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Mini-review

Mitochondrial calcium handling during ischemia-induced cell death in neurons

Yves Gouriou [a], Nicolas Demaurex [a,*], Philippe Bijlenga [b], Umberto De Marchi [a]

[a] Department of Cell Physiology and Metabolism, University of Geneva, rue Michel-Servet, 1, CH-1211 Genève, Switzerland
[b] Department of Clinical Neurosciences and Dermatology, University of Geneva, Switzerland

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ABSTRACT

Mitochondria sense and shape cytosolic Ca^{2+} signals by taking up and subsequently releasing Ca^{2+} ions during physiological and pathological Ca^{2+} elevations. Sustained elevations in the mitochondrial matrix Ca^{2+} concentration are increasingly recognized as a defining feature of the intracellular cascade of lethal events that occur in neurons during cerebral ischemia. Here, we review the recently identified transport proteins that mediate the fluxes of Ca^{2+} across mitochondria and discuss the implication of the permeability transition pore in decoding the abnormally sustained mitochondrial Ca^{2+} elevations that occur during cerebral ischemia.

1. Introduction

Stroke is one of the leading cause of death worldwide and a major cause of long-term disability [1,2]. Stroke can be classified into two major categories: ischemic and hemorrhagic. Ischemic strokes account for around 87% of all strokes [3] and are most frequently the result of the occlusion of a cerebral artery by a blood clot. The current management of stroke involves prevention by treating risk factors surgically (ie: carotid bifurcation sub-occlusions, permeable foramen ovale) or pharmacologically (prescription of acetylsalicylic acid to prevent thrombosis) or rescue attempts by early vessel repermeabilisation (ie: chemical clot dissolution using tissue plasminogen activator or endovascular mechanical clot removal). Unfortunately there is no screening to identify a population at risk before the first stroke and repermeabilisation is restricted to a short time window. Thus a majority of patients are still severely disabled or killed by the disease. Despite enormous investments, none of the drug strategies developed to protect ischemic brain tissue have proven to be of any clinical benefit for cardiac arrest or ischemic stroke. The failure has been accounted for by the complex interplay among multiple pathways including excitotoxicity, acidotoxicity, ionic imbalance, oxidative stress, inflammation and apoptosis, which can all lead to cell death and irreversible tissue injury [4]. Brain tissue has a high metabolic rate and thus is particularly vulnerable to ischemic damage. Reduction of the cerebral blood flow restricts the delivery of oxygen and glucose to the brain tissue and, within minutes, impairs the ability of neurons to maintain ionic gradients [5]. As the cells are unable to maintain a negative membrane potential, neurons depolarize, leading to the opening of voltage-gated calcium channels, and release of excitatory amino acids in the extracellular space. The cascade of events leads to a massive entry of calcium, which is well known to play an essential role in stroke-induced cerebral damage. The increase in free cytosolic calcium is transmitted to the matrix of mitochondria by Ca^{2+} channels and exchangers located on the inner mitochondrial membrane. Moderate calcium elevations within the mitochondrial matrix increase the activity of enzymes of the tri-carboxylic cycle, therefore boosting metabolism. Excessive increases in matrix [Ca^{2+}], however, alter the permeability of mitochondria, impair their ability to generate ATP, and cause the release of pro-apoptotic factors. The mitochondrial dysfunctions resulting from a calcium overload have been shown to be important in the process of ischemia-induced cell death [6]. The role of mitochondrial calcium in neurons in health and disease has been reviewed recently [7] and will only be briefly

Abbreviations: AMPAR, 2-amino-3-(3-hydroxy-5-methylisox-azol-4-yl) propionate receptors; ASICS, acid-sensing ion channels; Bcl-2, B-cell lymphoma 2; CaV1.2, L-type voltage-dependent Ca^{2+} channels; CsA, cyclosporin A; CypD, cyclophilin D; EF, helix–loop–helix structural domain; IP3, inositol triphosphate receptor; KAR, kainate receptor; Letm1, leucine zipper EF hand containing transmembrane protein 1; MCU, mitochondrial calcium uniporter; MICU1, mitochondrial calcium uptake 1; MPT, mitochondrial permeability transition; NMDAR, N-methyl-D-aspartate receptor; NCLX, mitochondrial Na^+/Ca^{2+} exchanger; NCX, plasma membrane Na^+/Ca^{2+} exchangers; PMCA, plasma membrane Ca^{2+} ATPase; PTP, permeability transition pore; RNAi, RNA interference; ROS, reactive oxygen species; t-PA, tissue plasminogen activator; TRM2, TRPM7, transient receptor potential ions channels; UCP2, UCP3, uncoupling protein 2, 3; WHS, Wolf-Hirschhorn syndrome.

* Corresponding author. Tel.: +41 22 379 5399; fax: +41 22 379 5338.
E-mail address: Nicolas.Demaurex@unige.ch (N. Demaurex).

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mentioned, with a focus on the alterations in mitochondrial calcium homeostasis that occur during ischemia-induced cell death. Here, we will review the transporters involved in the entry and the extrusion of calcium across the inner mitochondrial membrane, and discuss how the discovery of the mitochondrial Ca^{2+} handling proteins might provide new therapeutic strategies to protect neurons during ischemia.

2. Mechanisms of mitochondrial calcium influx and efflux

Mitochondria contain two membranes, an outer membrane permeable to solutes and an inner membrane impermeable to solutes that harbors the respiratory chain complexes. The respiratory chain pumps protons against their concentration gradient from the matrix of the mitochondrion into the inter-membrane space, generating an electrochemical gradient in the form of a negative inner membrane potential and of a pH gradient, the matrix being more alkaline than the cytosol [8,9]. The electrical and chemical components of the proton-motive force add up to energize the back-flux of protons down their electrochemical gradient across the ATP synthase, the enzyme that generates ATP. The negative m membrane potential ($\Delta\psi_m$) used to drive the entry of protons also favors the entry of calcium, a divalent cation, into the mitochondrial matrix. As a result, mitochondria can accumulate large amounts of calcium through a Ca^{2+}-selective channel known as the mitochondrial Ca^{2+} uniporter (MCU) [10,11]. The MCU has a relatively low Ca^{2+} affinity (Kd ~10 μM in permeabilized cells [8]), but Ca^{2+} uptake can be readily detected in intact cells because a significant fraction of mitochondria are located close to calcium release or calcium entry channels and therefore exposed to microdomains of high calcium concentrations [12–14]. Electrophysiological recordings of mitoplasts, small vesicles of inner mitochondrial membrane, revealed that the MCU is a highly Ca^{2+}-selective inward-rectifying ion channel [10]. The activity of the MCU had been known for decades to be inhibited by ruthenium red and its derivative Ru360 [15], but its molecular identity has only been unraveled very recently. In recent years, several molecules have been proposed to be either an essential or an accessory component of the MCU. In 2007, the uncoupling proteins (UCP) 2 and 3 [16] were proposed to be essential for the MCU, because overexpression and depletion of these two proteins increased and decreased mitochondrial calcium elevations, respectively, and because mice lacking UCP2 exhibited a reduced sensitivity to the calcium uptake inhibitor ruthenium red. However, these findings were disputed by another study that reported normal mitochondrial Ca^{2+} uptake in mice genetically ablated for UCP2 and UCP3 [17]. Furthermore, we recently showed that UCP3 modulates the activity of sarco/endoplasmic reticulum Ca^{2+} ATPases by decreasing mitochondrial ATP production [18]. The mitochondrial Ca^{2+} alterations associated with changes in UCP3 levels therefore reflect the exposure of mitochondria to abnormal cytosolic Ca^{2+} concentrations and do not reflect changes in MCU activity. These data indicate that UCP3 is not the mitochondrial Ca^{2+} uniporter. In 2009, the leucine zipper EF hand containing transmembrane protein 1(Letm1) [19] was identified by a genome-wide Drosophila RNA interference (RNAi) screen as a molecule that regulate both mitochondrial Ca^{2+} and H^+ concentrations. Letm1 was reported to be a high-affinity mitochondrial Ca^{2+}/H^+ exchanger able to import Ca^{2+} at low (i.e. submicromolar) cytosolic concentrations into energized mitochondria. Earlier studies had however linked Letm1 to mitochondrial potassium/protons exchange and to the maintenance of ionic mitochondrial balance, the integrity of the mitochondrial network and cell viability [20,21]. Importantly, deletion of Letm1 gene has been associated to seizures in Wolf-Hirschhorn syndrome (WHS), a severe human neurological disease [22]. The high-affinity of Letm1 for Ca^{2+} and its postulated $1Ca^{2+}/1\ H^+$ stoichiometry are at odds with the known properties of the MCU, and the recent identification of a protein that fits all MCU properties (see below) indicate that Letm1 is not the dominant mechanism of mitochondrial Ca^{2+} uptake. Instead, Letm1 might contribute to an alternate mode of mitochondrial Ca^{2+} uptake that was first reported in isolated rat liver mitochondria by Gunter's group. Using isotopic $^{45}Ca^{2+}$ measurements, these authors showed that the exposure of mitochondria to physiological calcium pulses was sufficient to produce significant mitochondrial Ca^{2+} sequestration via a rapid mode of uptake (RaM). The RaM occurred at the beginning of each pulse and was followed by a slower Ca^{2+} uptake characteristic of the MCU [23]. The RaM was transient and occurred predominantly at Ca^{2+} concentrations lower than 200 nM, indicating that it was mediated a high-affinity Ca^{2+} transporter. Subsequent studies using fluorescent probes reported mitochondrial Ca^{2+} uptake at nanomolar Ca^{2+} concentrations in a variety of cell types [24,25], and the implications of the coexistence of low and high-affinity modes of Ca^{2+} uptake have been recently reviewed [26]. Interestingly, the RaM can be modeled by a 4 state model whereby Ca^{2+} binds to an external trigger site to initiate a transient burst of high Ca^{2+} conductivity [27].

In 2010, Palmer and Mootha reported that a new mitochondrial EF hand protein MICU1 (for mitochondrial calcium uptake 1) was required for high capacity mitochondrial calcium uptake, and proposed that MICU acts as a calcium sensor that controls the entry of calcium across the uniporter [28]. Building up on this discovery, two groups simultaneously identified the mitochondrial calcium uniporter in June 2011 [29,30]. Using *in silico* analysis combined with phylogenetic profiling and analysis of RNA and protein co-expressed with MICU1, the group of Vamsi Mootha isolated a novel protein that co-immunoprecipitated with the exogenously expressed MICU1 [30]. Using the same database, the group of Rosario Rizzuto independently identified the same protein. These authors searched for proteins with two or more transmembrane domains (a defining transporter feature) whose expression differed in species known to exhibit or lack uniport activity (kinetoplastids and yeast, respectively) [29]. From the 14 proteins matching these criteria, one contained a highly conserved domain encompassing two transmembrane regions separated by a loop bearing acidic residues, as expected from a mitochondrial Ca^{2+} channel. Fonctional analysis confirmed that this protein behaves as expected for the mitochondrial uniporter, and it was therefore assigned the defining name of MCU. Mitochondrial Ca^{2+} uptake was strongly reduced by MCU silencing in cultured cells and in purified mouse liver mitochondria, whereas MCU overexpression enhanced ruthenium red-sensitive mitochondrial calcium uptake in intact and permeabilized cells. The MCU migrated as a large complex of 450 kD on blue native gels, and GFP-tagged MCU could be co-immunoprecipitated by V5-tagged MCU, indicating that the protein forms oligomers [30]. Both studies mapped the MCU to the inner mitochondrial membrane, but disagreed on whether the N and C termini face the matrix [30] of the inter-membrane space [29]. Mutations of conserved acidic residues within the short sequence linking the two transmembrane domains abrogated the ability of MCU to reconstitute mitochondrial Ca^{2+} uptake, whereas mutation of a nearby serine resitude (S259) conferred resistance to Ru360, indicating that the acidic residues are required for calcium uptake and that Ser 259 is critical for MCU sensitivity to ruthenium red [30]. Finally, and most convincingly, expression of the purified protein in planar lipid bilayers was sufficient to reconstitute ion channel activity in solutions containing only Ca^{2+} as the permeant ion [29]. The currents were carried by a channel of small conductance (6–7 pS),

fast opening/closing kinetics, and low opening probability, and were inhibited by ruthenium red, as expected for the MCU. Proteins mutated at two of the conserved acidic residues failed to generate Ca^{2+} currents when inserted into bilayers and acted as dominant negative when expressed in HeLa cells. These data conclusively show that MCU is the long sought-after mitochondrial Ca^{2+} uniporter, and open the way to the generation of animal models that will enable to test the role of mitochondrial Ca^{2+} uptake in cell and tissue physiology. Preliminary experiments are encouraging, as cells overexpressing MCU were more sensitive to apoptosis after treatment with ceramide and H_2O_2, supporting the notion that mitochondrial Ca^{2+} overload enhances the sensitivity to apoptosis [29].

Compared to the MCU, the proteins that catalyze the efflux of Ca^{2+} from mitochondria have received much less attention. The extrusion of Ca^{2+} from mitochondria is coupled to the entry of sodium across an electrogenic $1Ca^{+}$:$3Na^{+}$ exchanger [31] that is inhibited by the benzothiazepine derivative CGP-37157 [32], reviewed in [8]. The subsequent efflux of sodium ions by the mitochondrial $1Na^{+}$:$1H^{+}$ exchanger (mNHE)eventually results in the entry of three protons into the matrix for each Ca^{2+} ion that leaves mitochondria. Ca^{2+} extrusion thus has a high energetic cost, as it dissipates the proton gradient generated by the respiratory chain that is normally used to drive the synthesis of ATP by the F1F0 ATP synthase (reviewed in [33]). The molecule catalyzing mitochondrial Na^{+}/Ca^{2+} exchange has been recently identified as NCLX/NCKX6, a protein localized in mitochondrial cristae [34], whereas stomatin-like protein 2 (SLP-2), an inner membrane protein, was shown to negatively modulate the activity of the mitochondrial Na^{+}/Ca^{2+} exchanger [35]. Functional evidence from knock-down and overexpression studies indicate that NCLX is an essential part of the mitochondrial sodium calcium exchanger whereas SLP-2 is an accessory protein that negatively regulates mitochondrial Ca^{2+} extrusion. The proteins thought to regulate the fluxes of Ca^{2+} across the inner mitochondrial membrane are summarized in Fig. 1, along with commonly used inhibitors of mitochondrial Ca^{2+} entry and extrusion.

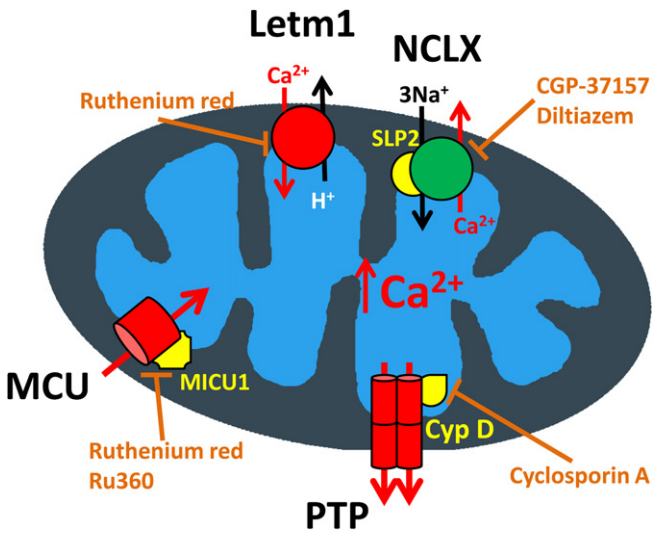

Fig. 1. Ca^{2+} transport proteins of mitochondria. In mammalian mitochondria, the uptake of Ca^{2+} into the matrix is mediated by a Ca^{2+}-selective channel, the mitochondrial Ca^{2+} uniporter (MCU), regulated by a calcium sensing accessory subunit (MICU1). Letm1 might mediate a slow Ca^{2+}/H^{+} exchange at low (nM) cytosolic Ca^{2+} concentration, driving Ca^{2+} entry, limited by pH gradient. Ca^{2+} is then extruded by the Na^{+}/Ca^{2+} exchanger NCLX, which is down-regulated by the protein SLP-2. High levels of matrix Ca^{2+} accumulation trigger the opening of the permeability transition pore (PTP), responsible for mitochondrial membrane permeabilization and neuronal cell death. PTP is also a reversible fast Ca^{2+} release channel. The mitochondrial matrix protein cyclophilin D (CypD) facilitates PTP opening by desensitizes PTP to Ca^{2+}. Common inhibitors of the mitochondrial proteins that control matrix Ca^{2+} uptake and release are indicated in orange.

3. Mitochondrial calcium overload during ischemia

During ischemia, neuronal calcium channels and transporters (including NCX, TRM2, TRPM7, ASICS, CaV1.2 and hemichannels) as well as glutamate receptors (NMDAR, AMPAR, KAR) are overactivated, a process known as excitotoxicity (reviewed in [36]). The increased activity of plasma membrane Ca^{2+} channels can then trigger the entry of Ca^{2+} into the cytosol of neurons, leading to larger than usual increases in the cytosolic calcium concentration. To avoid calcium overload, plasma membrane calcium pumps (PMCA) actively extrude calcium from the cytoplasm during neuronal activity. The increased turnover of PMCA increases the consumption of intracellular ATP that, in neurons, is mainly derived from oxidative phosphorylation occurring in the mitochondria. As discussed above, cytosolic Ca^{2+} elevations are rapidly transmitted to the mitochondrial matrix, where they amplify the activity of Krebs cycle enzymes and of the ATP synthase, thereby increasing the production of ATP [37,38]. During physiological Ca^{2+} elevations, the boost of ATP enables PMCA to extrude the cytosolic calcium and to sustain neuronal activity. During ischemia however, the levels of oxygen and glucose drop rapidly, impairing the production of ATP by mitochondria and by cytosolic glycolysis. As a result, ATP-dependent calcium extrusion mechanisms progressively come to a halt because the intracellular reservoir of ATP is depleted by the continuous activity of the Na^{+}/K^{+} ATPases. The importance of the Na^{+}/K^{+} ATPases in “stealing “ ATP from PMCA could be directly demonstrated as PMCA activity, which collapsed during metabolic depletion, could be rescued by inhibition of the Na^{+}/K^{+} ATPase [39]. PMCA inhibition amplifies the cytosolic calcium elevations that are transmitted to the mitochondrial matrix, and can then triggers a vicious sequence of mitochondrial calcium overload, mitochondrial dysfunction, release of mitochondrial pro-apotpotic factors, and the activation of death signals [6,40–42]. Mitochondria located near the plasma membrane (subplasmalemmal mitochondria) are more exposed to calcium overload due to their proximity to plasma membrane voltage-sensitive calcium channels and to the functionally incapacitated PMCA [43–45]. Subplasmalemmal mitochondria have been shown to take up calcium coming from voltage-gated calcium channels [46], and mitochondrial calcium overload and dysfunction has been linked to glutamate excitotoxicity mediated by the overactivation of NMDA receptors (NMDAs) at the plasma membrane [6,7,47,48]. The specific patterns of cell death triggered by the activation of ionotropic glutamate receptors during excitotoxicity has led to the "route specificity" hypothesis, which postulates that the neurotoxicity depends more on the routes of calcium entry rather than on the magnitude of the calcium overload [49–51], reviewed in [7]. Intracellular mitochondria are also at risk however, and calcium release from the endoplasmic reticulum has been associated to ischemia induced-cell damage [52–54]. Mitochondria are embedded within sheets of endoplasmic reticulum and the two organelles are maintained in very close proximity by linker proteins [55,56]. Because of this proximity, the release of calcium ions through IP3 receptor of the endoplasmic reticulum readily triggers an entry of calcium in adjacent mitochondria [12,13]. Thus, neuronal mitochondria are exposed both to Ca^{2+} ions entering across membrane channels and to Ca^{2+} released from endoplasmic reticulum Ca^{2+} stores. As shown in Fig. 2, an elevation in the mitochondrial matrix Ca^{2+} concentration can be readily recorded in intact PC-12 cells expressing a genetically encoded Ca^{2+} indicator and exposed to oxygen and glucose deprivation. Although

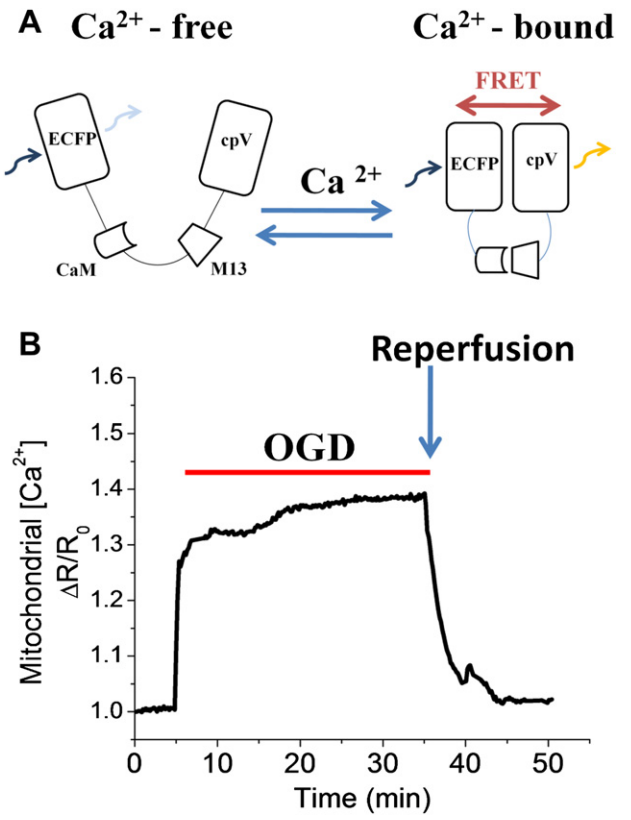

Fig. 2. Changes in mitochondrial matrix [Ca^{2+}] evoked by oxygen and glucose deprivation in PC-12 cells. A. Schematic representation of the genetically encoded "cameleon" Ca^{2+} indicator D3cpv. The probe consists of two cyan and yellow fluorescent proteins linked by a Ca^{2+}-sensing module (CaM). Binding of Ca^{2+} to the CaM brings the two fluorescent moieties in closer proximity, favoring fluorescence resonance energy transfer (FRET) and causing a shift in fluorescence emission from cyan to yellow. B. Measurement of mitochondrial calcium in PC-12 cells deprived of oxygen and glucose for 30 min and subsequently exposed to an oxygenated solution containing glucose for 15 min. Oxygen was replaced by nitrogen in the environmental chamber. Mitochondrial calcium rises abruptly upon oxygen/glucose deprivation (OGD), reaches a steady-state plateau, and rapidly returns to basal levels as soon as the normal oxygen tension is restored.

the magnitude of this ischemia-induced mitochondrial Ca^{2+} elevation is comparable to the responses evoked by the opening of membrane channels or by the addition of Ca^{2+}-mobilizing agonists, its duration far exceeds the physiological responses. To our knowledge, such a long-lasting elevation in the mitochondrial matrix free Ca^{2+} concentration has not been reported, but long-lasting cytosolic Ca^{2+} elevations are a defining feature of the ischemic process in neurons [57,58]. This Ca^{2+} overload reflects the failure of Ca^{2+} extruding systems to cope with the excess Ca^{2+} ions that enter cells across deregulated plasma membrane channels, following the cleavage of plasma membrane Ca^{2+} pumps [59,60], and Na^{+}/Ca^{2+} exchangers [61]. As a result of the global cytosolic Ca^{2+} elevation, mitochondria are exposed to micromolar Ca^{2+} concentrations for long durations in ischemic neurons, which favors Ca^{2+} uptake by the MCU. The impact of ischemia on the rates of mitochondrial Ca^{2+} uptake is controversial, with some studies reporting decreased mitochondrial Ca^{2+} uptake [62–64], whereas a more recent study reported that 15 min ischemia does not impact the rate of active Ca^{2+} uptake by isolated mitochondria [65]. Whether the long-lasting Ca^{2+} elevations taking place in the matrix of mitochondria during oxygen and glucose deprivation contribute to the neurotoxicity and can be prevented by MCU inhibition remains to be confirmed.

4. Mitochondrial permeability transition pore and cerebral ischemia

Currently, it is unclear whether the mitochondrial matrix Ca^{2+} elevations occurring during ischemia are causally related to the neuronal cell death that occurs after cerebral ischemia. The best established link between mitochondrial Ca^{2+} and cellular toxicity is the opening of a Ca^{2+}-activated channel located in the inner mitochondrial membrane, the permeability transition pore (PTP) [66–68], responsible for the so-called mitochondrial permeability transition (MPT). In this section we will discuss the molecular nature of PTP, its Ca^{2+}-dependence and its involvement in cerebral ischemia. The MPT [69] is a phenomenon induced by high levels of matrix Ca^{2+} accumulation and oxidative stress, responsible for a sudden increase in the mitochondrial inner membrane permeability to solutes with molecular masses up to 1500 Da. The MPT plays an important role in events ranging from tissue damage upon infarction to muscle wasting in some forms of dystrophy (see [67] for a recent review dealing with the involvement of permeability transition in pathological conditions). Importantly, the effect of cyclosporin A (CsA), the most commonly used inhibitor of the MTP, has implicated PTP-dependent mitochondrial dysfunction and Ca^{2+} deregulation in several diseases, including brain damage following ischemia/reperfusion [70–76] and hypoglycaemia [77]. The PTP is a Ca^{2+}-, ROS (reactive oxygen species)-, voltage-dependent and Csa-sensitive high-conductance channel, located in the inner mitochondrial membrane. Despite the great interest generated by this channel, which has been extensively characterized at the pharmacological and biophysical level, the molecular identity of the PTP is not known. The classical model envisions a supramolecular complex spanning the double membrane system of mitochondria, localized at contact sites [78]. Proteins of all mitochondrial compartments have been proposed to be part of the PTP [66,67,79], in particular cyclophilin D (CypD) in the mitochondrial matrix, the adenine nucleotide translocator in the inner membrane and mitochondrial porin VDAC in the outer membrane. Surprisingly, genetic studies have demonstrated that MPT can still be observed in mitochondria devoid of each of these proteins [80–82]. Some other proteins, including inter-membrane and cytosolic proteins and even the pro-apoptotic Bcl-2 family protein Bax, have been proposed to be part of the PTP under particular conditions [83,84], but Ca^{2+}-dependent MPT was shown to be independent of Bax [85]. PTP can be regulated by ligands of the outer mitochondrial membrane translocator protein TSPO, formerly known as the peripheral benzodiazepine receptor [86], suggesting that the PTP complex may include TSPO itself [87–90]. Recent results of Sileikyte and collaborators [91] show that MPT is an inner membrane event that is regulated by the outer membrane through specific interactions with TSPO, attesting a regulatory role for this protein. Although the proteins responsible for this important mitochondrial process have not yet been identified, the generation of CypD-knockout mice has now established an unquestionable role for CypD in facilitating PTP opening [80,92–95]. The biophysical properties (conductance, voltage-dependence, selectivity) of PTP were indistinguishable in mitochondria isolated from isogenic wild-type and engineered mice lacking CypD [95], but CsA only inhibited the PTP in wild-type mice [80,95], demonstrating that CypD represents the target for PTP inhibition by CsA. Importantly, mitochondria from CypD-knockout mice displayed a striking desensitization of the PTP to Ca^{2+}, such that pore opening required about twice the Ca^{2+} load necessary to open the pore in strain-matched, wild-type mitochondria [80].

That the permeability transition is triggered by an elevation in the free Ca^{2+} concentration within the mitochondrial matrix was discovered early [96–98]. Chelation of matrix Ca^{2+} induces a rapid closure of PTP and divalent cations such as Mg^{2+}, Mn^{2+}, Ba^{2+} and

Sr^{2+}, instead of inducing PTP opening, can act as inhibitors of Ca^{2+} trigger sites [99]. The Ca^{2+}-induced activation of the PTP has been well characterized in mitochondrial patch-clamp experiments [68], the probability of observing PTP activity in mitoplast patches increasing with the increasing [Ca^{2+}] in mitochondria isolated from mouse, rat or human cells [100]. Moreover, the time required for the channel to inactive was shown to be shorter at lower [Ca^{2+}], and single channel PTP recordings have demonstrated the competitive nature of the activation by Ca^{2+} and its inhibition by several agents [95,101,102]. Ca^{2+}-elicited PTP can be pharmacologically inhibited and then reactivated by increasing [Ca^{2+}] for several cycles. This behavior has been suggested to involve an overall equilibrium (instead of a limited number of binding sites on a protein). Cardiolipin, a lipid well-known to bind Ca^{2+}, has been proposed as "receptor" for this cation in this model [68]. Ca^{2+} is not the only regulator of PTP and many other factors (oxidative stress, voltage, pH, peptides and a wide array of small molecules) are able to modulate the activity of this pore [66,67,69]. Importantly, several regulators of PTP opening act by modulating the Ca^{2+} sensitivity of the pore [66,103]. In addition to being activated by Ca^{2+}, the PTP has also been proposed to act as a reversible fast Ca^{2+} release channel [104]. These functional studies have firmly established the relationship between [Ca^{2+}] and the PTP that was subsequently shown to be altered in CypD-knockout mice.

The relationship between MPT and cerebral ischemia inferred from the effects of CsA was nicely confirmed in CypD-knockout mice, by measuring the infarct size after cerebral ischemia/reperfusion injury induced by the occlusion of the middle cerebral artery [94]. In this study, Schinzel and colleagues demonstrated that isolated, CypD-deficent mitochondria showed an increased capacity to retain calcium and were resistant to Ca^{2+}-induced MPT in swelling experiments. When they induced ischemia/reperfusion, a dramatic decrease in infarct size (62%) was recorded in the brains of CypD-deficient mice, suggesting an essential role for CypD in cell death in the brain. A correlation between gene dosage and the extent of injury was elegantly established, by recording a partial protection in heterozygous mice (37% of reduction in infarct size). These data proved that conditions required for the activation of PTP were present during ischemia/reperfusion, as suggested by earlier pharmacological studies [70,73,74].

An important aspect of the ischemic process is the phenomenon referred as preconditioning, or ischemic tolerance, which offers a therapeutic opportunity to reduce tissue damage after cerebral or cardiac ischemia. This process consists in a short non-injurious ischemic insult that can greatly reduce the severity of a subsequent prolonged ischemia. This protocol was first described in the dog heart [105], but has since been confirmed in various animal models of brain ischemia [106] and in human stroke patients [107,108]. Different triggers are able to induce preconditioning and several mediating pathways have been characterized, but the final effectors remain unknown [108]. Pharmacological evidence [108,109] indicates that the activation of mitochondrial potassium channels might mediate preconditioning by inhibiting MPT activation during reperfusion [109]. Whether mitochondrial ATP-sensitive potassium channels are present in brain and play a role during cerebral ischemia is disputed [108–110], but electrophoretic potassium flux in brain mitochondria is well established [111] and a voltage-gated potassium channel (Kv1.3) has been electrophysiologically characterized in gerbil hippocampal mitochondria [112]. More relevant, two Ca^{2+}-activated potassium channels ($K_{Ca}1.1$ and $K_{Ca}3.1$) have been detected in inner mitochondrial membrane and characterized by mitochondrial patch-clamp [113,114]. The large conductance Ca^{2+}-activated potassium channels (BK_{Ca} or $K_{Ca}1.1$) is present in mitochondria from glioma cells [113] and from rat brain [115], and has been proposed to contribute to the cardioprotective effect of potassium influx into mitochondria [116]. Whether the recently discovered mitochondrial intermediate conductance Ca^{2+}-activated potassium channels (IK_{Ca} or $K_{Ca}3.1$) [114] is also present in brain tissue and contributes to the protection against ischemic insults remains to be determined.

Although the causal relationship between mitochondrial Ca^{2+} accumulation and PTP opening is well established, and despite the fact that MPT invariably leads to neuronal cell death, these relationships do not necessarily imply that matrix Ca^{2+} accumulation is directly responsible for the injuries related to cerebral ischemia. The group of Lemasters, for example, has proposed that mitochondrial Ca^{2+} overload is a consequence, rather than a cause, of the bioenergetic failure that follows MPT onset. In this view, the mitochondrial Ca^{2+} elevation is only a signature of diseased mitochondria and is not involved in the induction of the MPT, which occurs after reperfusion [117]. In this study of adult rat myocytes, ROS but not Ca^{2+} overload has been suggested to trigger pH- and MPT-dependent death after ischemia–reperfusion. Another important parameter to take into account is the timing of the PTP opening during ischemia/reperfusion. In the heart, there is a broad consensus that during ischemia the factors favoring PTP opening (increased matrix Ca^{2+} and depolarization) are balanced by PTP antagonists (intracellular acidosis, high levels of Mg^{2+} and ADP) that prevent PTP opening during ischemia [118]. Upon reperfusion, oxygen and substrate supplies are restored to the tissue, mitochondria re-energize, take up the Ca^{2+} that has accumulated in the cytosol during ischemia, and produce a burst in ROS. The combination of these factors provides ideal conditions for triggering PTP opening [103,118]. Direct methods to asses PTP opening in intact hearts support the concept that PTP is more likely to open upon reperfusion [119,120]. Whether the same sequence of events also occurs in ischemic brain is not known, and further studies are needed to determine the precise timing of the PTP opening during cerebral ischemia. The recent identification of the proteins involved in mitochondrial Ca^{2+} uptake and release provides new opportunities to study the role of mitochondrial Ca^{2+} in neuronal death during cerebral ischemia. The role of calcium in MPT activation and cell death can now be directly tested by modulating the expression levels of mitochondrial transport proteins. Targeting the proteins that control the fluxes of Ca^{2+} should reveal whether altered mitochondrial Ca^{2+} handling is causally related to ischemic neuronal death, and can potentially increase the repertoire of therapeutic tools to treat ischemic brain diseases.

5. Therapeutic strategies to protect neurons during ischemia by targeting mitochondrial Ca^{2+} handling proteins

Patients and therapists alike are eagerly awaiting new strategies allowing brain tissue to survive severe ischemia. Death following ischemia is not a fatality. Some species are remarkably resistant to hypoxia, non excitable cells can be cultured in anaerobic conditions, and various models of brain ischemia as well as human stroke patients' observations confirmed the possibility to greatly reduce the tissue damage after prolonged ischemia by preconditioning [121]. The optimal target to be modulated should be at the convergence of all ischemia signaling pathways but upstream to the irreversible triggers of apoptosis. The mitochondrion is a key organelle in this signaling integration process, and several strategies aiming to protect cells from ischemia have therefore focused on mitochondria. Modulation should allow the preservation of the mitochondrial proton gradient and avoid the opening of the mitochondrial permeability transition pore during ischemia and reperfusion. Strategies that target the PTP and its regulation by CypD have been shown to confer significant cardioprotection in isolated rat hearts [122], and the administration of cyclosporine

during percutaneous coronary intervention reduced infarct size in a cohort of patients [123]. Unfortunately cyclosporine causes immunosuppression and nephrotoxicity and the benefits of PTP inhibition are balanced by its adverse effects, as the loss of PTP-mediated Ca^{2+} efflux increases mitochondrial matrix Ca^{2+} [124], reviewed in [118]. Inhibition of mitochondrial Ca^{2+} uptake, on the other hand, is expected to reduce the long-lasting mitochondrial calcium elevations that occur during ischemia (Fig. 2) and to prevent PTP opening. The MCU is therefore a prime target as drugs that inhibit this Ca^{2+} uptake system should retain the beneficial effects conferred by PTP inhibition but not its adverse effects. Accordingly, inhibition of the MCU by ruthenium red protects hearts against ischemia injury [125]. Unfortunately ruthenium red is a very unspecific inhibitor that also inhibit several classes of ion channels and that interfere with the binding of Ca^{2+} to calmodulin (reviewed in [26]). The molecular identification of the MCU opens the way to the rational design of drugs targeting specifically the MCU. Ideally the drug should cross the blood brain barrier and act rapidly within a few seconds. It should be safe enough to be administered preventively when cerebral ischemia is expected during cardiac or cerebro-vascular surgeries, hemorrhagic shock, traumatic brain injuries or any other condition where cerebral blood flow is compromised. It should be easy and safe to handle to allow very early administration by paramedics when stroke is suspected. Such a medication would substantially increase the fraction of patients that would benefit from curative treatments and reduce disabilities and death.

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