant tert-butyl hydroperoxide (Brustovetsky *et al.*, 2002).

Although attempts to characterize the mPTP by means of various assays, such as mitochondrial swelling or mitochondrial Ca^{2+} accumulation were carried out at this time, only a few electrophysiological studies on the MMC were published. In one such study it was observed in patch-clamp experiments with rat liver mitochondria that ubiquinone 0 and decylubiquinone inhibited the activity of the MMC, in line with earlier observations for the mPTP (Fontaine, Ichas & Bernardi, 1998). Inhibition by these compounds was reversed by increasing [Ca^{2+}], a similar behaviour to that observed for several other MMC inhibitors. Classical MMC activity was observed in human hepatoma HepG2 cells. This channel had a high conductance of 1.23 nS (in 150 KCl), and frequently occupied a 640 pS sub-conductance level; it was active at high (1 mM) and closed at low (1 μM) Ca^{2+} and was inhibited by 10 μM Cyclosporin A (Loupatatzis *et al.*, 2002). Biochemical studies suggested that Bax cooperates with ANT in apoptotic events and thus was likely to be a component of the mPTP (Marzo *et al.*, 1998). However, other work (Campello *et al.*, 2005) showed that the activity of the MMC does not require Bax. In this study, using the human HCT116 cancer cell line, the MMC was found in 10–20% of patches from both Bax^+ and Bax^- cells, indicating that the mPTP was independent of the presence of Bax. MMC activity was recorded in 150 mM KCl, 0.5 mM $CaCl_2$, 1 mM Pi, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.35, and under these conditions had a conductance of about 1 nS (range 0.9–1.3 nS) with a hallmark flickering substate of about half this size. Weakly anionic or no selectivity ($PCl^-/PK^+ = 1.8$) for the high conductance state, and slight cationic selectivity ($PK^+/PCl^- = 2.8$) for the low conductance state was observed.

It was known that Cyclosporin A binds CypD, as described above. However, it was not clear whether CypD was a component of the pore itself. To solve this question, MMC channel properties from liver mitochondria from wild-type and CypD-deleted mice were compared in detail (De Marchi *et al.*, 2006). The pores observed in the two cases were indistinguishable, with the only clear difference being their sensitivity to Cyclosporin A. It was therefore concluded that CypD is a modulatory component of the PTP but is not part of the MMC pore (De Marchi *et al.*, 2006).

The work of Giorgio *et al.* (2013) proved that electrical mPTP activity was related to ATP synthase dimers. First, they identified the activity of monomers and dimers of ATP synthase after separation of mitochondrial proteins using blue native electrophoresis. Second, these gel-purified ATP synthase monomers or dimers, devoid of ANT, VDAC, and CypD, were incorporated into planar lipid bilayers made of purified soybean azolectin and channel activity was recorded. When the experimental medium contained 50 mM KCl, 1 mM Pi, and 0.3 mM Ca^{2+} no channel activity was observed when either monomer or dimer of ATP synthase were added. However, the addition of Bz-423, a proapoptotic agent (Boitano *et al.*, 2003) that was previously shown to target ATP synthase (Johnson *et al.*, 2005), elicited channel activity only when dimers but not monomers of ATP synthase were added to the bilayer. Bz-423 elicited similar activity in the presence of PhAsO, a sensitizer of the mPTP to Ca^{2+} (Krauskopf *et al.*, 2006). This channel activity could be inhibited by γ-imino ATP (AMP-PNP), a nonhydrolyzable ATP analog, and by ADP in the presence of Mg^{2+} ions; ADP also exhibited a partial inhibitory effect when present alone. Channel activity was not inhibited by Cyclosporin A, in agreement with the absence of CypD in the preparation and the fact that ATP synthase dimer was extracted in the presence of 10 mM Pi, which sensitizes the mPTP even in the absence of CypD. Channel opening was still observed in the presence of bongkrekic acid and could not be elicited by atractyloside, a selective inhibitor of ANT. These results have been further confirmed by recording the MMC activity using a highly purified preparation of bovine ATP synthase dimers (Urbani *et al.*, 2019).

Further evidence for the role of ATP synthase dimers in the formation of the pore came from the planar bilayer recordings of blue native polyacrylamide gel electrophoresis (BN-PAGE) purified yeast F_1/F_O-ATP synthase (Carraro *et al.*, 2014). The ATP synthase dimers did not elicit currents unless Ca^{2+}, PhAsO, and $Cu(OP)_2$ were added. Moreover, the channel activity was inhibited by Mg^{2+}+ ADP as found for the mammalian ATP synthase dimer. The unitary conductance of the channels formed by ATP synthase dimers

(Figure legend continued from previous page.)
anilide; JM-20, 3-ethoxycarbonyl-2-methyl-4-(2-nitrophenyl)-4,11-dihydro-1H-pyrido[2,3-b][1,5]benzodiazepine; MitoQ, 10-(6′-ubiquinonyl)decyltriphenylphosphonium bromide; $O_2^{\bullet-}$, superoxide anion; Pi, inorganic phosphate; PK11195, 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide; SfA, Sanglifehrin A; TMD#7538, N-phenethyl-6-phenyl-2, 3, 4, 9-tetrahydro-1H-carbazol-1-amine; TRO40303, 3,5-seco-4-nor-cholestan-5-one oxime-3-ol; $\Delta\Psi$, mitochondrial transmembrane potential.

ranged from 250 to 300 pS, and thus was lower than the conductance observed for the mammalian MMC. It should be noted that the dimer preparation did not contain translocase of outer mitochondrial membrane 20 (Tom20) or translocase of inner membrane 54 (Tim54) and therefore, that channel activity could not be due to the twin-pore translocase (Rehling *et al.*, 2003). The same approach showed that dimers of *Drosophila* Fo-ATPase form channels opened by Ca^{2+}, Bz-423, PhAsO, and $Cu(OP)_2$ with a single-channel conductance of only 53 pS (in 100 mM KCl) (von Stockum *et al.*, 2015).

This sequence of papers thus implicated ATP synthase dimers as the molecular entity responsible for mPTP activity. A subsequent study (Alavian *et al.*, 2014) showed that the purified human Fo subunit of ATP synthase alone, which is formed by an octameric ring of c-subunits (see Fig. 3D, E), when reconstituted into liposomes exhibited channel activity. This channel had a high conductance of around 100–300 pS, 500–750 pS, 1500 pS, and 1800–2000 pS and resembled the activity of the MMC. It was unselective with a permeability ratio $Na^+/K^+ = 1.5$. AMP, ADP, and ATP inhibited this c-subunit activity, and for ATP the half maximal effective concentration (EC50) was found to be 660 μM. The same study also determined the EC50 for ATP on MMC activity recorded in submitochondrial vesicles (SMVs), which was found to be lower by at least an order of magnitude (50 μM). An anti-pan-c-subunit antibody that inhibits the activity of the c-subunit ring also inhibited MMC activity induced by Ca^{2+} in SMVs. In contrast to purified ATP synthase monomers, the activity of the c-subunit was unaffected by Ca^{2+}. To demonstrate further that the c-subunit ring could create a pore, they substituted up to four highly conserved glycines within the first alpha-helical region of the c-subunit with valines, under the assumption that this would interfere with the tight packing of the c-subunit molecules within the ring structure. When reconstituted into liposomes, these mutants all demonstrated increased single-channel conductance compared to that of the wild-type, with the conductance of the quadruple mutant being the largest (Alavian *et al.*, 2014). This channel was also shown to be insensitive to blocking by ATP. They then hypothesized that F_1 binding to the c-subunit ring would inhibit the channel's activity, and applied purified individual F_1 proteins to reconstituted active c-subunit channels. Curiously, only the β subunit but not γ, δ, or ε subunits of ATP synthase had an inhibitory effect. Alavian *et al.* (2014) tested further the channel activity and regulation in the following preparations: (*i*) purified recombinant c-subunit lacking CypD and OSCP reconstituted into proteoliposomes (neither Ca^{2+} nor Cyclosporin A had an effect on channel activity); (*ii*) purified ATP synthase monomers containing OSCP but lacking CypD reconstituted into proteoliposomes (infrequent channel activity was observed, strongly enhanced by the addition of recombinant CypD protein either in the presence or absence of Ca^{2+}; this activity was inhibited by Cyclosporin A); (*iii*) mitochondria and SMV containing endogenous CypD and OSCP, and SMV exposed to urea to denature and remove extramembrane proteins, including F_1 components such as the OSCP, β subunit, and CypD (the activity of mitochondria and SMVs was regulated by Ca^{2+} and Cyclosporin A, but was completely absent from the urea-exposed SMVs; 1 mM ATP was still able to inhibit the activity of the channel). Mnatsakanyan *et al.* (2019) confirmed these initial conclusions by recording megachannel activity of purified porcine monomeric ATP synthase. Altogether, these patch-clamp experiments in which channel activity was recorded from reconstituted highly purified protein complexes indicated in a compelling way that ATP synthase, either as a dimer or as a monomer, is responsible for MMC activity.

A considerable controversy then arose as a result of studies showing that HAP1-A12 cells incapable of producing the ATP synthase c-subunit still show mPTP activity as measured by Ca^{2+} retention capacity of mitochondria (He *et al.*, 2017*b*). Recently, mitochondria derived from this cell line were further investigated by the patch-clamp technique (Neginskaya *et al.*, 2019). In contrast to the mitochondria of the wild-type HAP1 cells in which classic MMC activity of 1.3 ± 0.2 nS with a subconductance state of 0.4 ± 0.04 nS was detected, the mitochondria of HAP1-A12 cells contained a channel of a much smaller conductance of 0.3 ± 0.07 nS with a subconductance state of 0.13 ± 0.03 nS. Curiously, this channel was blocked by Cyclosporin A but also partially by ADP and bongkrekic acid. Similar features, including conductance and sensitivity to ADP and bongkrekic acid, were previously described for a purified ANT converted to a channel by Ca^{2+} treatment (Brustovetsky & Klingenberg, 1996) indicating that in the absence of a mPTP its function could be substituted by ANT.

Doubts about the involvement of the c-subunit in mPTP activity were replicated for other subunits, excluding from this phenomenon contributions of both subunits of the peripheral stalk of ATP synthase and of the whole enzyme once assembled. To reach these unexpected conclusions, clones were generated from HAP1-A12 cells *via* the disruption of *ATP5F1* and *ATP5O* genes encoding respectively for subunits b and OSCP (He *et al.*, 2017*a*). In both cases, the properties of the mPTP appeared unaltered, still opening following stress stimulation and being inhibited by Cyclosporin A, refuting a role of OSCP as an interactor between the mPTP and CypD. Using the same approach and in the same cell line, subunits e, f, g, 6.8PL and DAPIT were removed (Carroll *et al.*, 2019), leading to similar conclusions. Disruption of these proteins inevitably causes defects in ATP synthase assembly, thus arguing against the involvement of ATP synthase dimers in mPTP activity.

Nevertheless, a stronger case for the involvement of ATP synthase in MMC activity came from a study in which the activity of a single amino acid mutant of ATP synthase was studied by patch-clamp (Antoniel *et al.*, 2018). It was known from earlier work that MMC activity was blocked by protons (Szabo *et al.*, 1992). Antoniel *et al.* (2018) investigated whether a unique histidine in the OSCP subunit of the ATP synthase is important in this blockage by acidic pH. MMC activity from wild-type and from the OSCP H112Q mutant cells

was recorded in a standard symmetrical solution of 150 mM KCl, 0.1 or 0.2 mM $CaCl_2$, 10 mM Hepes, pH 7.3. Both channels exhibited a maximal conductance of 1 nS and several subconductance states with a prevalent substate of around 500 pS in agreement with previous observations for MMC activity. As observed previously for wild-type ATP synthase, acidification of the bath to pH 6.5 resulted in almost complete MMC activity inhibition. However, in mitoplasts from OSCP H112Q cells, a decrease in pH to 6.5 did not cause considerable changes to open probability but the MMC was still sensitive to the classical mPTP inhibitor Ba^{2+} (Szabo *et al.*, 1992).

Detailed information about the electrophysiological properties of the mPTP have recently been reviewed (Neginskaya, Pavlov & Sheu, 2021). This seminal paper discusses the evidence claiming that patch-clamp investigations can (*i*) discern among different mPTP pathways occurring in a given genetic or biochemical condition, and (*ii*) be used to understand the contribution of proteins or drugs in a highly controlled biophysical system.

V. THE mPTP – FROM MITOCHONDRIAL FRACTIONS TO INTACT CELLS

Since the 1990s, several methods have been developed to study the opening of the mPTP in intact cells, including cell imaging, the use of fluorescent dyes, and pharmacological inhibition of the mPTP (Petronilli *et al.*, 1998; Bonora *et al.*, 2016) (see Fig. 5). Depolarization of the mitochondrial transmembrane potential is a recognized consequence of the mPT (Petronilli *et al.*, 1993; Zamzami *et al.*, 1996), and thus several studies have measured variations in the mitochondrial transmembrane potential as an indicator of the opening of the mPTP (Fig. 5A) (Huser & Blatter, 1999; Rama Rao, Jayakumar & Norenberg, 2003; Briston *et al.*, 2017). However, since many other events besides the mPT can induce alterations in the mitochondrial transmembrane potential (including mitochondrial metabolism *per se*, lipid peroxidation, ion cycles, or activity of uncoupling proteins), it is unlikely that this is a useful method to investigate mPTP opening. Peroxidation of lipids within mitochondrial membranes can induce configurational changes that will alter membrane properties, including a progressive increase in membrane permeability, and consequently, depolarization of the membrane potential (Stark, 1991; Wong-Ekkabut *et al.*, 2007). When inducing the mPT with oxidant agents, one thus must eliminate artifacts caused by non-specific alterations in mitochondrial membrane permeability. Furthermore, transient opening of the mPTP is very difficult to follow by measuring the MMP using fluorescent dyes. The collapse of the MMP is not systematic and to detect measurable changes in the distribution of fluorescent dyes, extended periods of PTP opening are usually required (Petronilli *et al.*, 2001; Dumas *et al.*, 2009). Also, the use of Cyclosporin A as an inhibitor of mPT-induced mitochondrial depolarization can be unreliable, as Cyclosporin A also inhibits calcineurin in cells, which can cause artifacts. FK-506 (tacrolimus), which inhibits calcineurin but not the mPTP (Rodrigues-Diez *et al.*, 2016), or mitochondrial-directed Cyclosporin A (Malouitre *et al.*, 2009) can be used to account for the potential lack of specificity of Cyclosporin A in intact cells. For this reason, other approaches to measure mPTP opening *in situ* should be employed to complement the information obtained by measurement of MMP.

The 2-deoxyglucose method was developed to follow mPTP opening in intact cells (Fig. 5B). While there was indirect evidence that pore opening occurred during the reperfusion of hearts, there was a need for a method that would provide direct evidence of mPTP opening at critical time points during the reperfusion phase. Griffiths & Halestrap (1995) developed an elegant methodology to follow the opening of the mPTP that used 2-deoxy[^{3}H]glucose, which enters the cell through the glucose transporter and is phosphorylated to 2-deoxy[^{3}H]glucose-6-phosphate. This metabolite cannot be further metabolized and thus is trapped in the cell. However, it can not cross the mitochondrial inner membrane unless the mPTP is in an open state. Once the mPTP opens, 2-deoxy[^{3}H]glucose-6-phosphate enters the matrix of mitochondria. Subsequent treatment of isolated mitochondria with ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) will chelate Ca^{2+}, allowing the mPTP to close, trapping 2-deoxy[^{3}H]glucose-6-phosphate in the matrix. The measurement of disintegrations per minute (d.p.m.) in isolated mitochondrial fractions allows calculation of the uptake of 2-deoxy[^{3}H]glucose-6-phosphate by mitochondria and, therefore, demonstrates that the pore opened during that period. Since this method was developed, it has been used in several settings (Kerr, Suleiman & Halestrap, 1999; Rama Rao *et al.*, 2003; Ayoub, Radhakrishnan & Gazmuri, 2017).

Nieminen *et al.* (1995) developed a different fluorescent method using the fluorescent dyes calcein-acetoxymethyl (AM) and tetramethylrhodamine methyl ester (TMRM) to monitor the mPT in intact cells (Fig. 5C). Calcein is a hydrophilic fluorescein derivative, that when esterified with an AM group, acquires the ability to cross the cellular membrane and become entrapped in a non-fluorescent form. Once in the cytosol, intracellular esterases hydrolyse the AM group and the trapped calcein becomes fluorescent. Cleavage of AM moieties is a widely used strategy to entrap fluorescent probes inside cells. TMRM is a positively charged cell-permeant fluorescent compound that once inside the cell is sequestered by mitochondria, depending on the MMP. Nieminen *et al.* (1995) demonstrated that when cells are loaded with calcein at 37°C, this fluorescent dye accumulates predominantly in the cytosol, while mitochondria appear as dark spots when using confocal microscopy to image calcein fluorescence. However, due to its positive charge, TMRM accumulates in active mitochondria; hence the calcein-unlabelled dark spots now appear labelled with TMRM fluorescence. Once the mPTP opens, mitochondria lose TMRM fluorescence and the dark spots become filled with fluorescent

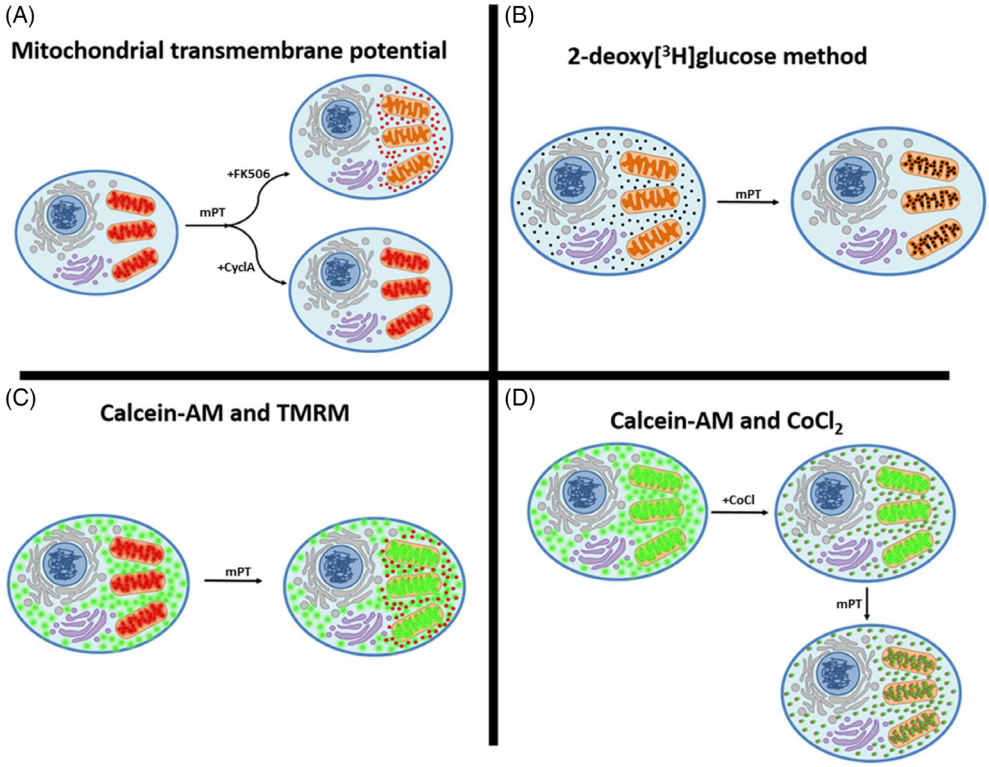

Fig 5. Techniques used to evaluate the mitochondrial permeability transition (mPT) in intact cells. (A) Evaluation of mitochondrial permeability transition pore (mPTP) opening by using a mitochondrial transmembrane potential ($\Delta\Psi$)-dependent dye such as tetramethylrhodamine methyl ester (TMRM) is based on its accumulation in mitochondria with a closed mPTP, in which the electric gradient is maintained. Because mPTP opening is not the only mechanism for loss of $\Delta\Psi$, proper controls need to be performed with Cyclosporin A (CyclA), a pore inhibitor. However, because this compound is not specific to cyclophilin D since it also inhibits calcineurin, a proper control with other calcineurin inhibitors is also needed (e.g. tacrolimus, also known as FK506). This method can be used with a fluorescent plate reader, flow cytometry or fluorescent microscopy. (B) A more specific method involves using 2-deoxy[^{3}H]glucose, which is retained in cells after phosphorylation, although it does not permeate intact mitochondria. Upon mPTP opening, it can permeate the mitochondria. Isolation of mitochondria and quantification of radioactivity in a scintillation counter allows measurement of the extent of mPTP opening. Although specific, this is a complex and often time-consuming and highly technical methodology. (C) The calcein-acetoxymethyl (AM) method involves loading cells with the dye under specific temperature conditions, in conjugation with TMRM. After cleavage of the AM moiety by esterases, green-fluorescent calcein labels the cytosol, while red-fluorescent TMRM labels polarized (intact) mitochondria. After mPTP opening, mitochondrial TMRM labelling is lost, while calcein now permeates the mitochondria. The most reliable methodology to follow these events is fluorescent microscopy. (D) An adaptation of the calcein-AM method in which cobalt chloride ($CoCl_2$) is used to quench calcein fluorescence. In this protocol, calcein is spread throughout the cell, including in mitochondria. As Co^{2+} does not permeate mitochondria, $CoCl_2$+calcein-treated cells show dark cytosol and green mitochondria. Upon mPTP opening, Co^{2+} permeates the mitochondria, and the green mitochondrial fluorescence is lost. This method can be used with a fluorescent plate reader, flow cytometry or fluorescent microscopy. In all methods, proper controls with mPTP inhibitors (as well as negative controls) must be performed to avoid artifacts.

calcein. By using laser-scanning confocal microscopy, this method allowed the authors to monitor the mPTP in intact hepatocytes after exposure to t-butylhydroperoxide (Nieminen *et al.*, 1995). Their method was, however, challenged by Petronilli *et al.* (1998) who pointed out caveats and advised caution in interpreting the results. Since the esterified form of calcein (calcein-AM) can cross intracellular membranes, diffusion among the different organelles can also occur and it may label other cell spaces beside the cytosol (Petronilli *et al.*, 1999). The esterified form of calcein can also be cleaved by mitochondrial esterases and the fluorescent calcein may become trapped inside the mitochondrial matrix. Regarding the dark spots observed by Nieminen *et al.* (1995) in confocal microscopy images of calcein fluorescence, Petronilli *et al.* (1998) suggested that TMRM could quench calcein fluorescence, or that the concentration of calcein inside the mitochondrial matrix could reach values high enough to cause calcein self-quenching. To overcome the flaws of this method, Petronilli *et al.* (1998, 1999) suggested loading cells with calcein-AM in the presence of 1 mM of $CoCl_2$ (Fig. 5D). In the presence of Co^{2+}, calcein fluorescence in the cytosol and nucleus is quenched, and because

Co^{2+} does not cross the mitochondrial inner membrane, mitochondria appear as green-fluorescent bodies against a dark background. Under a condition promoting mPTP opening, calcein can exit the mitochondria and Co^{2+} can flow to the mitochondrial matrix. A decrease in calcein fluorescence intensity in the mitochondrial matrix can be measured by fluorescence microscopy or in a regular multi-plate-based fluorescence assay. This method proved to be a useful tool for the *in situ* study of mPTP modulation and it available in the form of commercial kits.

As described in Section III, mitochondrial swelling is a valuable method to study mPTP opening in isolated mitochondria. However, the observation of mitochondrial swelling in intact cells after mPTP opening is still somewhat controversial. While some authors argue that morphological alterations observed in mitochondria in intact cells result from mitochondrial swelling (Minamikawa *et al.*, 1999), others believe that, in intact cells, mitochondrial swelling does not occur immediately after mPTP opening because of the presence of proteins in the cytosol that may inhibit osmotic water entry into mitochondria (Dumas *et al.*, 2009). Thus mitochondrial swelling is not a uniformly accepted end-point for measuring mPTP opening in intact cells.

The evaluation of the mPT in intact cells thus is not a straightforward process and remains subject to confounding factors and artifacts. There is still no single reliable method to measure *in situ* the dynamic of the mPT. Further difficulties arise when attempting to measure the low-conductance state of the mPTP, because most of the available methods are designed to evaluate its high-conductance form. Thus, for maximum reliability, a combination of different methods is required to evaluate mPTP dynamics in intact cells.

VI. THE mPTP IN CELL DEATH AND PATHOLOGY

As described in Section III, mPTP opening can lead to mechanical stress in the OMM due to swelling, leading to its rupture. It is of interest to consider whether this is a common phenomenon that takes place due to solute distribution between mitochondria and the cytosol in intact cells and tissues, or whether it is affected by the number of calcium-induced pores in a single mitochondrion. Neginskaya *et al.* (2020) calculated that the number of mPTP structures per mitochondrion was between one and nine, although they noted that this may be a possible underestimate. In any case, the mPTP is a central mechanism involved in cell death. Mitochondrial membrane depolarization resulting from mPTP opening leads to a deficit in mitochondrial ATP, a condition generally associated with necroptosis (Bauer & Murphy, 2020) or classical necrosis (Lemasters, 1999). Induction of the mPTP is also involved in Bax recruitment to mitochondria, an event that represents one of the first steps in the mitochondrial apoptosis pathway (Narita *et al.*, 1998; Precht *et al.*, 2005). The binding of Bax to the ANT increases IMM permeability and triggers cell death (Marzo *et al.*, 1998). It was also shown that the mPTP directs Bax translocation and multimerization in the OMM (De Giorgi *et al.*, 2002). This phenomenon was observed to be inhibited by binding of the anti-apoptotic protein Bcl-2 (Brenner *et al.*, 2000).

Further research demonstrated that mPTP induction is not always required for the apoptosis-inducing effects of Bax (Eskes *et al.*, 1998). Apoptosis caused by Bcl-2 homology 3 (BH3)-interacting domain death agonist Bid, a pro-apoptosis 'BH3-only' member of the Bcl-2 family, which is cleaved by caspase-8 and translocated to the OMM, was initially shown not to depend on mPTP opening or Bax translocation (Kim *et al.*, 2000), although later studies contradicted this (Zamzami *et al.*, 2000). One mechanism to explain these differences may involve the concentration of calcium, which in low concentrations may induce cytochrome c release without mPTP induction, whereas higher concentrations can activate the mPTP and trigger cytochrome c release (Gogvadze *et al.*, 2001).

Increased mPTP induction is associated with several pathological conditions. This is not surprising given its roles in cellular bioenergetic dysfunction and cell death and its association with redox and ionic disruption. We provide below a brief description of selected research in which the mPTP has been recognized as part of a pathophysiological mechanism.

The mPTP is a recognized mediator of neuronal death in several neurodegenerative diseases including amyotrophic lateral sclerosis (ALS) (Sun *et al.*, 2019), Parkinson's disease (Iravanpour *et al.*, 2021), hypoxic–ischemic encephalopathy (Chen *et al.*, 2021), and Alzheimer's disease (Du *et al.*, 2008) in different animal models. Interestingly, increased activation of the mPTP in Alzheimer's disease was recorded even in non-neuronal tissues such as skin fibroblasts from patients (Perez *et al.*, 2018).

Cardiac injury has been frequently associated with an increased activity of the mPTP. Anthracycline-induced cardiomyopathy, a pressing concern in anti-cancer therapy, has an important mitochondrial component with increased mPTP activation mediated by oxidative stress and CypD signalling (Oliveira *et al.*, 2004; Dhingra *et al.*, 2020; Wallace, Sardao & Oliveira, 2020). CypD hyperacetylation appears to be involved in increased mPTP opening during heart failure (Castillo *et al.*, 2019).

It is known that CypD gene silencing is protective against ischemic renal injury in a mouse model (Devalaraja-Narashimha, Diener & Padanilam, 2009). The protective effect of silencing CyPD and that of Cyclosporin A both evidence the role of the mPTP in kidney injury. Indirect evidence for kidney cell injury caused by mPTP opening is provided by Bendavia, a mPTP inhibitor, which inhibits damage caused by revascularization in experimental renal artery stenosis (Eirin *et al.*, 2012).

A recent study showed that hepatic steatosis in a mouse model was associated with mitochondrial swelling and depolarization, two mPTP-linked outcomes that were inhibited by Cyclosporin A (Li *et al.*, 2021). This result confirmed previous findings showing that CypD inhibition improved fatty liver in animal models (Wang *et al.*, 2018).

Aging is associated with progressive alterations in mitochondrial function, including altered mPTP regulation. Zhou *et al.* (2019) showed that reduced mPTP opening increases the lifespan of *Caenorhabditis elegans*. Their work also suggested an essential link between mPTP opening, IMM permeability, and autophagy in the regulation of yeast and mammal healthy aging (Zhou *et al.*, 2019). An earlier study showed that mitochondria from older (36-month-old) animals had a higher response to Ca^{2+} in terms of mPTP induction compared to younger (1-month-old) animals (Goodell & Cortopassi, 1998).

Questions still remain on how over-activation of the mPTP occurs in these and other pathologies. Increased oxidative stress is an obvious explanation, a condition that could be exacerbated by prolonged mPTP opening. Prolonged mPTP opening leads to mitochondrial depolarization, increased ROS production, and triggers cell death signalling, leading to tissue injury. Nevertheless, the situation is likely to be more complex. Some of the examples above include a role of CypD in potentiating mPTP opening, including post-translational modifications such as acetylation, resulting from the metabolic stress associated with different pathologies.

VII. NOT ALWAYS A BAD GUY: THE PHYSIOLOGICAL ROLES OF THE mPTP

In addition to the known pathological results of mPTP opening, it has been linked to diverse normal physiological processes, such as cell fate and differentiation (Hom *et al.*, 2011; Folmes *et al.*, 2012; Vega-Naredo *et al.*, 2014). Several studies have addressed the importance of mPTP regulatory molecules under physiological conditions that prevent the cell from deleterious changes to mitochondrial functions that could lead to cell death.

In the context of a physiological role of the mPTP, two possible forms of its open state were proposed: a full-conductance irreversible opening for permanent permeability, or an alternative transient and flickering short-term opening of the mPTP (Wang *et al.*, 2008; Hou *et al.*, 2014; Li *et al.*, 2020) (Fig. 6). The full-conductance open state leads predominantly to apoptosis and cell death, while the transient short-term open state with smaller and more variable conductance is likely to function during physiological conditions (Perez & Quintanilla, 2017).

Evidence for such physiological opening of the mPTP has been described, including a flickering opening activity (Crompton, 1999; Hausenloy *et al.*, 2004, 2010; Korge *et al.*, 2011); furthermore, an association between transient mPTP opening and 'superoxide flashes' was observed in striated muscle mitochondria (Wang *et al.*, 2008). Based on the suggestion that this transient opening of the mPTP may release mitochondrial matrix Ca^{2+} to maintain mitochondrial homeostasis, a model was proposed for a physiological function of the mPTP, in addition to its well-known role in cell death (Elrod *et al.*, 2010). The most prominent physiological role for the mPTP in mitochondrial and cellular Ca^{2+} homeostasis thus is apparently its capacity to act as a Ca^{2+} efflux mechanism (Altschuld *et al.*, 1992; Takeuchi *et al.*, 2015; Biasutto *et al.*, 2016; Hurst *et al.*, 2017; Krebs, 2017; Li *et al.*, 2020; Xu *et al.*, 2020).

Evidence for the transient opening of the mPTP as a physiological Ca^{2+} efflux pathway includes early demonstrations of the inhibition of Ca^{2+} release in the presence of mPTP inhibitor Cyclosporin A in isolated adult rat ventricular cardiomyocytes (Altschuld *et al.*, 1992). Transient or low-conductance opening of the mPTP was proposed to serve as an additional mode of Ca^{2+} efflux that mitigates sustained matrix Ca^{2+} overload (Ichas & Mazat, 1998; Bernardi & von Stockum, 2012). Numerous studies have supported this hypothesized physiological role (Elrod *et al.*, 2010; Korge *et al.*, 2011; Bernardi & von Stockum, 2012; Elrod & Molkentin, 2013; Gainutdinov *et al.*, 2015). However, in a recent study, mitochondrial Ca^{2+} efflux rates measured in intact HeLa cells were completely unaffected by mPTP inhibition either by Cyclosporin A or by small interfering RNA (siRNA)-mediated reduction of the ATP synthase c-subunit (De Marchi *et al.*, 2014*a*), suggesting that the mPTP may not always play a role in Ca^{2+} efflux under physiological conditions. Most experiments addressing the role of the mPTP in Ca^{2+} homeostasis use Cyclosporin A, which introduces inherent problems regarding its specificity. Notably, the inhibitory effect of Cyclosporin A depends on the expression level of CypD, which is rarely assessed (Bernardi *et al.*, 2015). Cyclosporin A inhibitory ability can vary significantly, with known differences in sensitivity to Cyclosporin A in different tissues; for example, NIH3T3 fibroblasts and HL60 cells show no sensitivity to this inhibitor (Li *et al.*, 2012). The relative expression of CypD and Fo-ATP synthase may also be crucial; cross-linking experiments in beef heart mitochondria indicate that there is much less CypD present than subunits b, d, and OSCP and that many Fo-ATP synthase channels are insensitive to Cyclosporin A even if CypD is expressed (Bernardi *et al.*, 2015). Thus the role of Cyclosporin A-sensitive/insensitive mPTP functions, particularly as a mechanism of mitochondrial Ca^{2+} efflux, remains unproven. Despite this continuing debate, most research does suggest that the mPTP appears to function as a Ca^{2+}-release mechanism required for proper metabolic regulation (Bernardi *et al.*, 2015).

Ca^{2+} remains the most important regulator and inducer of pore opening, given its numerous indirect roles regulating and modulating the mPTP (Biasutto *et al.*, 2016; Hurst *et al.*, 2017). At physiological levels, Ca^{2+} could stimulate transient opening of the pore, while Ca^{2+} overload changes the balance from physiology to pathology, leading to sustained mPTP opening with subsequent mitochondrial and cellular dysfunction (Hurst *et al.*, 2017; Mnatsakanyan *et al.*, 2017; Perez & Quintanilla, 2017; Nesci *et al.*, 2018; Lamb, 2020). Recent studies also highlighted a crucial role of the mPTP in cardiac, neurodegenerative and other pathologies and its involvement in cardiac and brain

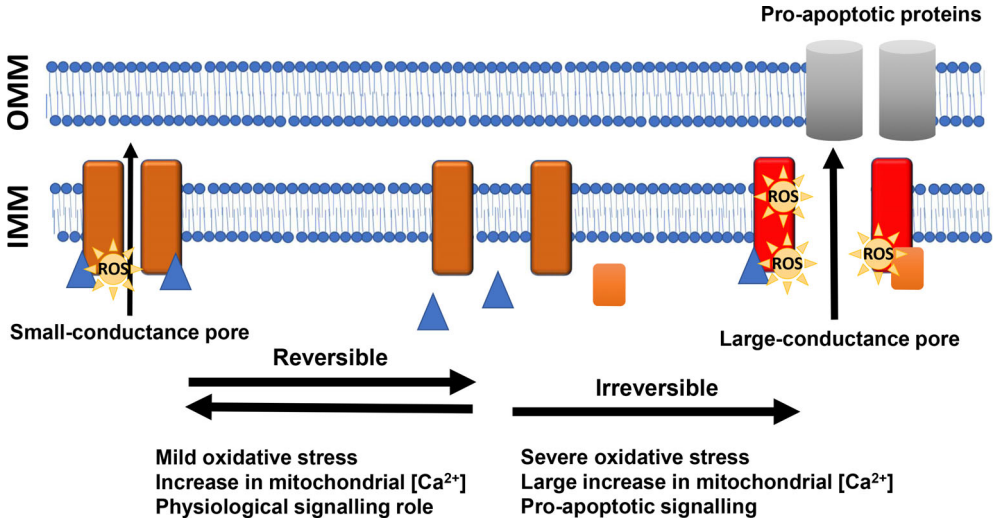

Fig 6. Reversibility of the mitochondrial permeability transition pore (mPTP). The mPTP has been described as undergoing different conformational changes, leading to alternative fates for the cell. Mild elevations in calcium and oxidative stress, as well as cytosolic signalling can trigger the formation of reversible low-conductance mPTP opening under conditions of normal mitochondrial functioning, and in particular during cell differentiation. Low-conductance mPTP opening can be useful to decrease mitochondrial membrane potential to avoid membrane hyperpolarization and excessive reactive oxygen species (ROS) production, as well as to discharge accumulated calcium. On the other hand, an excessive matrix calcium accumulation, together with persistent and elevated oxidative stress due to increased mitochondrial ROS production, as well as pro-apoptotic signalling may lead to the formation of an irreversible large-conductance form of mPTP opening, which can lead to bioenergetic collapse, and contribute to cell death. The small triangles and squares represent factors that regulate mPTP opening. One such protein factor is the matrix chaperon cyclophylin D (CypD), which has been previously shown to interact with putative pore components, including the ATP synthase. IMM, inner mitochondrial membrane; OMM outer mitochondrial membrane.

development (Folmes *et al.*, 2012; Perez & Quintanilla, 2017), neutrophil activation and ROS release (Vorobjeva *et al.*, 2020). The mPTP has been highlighted as a gating mechanism underlying differentiation in the developing heart and brain, potentially involving cross-talk between genetic and metabolic signalling (Folmes *et al.*, 2012). Transient mPTP opening directly regulates cellular energy metabolism as it uncouples oxidative metabolism from ATP synthesis, a mechanism that operates in concert with ROS flashes to promote cardiomyocyte differentiation (Folmes *et al.*, 2012). Knockout of the mPTP component cyclophilin D results in elevated mitochondrial matrix Ca^{2+}, enhancing the activation of Ca^{2+}-dependent dehydrogenases and thus reducing metabolic flexibility (Elrod *et al.*, 2010). It was demonstrated that mPTP opening in the early heart is a physiological event required for organ development. In the fetal heart, myocytes exhibit low MMP, high levels of ROS production, and opening of the mPTP. Inhibition of mPTP opening with Cyclosporin A led to maturation of mitochondrial structure and function, decreased intracellular ROS levels and increased MMP, which accelerated myocyte differentiation (Hom *et al.*, 2011).

Concerning the participation of the mPTP in neural development, it was reported that cultured embryonic mouse cortical neural progenitor cells demonstrate intermittent spontaneous bursts of mitochondrial superoxide generation that require a transient opening of the mPTP (Hou *et al.*, 2014). It was shown that both mitochondrial ROS scavengers and mPTP inhibitors such as Cyclosporin A reduced the frequency of mitoflashes and enhanced the proliferation of cortical neural progenitor cells, whereas prolonged mPTP opening and superoxide generation increased the incidence of mitoflashes and promoted differentiation of these neuronal cells (Hou *et al.*, 2012). The evidence that the mPTP participates in cardiac and brain development is thus convincing, but there remains much to understand regarding its roles in other cell types [although for haematopoietic progenitor cells and vascular progenitor cells see Arnold *et al.* (2000) and Davies *et al.* (2005)]. The physiological participation of the mPTP in cell development and differentiation appears to depend on the specific cell type, the subcellular localization of mitochondria and on the developmental stage (Mnatsakanyan *et al.*, 2017; Perez & Quintanilla, 2017). Transient pore opening has been shown to be associated with a transient depolarization of the MMP (Jou, 2011; Hurst *et al.*, 2017). While the exact mechanism responsible is still unclear, the physiological roles are beginning to be elucidated. The frequency of these transient openings has been associated with metabolism, aging, wound healing, and an essential role in cell differentiation (Shen *et al.*, 2014; Vega-Naredo *et al.*, 2014; Ding *et al.*, 2015).

VIII. THE ROAD TO THE CLINIC: FUTURE PERSPECTIVES IN mPTP REGULATION

The potentially dual physiological and pathological role of the mPTP has attracted attention in terms of drug discovery, with several pre-clinical trials already published.

The mPTP has been extensively studied as a target for therapies aimed at blocking ATP synthesis, especially that of pathogens causing infective pathologies in humans (Andries *et al.*, 2005). By binding the F_O-ATP synthase c-subunit and mycobacterial subunit ε, some antimicrobials can selectively and effectively eliminate strains of microorganisms that are resistant to conventional drugs. Another example of the use of F_1F_O-ATP synthase as a pharmacological target is 1,4-benzodiazepine (Bz-423), which induces a selective, ROS- and mPTP-dependent apoptotic cell death in B lymphocytes *via* its interaction with the OSCP subunit (Johnson *et al.*, 2005), the same site used by CypD to modulate mPTP. The discovery of Bz-423 has opened the way for potential treatments for autoimmune disorders such as lupus erythematosus.

Subunit c, together with CypD, have been defined as critical components of the mPTP. However, there are conceptual and technical difficulties in the use of both of these as targets for screening for inhibitors of mPTP opening: CypD is considered a regulator and not a pore component, thus, its targeting may only desensitize pore activity. Subunit c is part of the membrane-bound F_O portion (see Fig. 3D, E) and involved in proton translocation for ATP generation. Therefore, its inhibition may lead to unwanted side effects, as is the case for oligomycin, an antibiotic that targets the F_O component, and *N*,*N*-dicyclohexylcarbodiimide (DCCD), for example. Oligomycin is a natural macrolide that acts as a potent inhibitor of both the synthesis and hydrolysis of mitochondrial ATP. It is produced from *Streptomyces* and exists in six different isoforms, A to F, based on the R group attached to the macrolide. Oligomycin binds side chains of amino acids located on two consecutive c subunits and can inhibit 50% of mPTP opening in living cells at a concentration of 10 μM (Bonora *et al.*, 2017). DCCD is a lipid-soluble carbodiimide with strong inhibitory properties of both portions of F_1F_O-ATP synthase, depending on its use. At low concentrations (about 50 μM), it interacts covalently with the c-ring through an essential carboxyl amino acid (Asp61) of subunit c and inhibits mPTP opening by 45% if used in the range 7.5–15 μM; at higher concentrations, it also interacts with the F_1 portion through a glutamine residue in the ß subunit. However, the toxic nature of these drugs makes them unacceptable for use: despite significant inhibition of mPTP activity *in vitro*, they also deplete mitochondrial ATP, causing toxic side effects in more complex disease models.

Considering the importance of the c-ring in mPTP modulation, many efforts are ongoing to find less toxic c-subunit inhibitors. This goal could be achieved by identifying the essential core of a drug required to recognize subunit c, and adapting the surrounding chemical structure to minimize unwanted side effects while maintaining its inhibitory potential. A small-molecule library of c-subunit inhibitors has been obtained by modifying the oligomycin functional core, leading to new compounds that strongly reduce reperfusion damage in animal models of global ischemia without interfering with ATP production (Morciano *et al.*, 2018). For example, compound 10 inhibited mPTP opening *in vitro* by 40–50% at very low concentrations (0.5–1 μM) and reduced cardiac apoptotic cell death by 40% when administered during reperfusion for 10 min. Compound 10, together with compounds 5c and 6g, showed low toxicity, probably due to their exclusive localization in mitochondria and their reversible binding.

Danshensu (DSS), the main constituent of *Salvia miltiorrhiza* (Danshen), a traditional Chinese herb, provided substantial cardioprotection against myocardial ischemia/reperfusion injury as measured by cell viability loss, and creatine kinase-isozyme MB, cardiac troponin and lactate dehydrogenase (LDH) release. This cardioprotection was dependent of the modulation of subunit c protein (Yin *et al.*, 2013). DSS acted by downregulating subunit c messenger RNA (mRNA) and protein levels in reperfused rat hearts, and inhibited mPTP opening and the consequent cardiac ischemia/reperfusion injury, improving heart parameters and cardiomyocyte survival (Gao *et al.*, 2017).

Genetic studies in animals have highlighted the importance of CypD in mPTP-mediated cardioprotection by modifying its *Ppif* gene expression. CypD can be manipulated by a long list of drugs, almost all derived from Cyclosporin A. Cyclosporin A was first isolated from a fungus and entered clinical practice some years later; its binding to CypD is due to a tryptophan (Trp-121) within a short α-helical region of the protein (Davis *et al.*, 2010). Its excellent activity in inhibiting mPTP opening at low concentrations (0.2–1.2 μM) and its unquestionable results in *in vitro* and preclinical models, have made Cyclosporin A one of the most promising positive controls for assessing mPTP function. However, Cyclosporin A also has non-mPTP-related effects. Probably due to its diffuse localization pattern inside cells, in both cytosol (Youn *et al.*, 2002; Abikhair *et al.*, 2016) and nucleus (Le Hir *et al.*, 1995), Cyclosporin A can act as an immunosuppressant. Its incorporation into poly-lactic/glycolic acid (PLGA) nanoparticles (CsA-NP) allows better mitochondrial localization; treatment with CsA-NP at the time of reperfusion increased cardioprotection with a significant reduction in infarct size using lower concentrations compared to Cyclosporin A alone (Ikeda *et al.*, 2016). Cyclosporin A derivatives with a significantly decreased (several thousand-fold less) immunosuppressant effect have also been developed. Examples include N-methyl-isoleucine-4-cyclo-sporin (NIM-811) and N-methyl-D-alanine-3-N-ethyl-valine-4-cyclosporin (Debio025). NIM-811 and Debio025 are both semisynthetic analogs of CsA in which cytosolic side effects have been partially abolished by eliminating the calcineurin-binding motif (Waldmeier *et al.*, 2002; Hansson *et al.*, 2004). In assays targeting the mPTP induced with Ca^{2+} overload and Pi in both living cells and isolated mitochondria, NIM-811 showed the same potency as Cyclosporin A. The reduction in its immunosuppressant effects offers an additional advantage since Cyclosporin A has a limited window of action in terms of concentrations (0.2 to 1.2 μM). NIM-811 does not show the same toxicity. Debio025, a NIM-811 derivative, in a comparative study performed in isolated brain and heart mitochondria, showed a 10-fold more potent activity than Cyclosporin A. Multiple studies have reported beneficial effects of these two drugs in

reduced cell death, recovery of left ventricle contractile function, and improved survival (Gomez *et al.*, 2007, 2009; Cour *et al.*, 2011).

Despite the preclinical potential of its derivatives, only Cyclosporin A has so far entered clinical trials, which ended after 15 years with a phase III failure against cardiac reperfusion injury. Indeed, CIRCUS (Cung *et al.*, 2015) and CYCLE (Ottani *et al.*, 2016) trials, consisting of a single intravenous bolus of Cyclosporin A (2.5 mg/kg) before revascularization, showed no improvement in clinical outcome. These findings did not confirm the results of a pilot study (Piot *et al.*, 2008), which initially raised hope for the use of Cyclosporin A to treat reperfusion injury.

Another non-selective inhibitor of CypD is Sanglifehrin A (SfA) (Clarke, McStay & Halestrap, 2002). SfA is as potent as Cyclosporin A regarding mPTP opening; recovery of cardiac performance upon ischemia/reperfusion and a significant reduction of LDH release was observed after SfA treatment (Hausenloy *et al.*, 2003). Since SfA differs structurally from Cyclosporin A, the formation of calcineurin–SfA complexes is avoided, while the ability to bind CypD although at a different site, is maintained, giving this molecule discrete immunosuppressant activities. Similar to the effects of DCCD on ATP synthase subunit c, SfA strongly binds CypD, failing to detach upon washing. Together with its immunosuppressant activity, this may discourage its use in clinical practice, as prolonged residence inside cells may lead to side effects.

Small-size molecule inhibitors also exist for CypD, such as C-9 and C-19. C-9 was initially identified as a therapeutic agent to delay Alzheimer's disease symptoms by preventing the interaction between CypD and amyloid-beta (Aβ) to decrease mitochondria-dependent neuronal stress (Valasani *et al.*, 2014). C-9 was also found *in vitro* to show potential for the treatment of many other diseases such as acute pancreatitis, ultraviolet radiation damage, and in other neurodegenerative diseases, due to its mPTP-inhibiting properties. The most potent inhibitor in this category is C31 (Panel *et al.*, 2019), which was able to restore mitochondrial parameters following hepatic injury.

Suggested initially as mPTP regulator and most commonly known as the peripheral benzodiazepine receptor for its high affinity in binding benzodiazepines, TSPO has been proposed as a potent inducer of mPTP opening upon interaction with protoporphyrin IX (PPIX) (Pastorino *et al.*, 1994). Targeting of TSPO by 3,5-seco-4-nor-cholestan-5-one oxime-3-ol (TRO40303) showed promising cardioprotective effects in a rat model of cardiac ischemia; its administration prior to reperfusion reduced infarct size (IS) and concomitant cell death by about 40%. However, desensitization of mPTP opening seems to be secondary to its remarkable antioxidant properties (Schaller *et al.*, 2010). Further supporting an indirect mechanism in the modulation of mPTP, studies on TSPO KO mice have excluded the possibility that TSPO ligands, and TSPO itself, may regulate mPTP activity by showing that the presence of the protein in hearts subjected to ischemia and reperfusion was dispensable (Sileikyte *et al.*, 2014). The safety and efficacy of TRO40303 were evaluated in myocardial infarction patients undergoing percutaneous coronary intervention. This multicenter, double-blinded, phase II study (MITOCARE), in which TRO40303 was administered just before revascularization failed to show efficacy in reducing or limiting reperfusion injury (Atar *et al.*, 2015). In this study, infarct size, left ventricle (LV) ejection fraction evaluation, creatine kinase, and troponin I dosage did not differ between placebo and treated patients.

Investigations of the properties of the mPTP have also highlighted compounds derived from cinnamic anilide, such as GNX-4728 and GNX-4975. These molecules modulate the mPTP in a CypD- and subunit c-independent way, showing beneficial effects in an ALS transgenic mouse model (Martin *et al.*, 2014) with delayed onset of symptoms, increased lifespan, and reduced inflammation. It has been hypothesized that GNX-4975 shares the same binding site as calcium in mPTP opening; once open, ANT and PiC would be subject to conformational changes to form an interface in the inner membrane to which the compound binds (Richardson & Halestrap, 2016).

Screening performed on isolated mitochondria with thousands of compounds in the National Institute of Health repository identified many other small-molecule inhibitors, based on an isoxazole functional core. Of these compounds, ML-404 selectively inhibited mPTP opening without side effects in concentrations up to 100 μM; this compound was classified with twice the potency of GNX-series compounds (Sileikyte *et al.*, 2010). Having a synergistic effect with Cyclosporin A, ML-404 and other isoxazole-based compounds (such as compound 60) do not act through CypD binding (Roy *et al.*, 2015).

Another strategy to inhibit mPTP opening *via* CypD-independent interactions involved compounds based on the N-phenylbenzamide scaffold; these have pharmacological relevance due to their complete inhibition of the pore at low concentrations without interfering with ATP synthase production (Roy *et al.*, 2016).

Similar to TRO40303, many compounds that promote mPTP desensitization act primarily as potent oxidant scavengers: these include a gallic acid-derivative, the mitochondriotropic antioxidant AntiOxBEN$_3$ (Teixeira *et al.*, 2018) and Agomelatine (AGO), a melatonin receptor agonist. These two molecules showed low toxicity, particularly for AGO which protected the rat ischemic myocardium by acting on targets upstream of the mPTP, including the enhanced phosphorylation of GSK3β. Melatonin itself, which has antioxidant activity, also modulates mPTP opening. New evidence and ongoing work has focused on additional and direct effects on the pore (Zhou *et al.*, 2017; Tarocco *et al.*, 2019). Currently, melatonin is considered one of the safest drugs available for use as a mPTP inhibitor. Melatonin is a chronobiotic indolamine mainly synthesized and secreted in the pineal gland with a marked circadian rhythm. Given its high level of lipophilicity, pineal melatonin can diffuse across cell membranes, allowing the distribution of this molecule throughout all cells of the body and influencing organ function (Tan *et al.*, 1999). However, increasing evidence supports

that rather than a passive diffusion process, there are active or facilitated mechanisms that favour melatonin uptake and its cellular internalization, probably *via* members of the solute carrier family 2 (SLC2)/glucose transporter (GLUT) and peptide transporter 1 (PEPT1) families (Hevia *et al.*, 2008; Huo *et al.*, 2017; Mayo *et al.*, 2018). Indeed, some cellular organelles, especially mitochondria, were found to accumulate high melatonin concentrations (Leon *et al.*, 2004). A recent study also showed that large amounts of melatonin are synthesized by mitochondria during oocyte maturation, being essential for the maintenance of energy metabolism, mitochondrial function, and the quality of oocytes (He *et al.*, 2016). Enzymes involved in the synthesis of melatonin were found in mitochondria and isolated mitochondria retain the capacity to produce melatonin (Coelho *et al.*, 2015; He *et al.*, 2016). These findings underline the importance of melatonin for the mitochondria and raise questions regarding the physiological and protective effects of this neurohormone on these organelles. In addition to its antioxidant role, melatonin has a protective effect against neural disorders and other diseases *via* modulation of the activity of the mPTP and apoptosis responses (Petrosillo *et al.*, 2009; Espino *et al.*, 2010). Likewise, melatonin stimulates uncoupling proteins (UCPs), contributing to dissipation of the electrochemical proton gradient across the IMM and the consequent reduction of the MMP (Tan *et al.*, 2016; Pan *et al.*, 2018).

mPTP modulation by melatonin is a novel role within the broad spectrum of protective actions of melatonin in diverse diseases, especially in neurological disorders. Early observations in rat brain astrocytes showed that melatonin seems to suppress mitochondrial ROS formation and to target Ca^{2+}-mediated mPT to protect against cell death (Jou *et al.*, 2010). A later study using fluorescence laser scanning microscopy found that melatonin prevents mitochondrial depolarization and mPTP neurotoxicity under disturbed Ca^{+2} homeostasis, preserving the protective conformation of the mPTP (Jou, 2011). A recent study demonstrated that melatonin addition to isolated brain mitochondria inhibits the opening of the mPTP, probably hindering mPTP-mediated mitochondrial dysfunction (Waseem, Tabassum & Parvez, 2016). In this study, isolated mitochondria were incubated with Ca^{2+} and 5-hydroxydecanoate to stimulate mitochondrial swelling and induce mPTP opening. Melatonin administration significantly reduced mitochondrial swelling and MMP and improved mitochondrial respiration. Benefits of melatonin are also known in the heart from aged mice and in in frozen–thawed sperm, where melatonin administration inhibited mPTP opening and improved cellular respiration and ATP production (Sahach *et al.*, 2008; Fang *et al.*, 2019). A recent mechanistic study revealed that a melatonin receptor 1 (MT1) antagonist eliminated the mPTP opening inhibition generated by melatonin, whereas a MT1 agonist had the opposite effect (Fang *et al.*, 2020). However, the exact mechanism of action by which melatonin regulates the mPTP still remains unclear, although related molecular mechanisms have been proposed. Interestingly, promising results may provide evidence that melatonin can regulate the mPTP *via* inhibition of CypD (Zhou *et al.*, 2018). This study transfected CypD mutants (mimicking permanent phosphorylation) into melatonin-treated endothelial cells and demonstrated that melatonin represses the receptor-interacting serine/threonine-protein kinase-3 (Ripk3)–phosphoglycerate mutase family member 5 (PGAM5)–CypD cascade, attenuating necroptosis and cardiac ischemia–reperfusion injury. Another study used recordings of IMM potentials with a patch-clamp approach in liver mitoplasts from rodents to evaluate the direct effects of melatonin on the mPTP (Andrabi *et al.*, 2004). This work showed that melatonin inhibits mPTP opening in a dose-dependent manner, although there was no evidence regarding its target protein. Together, these publications show that melatonin can modulate mPTP activity and preserves the optimal MMP and mitochondrial integrity, contributing to the maintenance of cell functions and survival.

IX. CONCLUSIONS

(1) Advances in methodologies, including genetic manipulation, have accelerated our understanding of the mPTP as a constantly evolving entity present in many species, although with different forms of regulation.
(2) The mPTP plays crucial roles in cell physiology and pathology; excessive mPTP opening is involved in the pathophysiology of different human diseases, but regulated mPTP opening is critical for regulating cell and mitochondrial ionic and redox balance, and plays an apparently important role in fetal development.
(3) Opening of the mPTP increases the permeability of mitochondrial membranes to different solutes, and can switch from a lower to a higher conductance state according to different stress or physiological stimuli.
(4) The mPTP is notoriously activated by high levels of oxidative stress and increased Ca^{2+} concentrations, but it is also precisely regulated by several other metabolites.
(5) No single methodology can accurately measure mPTP opening rates.
(6) More recent models suggest that ATP synthase is a structural component of the mPTP, although doubt still exists on the exact subunit(s) responsible for channel activity.

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