Title	**Modulation of mitochondrial calcium as a pharmacological target for Alzheimer's disease**
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Citation	**Ageing Research Reviews, 2010, v. 9 n. 4, p. 447-456**
Issued Date	**2010**
URL	**http://hdl.handle.net/10722/124475**
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Elsevier Editorial System(tm) for Ageing Research Reviews
Manuscript Draft

Manuscript Number: ARR-D-10-00011R1

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Article Type: Review Article

Keywords: mitochondria; calcium; Alzheimer's disease; voltage dependent anion channel; mitochondrial membrane potential

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Modulation of mitochondrial calcium as a pharmacological target for Alzheimer's Disease

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Running Title: Mitochondrial calcium in AD

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Abstract

Perturbed neuronal calcium homeostasis is a prominent feature in Alzheimer's disease (AD). Mitochondria accumulate calcium ions (Ca^{2+}) for cellular bioenergetic metabolism and suppression of mitochondrial motility within the cell. Excessive Ca^{2+} uptake into mitochondria often leads to mitochondrial membrane permeabilization and induction of apoptosis. Ca^{2+} is an interesting second messenger which can initiate both cellular life and death pathways in mitochondria. This review critically discusses the potential of manipulating mitochondrial Ca^{2+} concentrations as a novel therapeutic opportunity for treating AD. This review also highlights the neuroprotective role of a number of currently available agents that modulate different mitochondrial Ca^{2+} transport pathways. It is reasoned that these mitochondrial Ca^{2+} modulators are most effective in combination with agents that increase the Ca^{2+} buffering capacity of mitochondria. Modulation of mitochondrial Ca^{2+} handling is a potential pharmacological target for future development of AD treatments.

1. Introduction

As the average life span of human population gradually increases, the prevalence of age-related diseases has significantly increased. Alzheimer's disease (AD) is a fatal neurodegenerative disorder, affecting approximately 35.6 million people worldwide (Prince and Jackson, 2009). AD is the most common form of dementia. The disease is characterized by progressive synaptic dysfunction and neuronal loss in various brain regions, especially in the cortex and hippocampus. Severe neurodegeneration in these brain regions results in cognitive, emotion, social and motor impairments. With more than a hundred years of research, the underlying mechanism of this incurable disease still remains elusive. Perturbed neuronal calcium (Ca^{2+}) homeostasis is a common feature in many neurodegenerative diseases including AD, amyotrophic lateral sclerosis (ALS), ischemic stroke and Parkinson's disease (PD) (Mattson and Chan, 2003). Increasing lines of evidence support the idea that Ca^{2+} dysregulation plays a key role in AD pathogenesis (Bezprozvanny, 2009; Bojarski et al., 2008; LaFerla, 2002; Mattson and Chan, 2003; Yu et al., 2009).

2. Neuronal Ca^{2+} dysregulation and Alzheimer's disease

Ca^{2+} signaling is essential for life and death processes including neuronal excitability, synaptic plasticity, gene transcription and apoptosis (Berridge, 1998; Berridge et al., 1998). The Ca^{2+} dysregulation hypothesis postulates that sustained increase in cytosolic Ca^{2+} concentrations can lead to neurodegeneration in AD (Khachaturian, 1994; Toescu and Verkhratsky, 2007). Disturbances in Ca^{2+} signaling has been found in both sporadic and familial cases of AD (LaFerla, 2002). Several age-related perturbations in pathways regulating Ca^{2+} homeostasis have been reported, suggesting a possible linkage between aging and the development of sporadic AD (Bezprozvanny, 2009). A small proportion of AD patients (~5%) suffer from an early-onset familial form that occurs under age of 65 (Hardy, 2006). The genes involved in familial AD include presenilins (presenilin 1 and 2) and amyloid precursor protein (APP) (Hardy and Gwinn-Hardy, 1998). Both have been shown to play important roles in Ca^{2+} signaling (LaFerla, 2002). The mechanisms of how Ca^{2+} homeostasis is disrupted in AD have been extensively reviewed (Bezprozvanny, 2009; Bojarski et al., 2008; LaFerla, 2002; Mattson and Chan, 2003; Yu et al., 2009). In the following sections, we will briefly discuss this issue for readers to understand how Ca^{2+} dyshomeostasis is linked with AD.

2.1 APP mutation induces Ca^{2+} influx and elevates cytosolic Ca^{2+} concentrations

Accumulation of senile plaques and neurofibrillary tangles are two important pathological hallmarks in AD brains. Senile plaques are made of beta-amyloid (Aβ) peptides which are derived from APP. Mutations associated with familial AD result in increased production of the amyloidogenic Aβ fragments (Mattson, 1997). APP derivatives such as secreted forms of APP (sAPP), Aβ-containing fragments, and APP intracellular domain (AICD) have been shown to modulate cellular Ca^{2+} signaling (Leissring et al., 2002; Mattson et al., 1993; Mattson et al., 1992). Aβ aggregates have been found to form cation-selective ion channels in the plasma membrane, resulting in increased cytosolic Ca^{2+} concentrations (Arispe et al., 1993a; Arispe et al., 1993b; Kagan et al., 2002). Nevertheless, how Aβ-induced membrane pores are related to human AD is still unclear. Oxidative damage is another mechanism by which Aβ causes disruption in Ca^{2+} homeostasis and neurotoxicity (Hensley et al., 1994; LaFerla, 2002). Accumulation of Aβ leads to formation of reactive oxygen species (ROS), which promotes DNA damage, lipid peroxidation, protein carbonylation and nitrosylation. Lipid peroxidation modifies functions of membrane transporters and ion channels (Mark et al., 1995), which in turn further elevates basal cytosolic Ca^{2+} concentrations, forming a vicious cycle (LaFerla, 2002; Mattson and Chan, 2003).

2.2 Presenilins modulate ER Ca^{2+} signaling and enhances ER Ca^{2+} release

Presenilins (PS1 and PS2) are components of the γ-secretase complex which are involved in the proteolytic cleavage of APP. PS1 and PS2 are located in various intracellular compartments such as the endoplasmic reticulum (ER) (Annaert et al., 1999), Golgi apparatus (Annaert et al., 1999), mitochondria (Ankarcrona and Hultenby, 2002). Notably, presenilins are highly enriched in a specific region where the ER membranes are in close contact with mitochondria namely the ER-mitochondrial-associated membranes (MAM) (Area-Gomez et al., 2009).

FAD-linked presenilin mutations are believed to alter the activity of γ-secretase such that more Aβ are produced, especially the fibrillogenic $A\beta_{1-42}$ peptides (Xia et al., 1997). FAD-related mutant presenilins can also affect ER Ca^{2+} handling independent of Aβ by exaggerating Ca^{2+} release from the ER in response to agonist stimulation. FAD mutant PS1 and PS2 have been shown to interact with the inositol 1,4,5-triphosphate receptor ($InsP_3R$) Ca^{2+}-releasing channels and enhance their gating activity by a gain-of-function effect (Cheung et al., 2010; Cheung et al., 2008). $InsP_3Rs$ are more likely to be in a high-probability burst mode, resulting in enhanced ER Ca^{2+} release (Cheung et al., 2010). However the molecular mechanism of this modulation remains elusive.

Depletion of ER Ca^{2+} store triggers Ca^{2+} influx from extracellular space via store-operated Ca^{2+} channels (Putney, 1986). This is known as capacitive Ca^{2+} entry (CCE or store-operated Ca^{2+} entry). Stromal interacting molecule 1 (STIM1) protein acts as Ca^{2+}-sensors on the ER which interact with Orai1/TRPC channels in the plasma membrane and activate store-operated channels for Ca^{2+} entry (Ong et al., 2007; Zhang et al., 2005). CCE has been shown to be attenuated by PS mutants, possible due to increased Ca^{2+} in the ER store (Herms et al., 2003; Leissring et al., 2000; Yoo et al., 2000). Moreover, increased levels of STIM1 have been found in mouse embryonic fibroblast lacking presenilins, implicating that expression of STIM1 may be presenilin-dependent (Bojarski et al., 2009).

2.3 Ca^{2+}-dependent tau phosphorylation and dephosphorylation

Neurofibrillary tangles formed by hyperphosphorylation of the microtubule-associated protein tau are another hallmark in AD. The phosphorylation state of tau is highly Ca^{2+}-dependent. Tau phosphorylation is regulated by Ca^{2+}-dependent calmodulin-dependent protein kinase II (CaMKII) and calpain (Litersky et al., 1996; Maccioni et al., 2001). Activation of cyclin-dependent protein kinase 5 (Cdk5) by calpain via p25 has been suggested to play a role in tau hyperphosphorylation (Maccioni et al., 2001). On the

other hand, calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase is involved in tau dephosphorylation (Fleming and Johnson, 1995). Tau dephosphorylation was completely attenuated in rat cerebral-cortical slice pre-treated with the calcineurin inhibitor Cyclosporin A (Fleming and Johnson, 1995). Injection of FK506 (a calcineurin inhibitor) has been reported to enhance tau phosphorylation at various phosphorylation sites in mouse brain (Luo et al., 2008). On the other hand, calcineurin inhibitors have also been shown to increase phosphorylation of glycogen synthase kinase-3 beta (GSK-3β) at serine-9 (Kim et al., 2009). Phosphorylation of GSK-3β at serine-9 inhibits tau phosphorylation by GSK-3β (Hughes et al., 1993). Hence, both increase and decrease cytosolic Ca^{2+} concentrations contribute to tau phosphorylation, therefore perturbed Ca^{2+} homeostasis may associate with the tau pathology in AD.

2.4 Sporadic AD: ApoE4 and CALHM1

Apolipoprotein E is involved in transporting cholesterol from the blood to the cells. Individuals with the allele for the E4 isoform of apolipoprotein E (ApoE4) have an increased risks of sporadic AD (Mahley et al., 2006). ApoE 4 was found to disrupt Ca^{2+} homeostasis by triggering extracellular calcium influx and amplifying neuronal Ca^{2+} responses (Hartmann et al., 1994; Tolar et al., 1999). Recent research has identified

polymorphism of a gene called calcium homeostasis modulator 1 (CALHM1) that may link with sporadic AD. CALHM1 encodes for a protein which forms a Ca^{2+} channel on the plasma membrane and controls Aβ levels (Dreses-Werringloer et al., 2008). Since then several studies have shown that the P86L polymorphism of CALHM1 is associated with AD (Boada et al., 2010; Cui et al., 2010), whilst other studies failed to find a link between CALHM1 and risk of AD (Bertram et al., 2008; Minster et al., 2009; Nacmias et al., 2010; Sleegers et al., 2009). The relevance of CALHM1 in AD remains unclear.

As illustrated above, it is clear that Ca^{2+} signaling pathways are highly involved in AD pathogenesis. Several FAD-approved drugs and drugs tested in clinical trials therefore aim to target different Ca^{2+} signaling pathways in order to re-establish the cytosolic Ca^{2+} homeostasis. Memantine (Namenda) is the most common drug for moderate to severe AD. Memantine is a non competitive N-methyl D-aspartate (NMDA) antagonist. It inhibits Ca^{2+} entry into neurons through the NMDA receptors and therefore reduces excitotoxicity (Bezprozvanny, 2009). However, currently it only provides limited benefits for AD patients. Hu et al. found that specific antagonists targeting at NMDA receptors containing the GluN2B subunit e.g. ifenprodil and Ro 25–6981, might be effective in protecting neurons from Aβ-induced inhibition of synaptic plasticity *in vivo* (Hu et al., 2009). EVT-101 (Evotec AG, Hamburg, Germany; http://www.evotec.com/) is

a newly developed NMDA receptor subunit 2B specific antagonist. Phase I trial of EVT-101 has now completed and cognitive performance of patients was improved (NCT00526968). This specific NMDA receptor antagonist is believed to greatly reduce the chance of side effects caused by the unspecific NMDAR antagonist memantine.

Nimodipine is an isopropyl Ca^{2+} channel blocker which has been shown to improve cognitive performance of dementia patients including AD (Lopez-Arrieta and Birks, 2002). MEM-1003 (Memory Pharmaceuticals, Montvale, New Jersey, USA; http://www.Memrypharma.com/) is a nimodipine-related neuronal L-type calcium channel antagonist. Phase IIa clinical trial has recently been completed (NCT00257673), but failed to show significant improvements in patients (Hareyan, 2007). Evidence from NMDA receptor antagonists and Ca^{2+} channel blockers indicates that decreased Ca^{2+} flux into neurons may benefit AD patients.

Indeed, classic therapies that are currently used in AD patients aim to compensate the level of acetylcholine also cause alteration in Ca^{2+} homeostasis. FAD-approved acetylcholinesterase (AChE) inhibitors e.g. Donepezil, Galatamine, and Rivastigmine inhibit degradation of acetylcholine and therefore increase acetylcholine concentrations in the brain which is believed to associate with improvement in cognitive functions. In fact, the AChE inhibitors will cause an increase opening of acetylcholine receptors,

which are receptor-activated Ca^{2+} channels themselves. The two major classes of FAD-approved AD drugs (NMDA receptor antagonists and AChE inhibitors) apparently will have opposite effects on cytosolic Ca^{2+} concentration, implying that there is evidence for both increased and decreased cytosolic Ca^{2+} in AD.

Dimebon (Latrepirdine) (Medivation Inc., San Francisco, CA) is an antihistamine drug used in Russia (Bachurin et al., 2001). Recent studies have discovered the novel role of Dimebon as a neuroprotective agent as well as a cognition-enhancing agent (Bachurin et al., 2001). As an antagonist of NMDAR and Ca^{2+} channels, Dimebon protects neurons by preventing NMDA and Ca^{2+}-induced neurotoxicity (Bachurin et al., 2001). On the other hand, it also increases the level of acetylcholine by inhibiting the acetylcholinesterase (Bachurin et al., 2001). Phase II clinical trial reported that Dimebon is well tolerated and exhibit significant improvements in patients with mild to moderate AD (Doody et al., 2008). However, a recent Phase III clinical trial failed to show the same promising results (Neale, 2010). Additional Phase III clinical trials of Dimebon are still on-going at the moment; therefore the effectiveness of Dimebon in AD remains debatable.

Most of the current AD treatments such as AChE inhibitors can provide a one-time elevation of cognitive performance. However, the decline of cognitive ability from

this elevated level will occur with the same speed as in non-treated patients. This urges researchers to seek for disease-modifying drugs.

3. Mitochondrial Ca^{2+} governs neuronal life and death pathways

Mitochondria are important in maintaining neuronal Ca^{2+} homeostasis. Normal mitochondrial functions are extremely important for neurons, as neuronal activities such as synaptic transmission and axonal transport require high level of energy. In particular, mitochondrial Ca^{2+} levels are crucial for maintaining cellular functions including bioenergetic metabolism. Excessive Ca^{2+} uptake into mitochondria results in rupture of outer mitochondria membrane, which may then lead to initiation of apoptosis. However, this phenomenon is likely to occur only *in vitro*. The regulatory systems maintaining the mitochondrial Ca^{2+} homeostasis thus provide an attractive therapeutic target in treating AD. In the following sections we will explain how mitochondrial Ca^{2+} is involved in life and death pathways of the cell (Fig.1), and how mitochondrial Ca^{2+} is linked to AD.

3.1 The cell life pathway: Physiological roles of mitochondrial Ca^{2+} uptake

Ca^{2+} uptake into mitochondria plays a key role in cellular ATP production and mitochondrial motility. Bioenergetic metabolism in mitochondria highly relies upon Ca^{2+}.

In the mitochondrial matrix, activity of the metabolic enzymes involved in the Krebs cycle (pyruvate, α-ketoglutarate, and isocitrate dehydrogenases) are all Ca^{2+}-dependent (Rizzuto et al., 2000). Ca^{2+} directly regulates α-ketoglutarate and isocitrate dehydrogenases, whilst pyruvate dehydrogenases are activated by Ca^{2+}-dependent phosphatases (Rizzuto et al., 2000). Ca^{2+} concentration in mitochondria therefore determines the rate of ATP synthesis for the cell.

Mitochondria are mobile organelles which travel along the axons to regions of increased energy need in the cell, such as synapses (Chang et al., 2006; Hollenbeck and Saxton, 2005). Microtubules-dependent mitochondrial motility is regulated by the kinesin1/Miro/Milton complex (Glater et al., 2006; Guo et al., 2005; Stowers et al., 2002). Miro (mitochondrial Rho GTPase) is a mitochondrial outer membrane protein. The activity of Miro is Ca^{2+}-dependent due to the presence of a pair of Ca^{2+}-binding EF hand motifs (Frederick et al., 2004). Milton is a cytoplasmic protein which binds with Miro to form a protein complex that links kinesin-1 to mitochondria for anterograde transport (Glater et al., 2006; Guo et al., 2005; Stowers et al., 2002). The Ca^{2+}-binding EF-hand domain of Miro is essential for Ca^{2+}-dependent mitochondrial movement and elevated Ca^{2+} causes kinesin heavy chain to dissociate with microtubules, suppressing mitochondrial motility (Wang and Schwarz, 2009). Ca^{2+}-dependent mitochondria motility

is crucial for distribution of mitochondria in neurons. It recruits mitochondria to cellular regions with the need of ATP supply and Ca^{2+} buffering e.g. activated synapses (Macaskill et al., 2009).

In addition, Miro is essential for regulation of mitochondrial morphology. At resting low cytosolic Ca^{2+} levels, it facilitates the formation of elongated mitochondria by inhibiting dynamin-related protein 1 (Drp-1 or dynamin-like protein 1, DLP-1)-mediated fission (Saotome et al., 2008). On the other hand, high cytosolic Ca^{2+} triggers fragmentation and shortening of mitochondria (Saotome et al., 2008). Miro-mediated redistribution of mitochondria has also been shown to increase their ability to accumulate Ca^{2+} (Saotome et al., 2008). Evidence from the above studies demonstrates that Miro acts as a cytosolic Ca^{2+}-dependent regulator of mitochondrial dynamics. Meanwhile, calcineurin, a Ca^{2+}-dependent phosphatases, has been shown to regulate the translocation of cytosolic Drp-1 via dephosphorylation during fission (Cereghetti et al., 2008).

Clearly, Ca^{2+} regulates motility, distribution, morphology and functions of mitochondria in physiological conditions. It is therefore crucial to maintain mitochondrial Ca^{2+} homeostasis for normal cellular functioning. If this homeostasis is disrupted, a death signal can be resulted.

3.2 The cell death pathway: mitochondrial Ca^{2+} overload triggers intrinsic apoptosis

The physiological Ca^{2+} signal can switch to a death signal when the Ca^{2+} level is beyond the physiological threshold. Hence, excessive Ca^{2+} uptake into mitochondria can be lethal. The intrinsic (mitochondrial) pathway of apoptosis is triggered by intracellular stress, such as Ca^{2+} overload and oxidative stress (Galluzzi et al., 2009). Mitochondria integrate pro- and anti-apoptotic signals and determine the fate of the cell. If death signals predominate, mitochondrial-membrane-permeabilization (MMP) occurs, and large conductance permeability-transition-pores (PTP) opens (Galluzzi et al., 2009). PTP opening allows uncontrolled entry of solutes and water into the mitochondrial matrix by osmotic forces (Galluzzi et al., 2009). This causes mitochondria to swell and leads to rupture of the outer mitochondria membrane, releasing proteins from the intramembrane space e.g. cytochrome c into the cytosol (Galluzzi et al., 2009). MMP results in mitochondrial depolarization, uncoupling of oxidative phosphorylation, overproduction of ROS and release of pro-apoptotic proteins to the cytosol, eventually leading to cell death. When MMP is permanent and numerous mitochondria are continuously affected, neurons can no longer cope with the stress and apoptosis is initiated (Galluzzi et al., 2009). Physiological mitochondrial Ca^{2+} concentrations do not induce PTP opening, but

will work in synergy with pro-apoptotic stimuli (Rizzuto et al., 2009). The "double hit" hypothesis proposes that apoptotic stimuli have dual targets (Pinton et al., 2008). On one hand, it causes Ca^{2+} release from the ER and subsequent Ca^{2+} uptake by mitochondria. On the other hand, it makes mitochondria more sensitive to potential Ca^{2+} damaging effects (Pinton et al., 2008).

The above pathways are summarized in Fig. 1. Given the dual roles of mitochondria Ca^{2+} in neurons, we will critically discuss the possibility of modulating Ca^{2+} in mitochondria as a potential pharmacological target for AD in this review.

4. Mitochondrial Ca^{2+} handling and AD

Mitochondrial dysfunction is a prominent feature in AD. Aβ has been found in mitochondria of AD brain and transgenic mouse model of AD overexpressing Aβ. Aβ peptides accumulate in mitochondria and are associated with oxidative stress, disrupted Ca^{2+} homeostasis, impaired energy metabolism and induction of apoptosis (Mattson et al., 2008). Mitochondria from aged cerebellar granular neurons are depolarized and less efficient in handling Ca^{2+} load (Toescu and Verkhratsky, 2007). Cortical mitochondria from 12 month-old mice also show a reduced capacity for Ca^{2+} uptake when challenged with $CaCl_2$ pulses, compared to that of 6-month-old mice (Du et al., 2008). Mitochondria

isolated from fibroblasts of AD patients shows reduced Ca^{2+} uptake compared to age-matched control, suggesting that Ca^{2+} buffering ability may be impaired in the mitochondria of AD fibroblasts (Kumar et al., 1994). Following oxidative stress, the increase in Ca^{2+} uptake in mitochondria of AD fibroblasts is much greater than that in control, implicating that mitochondria from AD fibroblast have a higher sensitivity towards oxidative stress (Kumar et al., 1994). Mitochondria with overexpression of human APP also show a lower Ca^{2+} capacity compared to non-transgenic mitochondria (Du et al., 2008). $A\beta_{1-42}$ oligomer induces Ca^{2+} overload in mitochondria in both cerebellar granule and cortical neurons (Sanz-Blasco et al., 2008). The increase is limited to a pool of mitochondria close to the sites of Ca^{2+} entry and release (Sanz-Blasco et al., 2008). Ca^{2+} overload in mitochondria causes increased ROS production and the impairment of bioenergetic metabolism which eventually leads to cell death. Mutations in presenilins may promote mitochondrial dysfunction by perturbing ER Ca^{2+} handling, which promotes synaptic mitochondrial Ca^{2+} overload and in turn triggers apoptosis. A recent study has also shown that mutated CALHM1 may cause slower kinetics of mitochondrial Ca^{2+} uptake and release, increasing the risk of mitochondrial Ca^{2+} overload (Moreno-Ortega et al., 2010).

The importance of mitochondrial Ca^{2+} in apoptosis has been emphasized in neuronal death in AD. However, mitochondrial Ca^{2+} is also important in earlier stages of the disease. The rupture of mitochondrial membrane caused by Ca^{2+} overload reduces the number of "healthy" mitochondria, and this will affect crucial neuronal functions including synaptic transmission and axonal transport. This could perhaps account for some of the early symptoms of the disease e.g. memory impairment. In this notion, the maintenance of mitochondrial Ca^{2+} homeostasis is important for both early and later stages of the disease. In the following paragraphs, we will illustrate different influx and efflux pathways regulating the mitochondrial Ca^{2+} homeostasis, and how different agents targeting these pathways can provide neuroprotection in AD.

5. Mitochondria in neuronal Ca^{2+} signaling

Ca^{2+} signaling causes transient changes in cytosolic Ca^{2+} concentration. Mitochondria rapidly take up Ca^{2+} when a physiological stimulus elicits an increase in cytosolic Ca^{2+} concentrations. This uptake machinery allows mitochondria to act as "Ca^{2+} buffers" to maintain the normal homeostasis. At the same time, it also provides Ca^{2+} for various mitochondrial functions. Mitochondrial Ca^{2+} signaling therefore plays an

important role in determining the fate of neurons. Mitochondria possess various Ca^{2+} influx and efflux pathways (Fig.2), which provide attractive targets for manipulation of Ca^{2+} concentrations within the organelle (Table 1).

5.1 Pathways for Ca^{2+} uptake

5.1.1 Voltage-gated anion channel regulates Ca^{2+} uptake in the outer mitochondrial membrane

The outer mitochondrial membrane (OMM) is relatively permeable to Ca^{2+} due to the high conductance voltage dependent anion channel (VDAC) located in this membrane. Overexpression of VDAC has been shown to promote Ca^{2+} uptake into mitochondria (Rapizzi et al., 2002). Closure of enhances Ca^{2+} influx into mitochondria, thereby promotes mitochondrial permeability transition and subsequent cell death (Rizzuto et al., 2009; Rostovtseva et al., 2005; Tan and Colombini, 2007).

5.1.2 Mitochondrial membrane potential regulates Ca^{2+} entry via the uniporter in the inner mitochondrial membrane

In the inner mitochondrial membrane (IMM), the mitochondrial Ca^{2+} uniporter regulates Ca^{2+} entry into mitochondria. The uniporter is a highly selective divalent cation

channel (Kirichok et al., 2004). The electron transport chain (ETC) in the IMM consists of five protein complexes for the production of ATP. The ETC maintain an electrochemical gradient of -180 mV across the IMM, and is known as the mitochondrial membrane potential ($\Delta\Psi_m$). $\Delta\Psi_m$ provides a driving force for Ca^{2+} to enter the mitochondria via the uniporter. Given that mitochondrial Ca^{2+} overload can lead to cell death, depolarization of $\Delta\Psi_m$ (hence reduced driving force for Ca^{2+} entry) can be a drug target for stopping excessive Ca^{2+} from entering mitochondria.

5.2 Pathways for calcium efflux

5.2.1 Antiporters and permeability transition pores for mitochondrial calcium sequestration

Besides various Ca^{2+} uptake systems mentioned, there are also a few pathways for Ca^{2+} efflux. The Na^+/Ca^{2+} and H^+/Ca^{2+} antiporters are two main routes for Ca^{2+} release from mitochondria. Generally, $3Na^+$ and $3H^+$ enter mitochondria via the respective antiporters when a Ca^{2+} is extruded (Fig.2). Hence, concentrations of Na^+ and H^+ can affect Ca^{2+} concentration in the mitochondria. These efflux pathways can become saturated when there is high Ca^{2+} concentration in the matrix, which can lead to mitochondrial Ca^{2+} overload (Rizzuto et al., 2009). As mentioned earlier, mitochondrial

Ca^{2+} overload triggers opening of PTP which locates across the OMM and IMM. The molecular identity of PTP is still uncertain, but it is suggested to be a multimeric complex composed of the VDAC, an integral protein called adenine nucleotide translocase (ANT) on the IMM, and a matrix protein called cyclophilin D (CypD). However, mitochondria lacking VDAC (Szalai et al., 2000) and ANT (Kokoszka et al., 2004) have been shown to undergo Ca^{2+}-induced PTP opening, implying that the two components may not be prerequisite for MPT (Rizzuto et al., 2009). PTP is a non-selective channel of which operation is dependent on the mitochondrial matrix Ca^{2+}. High Ca^{2+} levels in the mitochondrial matrix activate transolocation of CypD to the IMM. CypD binds to ANT and inhibits ATP/ADP binding, thereby inducing opening of PTP (Rizzuto et al., 2009).

5.3 ER/mitochondria calcium crosstalk is important for efficient mitochondrial calcium signaling

Mitochondria rapidly take up Ca^{2+} released from the ER. The proximate juxtaposition between these two organelles ensures efficient Ca^{2+} transfer (Rizzuto et al., 1993; Rizzuto et al., 1998). In fact, the contact between the ER and mitochondria is estimated to be 5-20% of the total mitochondrial surface (Rizzuto et al., 1998). MAM is a region between the ER and mitochondria enriched with enzymes and proteins involved in

lipid biosythesis and Ca^{2+} signaling between the organelles (Vance, 1990). Indeed, VDAC on the OMM is located in the interface between the ER and mitochondria. Hence, MAM also involves in intracellular communication and delivery of Ca^{2+} between the organelles. Outside the mitochondria, glucose-regulated protein 75 (grp75) mediates the interactions of VDAC and IP_3R on the ER membrane to regulate Ca^{2+} uptake into mitochondria (Szabadkai et al., 2006). The interaction of sigma-1 localizes on the MAM and grp 78 (BiP) is crucial in regulating the integrity between the ER and mitochondria (Hayashi and Su, 2007). A family of fission and fusion proteins regulating mitochondrial morphology is also important for maintaining ER-mitochondrial Ca^{2+} coupling. Genetic ablation of mitofusin 2 causes an increase in distance between the ER and mitochondria, resulting in less efficient mitochondrial Ca^{2+} uptake (de Brito and Scorrano, 2008). This provides genetic evidence supporting the Ca^{2+} microdomains theory, which proposes that mitochondria preferentially accumulate at "microdomains" of high Ca^{2+} concentrations (Rizzuto and Pozzan, 2006). Ca^{2+} microdomains refer to localized areas with increased cytosolic Ca^{2+} that does not generalize to the whole cell cytoplasm (Rizzuto and Pozzan, 2006). Microdomains enriched in IP_3Rs and can be found between mitochondria and the cytosolic mouth of Ca^{2+} channels, localized either in the neighboring ER or in the plasma membrane (Rizzuto and Pozzan, 2006). These microdomains allow efficient Ca^{2+} uptake

into mitochondria. Increased levels of Ca^{2+} in those contact points will then be rapidly diffused into other mitochondria.

6. Potential targets for mitochondrial Ca^{2+} modulation

6.1 Modulating mitochondrial calcium uptake via VDAC to attenuate calcium overload

VDAC is highly permeable at low potentials (10 mV) (Shoshan-Barmatz and Gincel, 2003), and is relatively "closed" at higher potentials. VDAC can also be modulated by various proteins and cytosolic compounds, including Bcl-2 family of proteins (Shimizu et al., 2000; Shimizu et al., 1999; Vander Heiden et al., 2001), metabolic enzymes such as hexokinase (Pastorino and Hoek, 2008), and the cytoskeletal protein tubulin (Rostovtseva et al., 2008).

Minocycline is an antibiotic derived from tetracycline and is a potential therapeutic agent in various neurological diseases (Garcia-Martinez et al., 2010). It has been shown that minocycline can act as a modulator of VDAC (Garcia-Martinez et al., 2010). Minocycline reduces the conductance and voltage dependence state of VDAC (Garcia-Martinez et al., 2010). However, it is unclear if these modulations can reduce Ca^{2+} influx via VDAC.

6.2 Reduce mitochondrial Ca^{2+} uptake by mitochondrial membrane depolarization to inhibit calcium overload

As mentioned earlier, Ca^{2+} entry to the mitochondria is highly dependent on $\Delta\Psi_m$. FCCP [carbonyl cyanide-p-(trifluoromethoxy) phenylhydrazone] is a protonophore and potent uncoupler of oxidative phosphorylation. It depolarizes the mitochondrial membrane and inhibits mitochondrial Ca^{2+} uptake. FCCP has been shown to inhibit mitochondrial Ca^{2+} elevation triggered by $A\beta_{1-42}$ oligomers (Sanz-Blasco et al., 2008). FCCP-induced inhibition of mitochondrial Ca^{2+} uptake also attenuates both cytochrome c release and cell death without affecting cellular levels of ATP (Sanz-Blasco et al., 2008). These results suggest a possible neuroprotective mechanism against Aβ-induced neurotoxicity by depolarizing the mitochondrial membrane, thereby attenuating mitochondrial Ca^{2+} overload. Indeed, uncouplers such as FCCP and 2-4 dinitrophenolas are dangerous drugs due to their high risk of intoxication. Allosteric modulators of uncoupling proteins would be a much safer alternative approach to induce pharmacological reduction of mitochondrial membrane potential.

An early report showing that patients suffering from rheumatoid arthritis has a low risk of developing AD leads to a hypothesis that there is chronic neuroinflammation

in AD brains and anti-inflammatory agents maybe neuroprotective (McGeer et al., 1990). Non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin have been shown to reduce the degree of cognitive decline in AD patients (Rogers et al., 1993). The effectiveness of NSAIDs in AD has been challenged by negative results from clinical trials (McGeer et al., 2006). Nevertheless, a recent study has shown a novel neuroprotective mechanism by NSAIDs. Salicylate, R-flurbiprofen and indomethacin induce depolarization of the mitochondrial membrane, which then reduce Ca^{2+} entry into mitochondria (Sanz-Blasco et al., 2008). However, a direct action of NSAIDs on mitochondrial membrane potential has not been well established. In addition, a recent Phase III clinical trial with R- flurbiprofen showed negative results to treat AD patients. The failure from using NSAIDs as an AD treatment may suggest that a more specific but mild potent compound which modulates uncoupling proteins may be the future therapeutic target.

KB-R7943 is a selective inhibitor of the Na^{+}/Ca^{2+} exchanger. It causes depolarization of isolated brain mitochondria and reduces mitochondrial Ca^{2+} uptake (Storozhevykh et al., 2009). Furthermore, KB-R7943 has been shown to inhibit mitochondrial Ca^{2+} uptake in permeablized HeLa cells (Santo-Domingo et al., 2007). However, the mechanism of how KB-R7943 induces depolarization is not clear

(Storozhevykh et al., 2009). Similarly, minocyline has also been shown to induce depolarization of the mitochondrial membrane which reduces NMDA-induced Ca^{2+} overload in the mitochondria (Garcia-Martinez et al., 2010).

Taken together, depolarization of the mitochondrial membrane can be a possible way to inhibit Ca^{2+} uptake into the mitochondria. By reducing Ca^{2+} entry, the risk of mitochondrial Ca^{2+} overload can be lowered. However, the underlying mechanism of the depolarizing effect by the above drugs is still awaited to be elucidated. A possible target would be the components of the ETC which regulate $\Delta\Psi_m$. Further studies on how the membrane is depolarized by the drugs are required.

6.3 Modulation of uniporter calcium uptake efficiency attenuates excessive calcium entry

$\Delta\Psi_m$ establishes a driving force for Ca^{2+} entering mitochondria via the uniporter on the IMM. The activity of uniporter is regulated by extra-mitochondrial Ca^{2+} (Kroner, 1986), and increase in cytosolic Ca^{2+} can both activate and inactivate mitochondrial Ca^{2+} uptake (Rizzuto et al., 2009). The uniporter is readily inhibited by Ruthenium Red and is also regulated by adenine nucleotides (Bernardi, 1999; Litsky and Pfeiffer, 1997) and plant flavonoids (Montero et al., 2004). Protein kinases are also important regulators of

the uniporter. Treatment with SB202190, a specific inhibitor of α and β isoforms of p38 mitogen-activated protein (MAP) kinase has been shown to increase the rate of Ca^{2+} uptake by mitochondria (Montero et al., 2002). The results suggest that p38 MAP kinase may inhibit the opening of uniporter. Protein kinase C has dual effects on Ca^{2+} uptake by the uniporter: while the ζ isoform activates the uniporter, the β and δ isoforms inacitvate it. Taken the above reports together, uniporter on mitochondria is able to be modulated by numerous pharmacological interventions. Careful consideration has to be taken regarding the specificity of these interventions.

6.4 Inhibition of permeability transition pore opening to inhibit induction of apoptosis

Dimebon has been shown to inhibit the opening of PTP induced by $A\beta_{25-35}$ (Bachurin et al., 2003). However, the mechanism of Dimebon is not specific to mitochondria (Bachurin et al., 2001). In addition, it is not known whether inhibition of PTP opening would have any effect on mitochondrial Ca^{2+} homeostasis.

The abundance of CypD is associated with the vulnerability of the mitochondrial PTP to Ca^{2+} (Du et al., 2008). The immunosuppressant Cyclosporine A (CsA) binds to CypD and inhibit its translocation to the IMM and subsequent induction of PTP opening

(Rizzuto et al., 2009). Pre-treatment of CysA has been shown to increase mitochondrial Ca^{2+} buffering capacity in wild type and mutant amyloid precursor protein (mAPP) transgenice mice (Du et al., 2008). Moreover, mitochondria isolated from CypD deficient mAPP mice have a higher Ca^{2+} uptake capacity than that of mAPP mice (Du et al., 2008). CypD deficient mitochondria are resistant to both Aβ- and Ca^{2+}-induced mitochondrial swelling and PTP opening (Du et al., 2008). This result shows that the absence of CypD protects neurons from Aβ-induced cell death. Blockade of CypD also improves learning and memory in AD mice (Du et al., 2008), implying that inhibition of CypD can be a potential therapeutic target for treatment of AD.

6.6 Modifying calcium release from the ER to reduce calcium uptake into mitochondria

Bcl-2 family proteins can form ion channel in membranes (Minn et al., 1997; Schendel et al., 1998), and affect Ca^{2+} homeostasis in the ER and mitochondria. Over-expression of Bcl-2 causes an increase in Ca^{2+} leak and thereby reduces the amount of Ca^{2+} stored in the ER (Pinton et al., 2000). This in turn reduces the risk of Ca^{2+} overload in mitochondria as there is less Ca^{2+} available for uptake. Agents reducing ER Ca^{2+} release may thus reduce the risk of mitochondrial Ca^{2+} overload.

6.7 Enhancement of mitochondria activity as a drug target for AD

Mitochondrial defects are implicated in many neurodegenerative diseases including PD and AD. New therapeutic approaches have now begun to target mitochondria as a potential drug target (Chaturvedi and Beal, 2008). So far, we have mentioned different ways to reduce Ca^{2+} uptake in order to prevent excessive Ca^{2+} from entering mitochondria. As mitochondria act as Ca^{2+} buffers in the cell, a second approach to prevent Ca^{2+} overload is to increase the buffering capacity of mitochondria.

Agents such as Creatine protect neurons from glutamate- and Aβ-induced toxicity by providing energy reserves (Brewer and Wallimann, 2000). In PD animal models, antioxidants such as mitoQ (mitoquinone) and Coenzyme Q10 (CoQ10) selectively prevent mitochondrial oxidative damage (Chaturvedi and Beal, 2008). CoQ10 has also been shown to exhibit anti-amyloidogenic effects (Chaturvedi and Beal, 2008). These antioxidant agents may enhance the efficiency of ETC, hence results in better maintenance of mitochondrial membrane potential and therefore ATP production. Mitochondrial Ca^{2+} overload is not just dependent on mitochondrial Ca^{2+} concentration but may also depends on mitochondrial energy and redox state. These antioxidants may therefore indirectly increase the mitochondrial buffering capacity by indirectly preventing

the induction of PTP opening through increased mitochondrial calcium. Taurine is another example that can increase the capacity of mitochondria to sequester Ca^{2+} when the cells are challenged by agents that cause an increase in cytosolic Ca^{2+} (El Idrissi, 2008). As all the proposed drug candidates above have not gone through Phase III clinical trials of PD or AD, their relevance to the diseases remains obscure.

6.8 Other potential agents

Tournefolic acid B (TAB) is a polyphenolic anti-oxdative compound extracted from *Tournefortia sarmentosa* Lam, which is widely used as deoxicants and anti-inflammatory agents in Taiwan (Chi et al., 2008). TAB significantly decreases the $A\beta_{25-35}$-induced elevation of mitochondrial Ca^{2+} in cortical neurons (Chi et al., 2008). TAB also blocks the $A\beta_{25-35}$-induced cytochrome c release from mitochondria and the generation of mitochondrial protein tBid (Chi et al., 2008). The exact mechanism of how TAB attenuates mitochondrial Ca^{2+} uptake remains unclear.

7. Discussions and future directions

Mitochondria play a crucial role in determining the fate of cells. When mitochondrial Ca^{2+} concentration is within the physiological limit, Ca^{2+} activates ATP

production and regulates other mitochondrial functions. However, when the cell is challenged by apoptotic stimuli and mitochondria become overwhelmed with Ca^{2+}, the intrinsic pathway of apoptosis is initiated. Mitochondrial Ca^{2+} concentration therefore plays a key role in switching between life and death signal. There is evidence that mitochondrial Ca^{2+} homeostasis is altered in AD and may even contribute to the cognitive deficits in AD. This leads to the hypothesis that modulating the level of mitochondrial Ca^{2+} by various pathways can be beneficial for patients suffering from AD. Mitochondrial Ca^{2+} handling provides an exciting and interesting drug target. Of all the drugs we have discussed so far, up-to-date, FCCP and cyclosporine are the drugs which have a specific and clearly identified action on mitochondria.

Nonetheless, at the moment it is not clear whether altering mitochondrial Ca^{2+} homeostasis represents a viable therapeutic strategy for AD. The biggest challenge now is to understand more about mitochondrial Ca^{2+} homeostasis at a molecular level, especially the molecular identity of the Ca^{2+} uniporter, Na^{+}/Ca^{2+} and H^{+}/Ca^{2+} exchangers. Moreover, the molecular composition of PTP is unclear. Additional Ca^{2+} uptake mechanisms such as the rapid mode of Ca^{2+} uptake and mitochondrial ryanodine receptors have been demonstrated in mitochondria from other tissues in the body e.g. the heart. Nevertheless, the role of these Ca^{2+} uptake modes in neuronal mitochondria is yet to be explored.

Regarding the role of Ca^{2+} in mitochondria, there is so much to be explored: e.g. how Ca^{2+} can be switched from physiological to pathological and how mitochondrial Ca^{2+} signaling is affected when the tethering between ER and mitochondria is disrupted? With more research in these areas, it is more likely for us to design viable drugs targeting the mitochondrial Ca^{2+} pathways. Designing drugs that can specifically target mitochondrial Ca^{2+} homeostasis in neurons is challenging. It is important that the drug can be specifically delivered to neurons; otherwise it is likely to alter mitochondrial Ca^{2+} homeostasis in other tissues as well, including heart, muscle and liver. This will result in severe side effects. In this case, special central nervous system (CNS) drug delivery systems such as intranasal administration provides a potential drug delivery method (Illum, 2004).

Majority of the studies investigating the neuroprotective effect of modulating mitochondrial Ca^{2+} handling are based on AD models induced by Aβ. Future studies on the molecular basis of mitochondrial Ca^{2+} handling in other areas in AD e.g. tau and AD animal models will definitely give us a clear picture.

In addition to the points above, there are some important questions we have to critically consider when designing drugs that alter mitochondrial Ca^{2+}:

Decrease or increase mitochondrial Ca^{2+} uptake?

A number of studies mentioned have shown that by reducing Ca^{2+} influx into mitochondria, the risk of Ca^{2+} overload is lowered and induction of apoptosis can be attenuated (Garcia-Martinez et al., 2010; Sanz-Blasco et al., 2008). However, other studies have shown that by increasing the Ca^{2+} buffering capacity of mitochondria, more Ca^{2+} are sequestered from the cytoplasm, and thus neurons can be protected (Du et al., 2008; El Idrissi, 2008). It is still unclear whether increasing or reducing mitochondrial Ca^{2+} uptake is a better approach for neuroprotection. Both approaches have their own reasons, but there are a few points we have to carefully consider. If the modulations allow less Ca^{2+} entering the mitochondria, it is important to make sure that the reduced Ca^{2+} uptake will not affect Ca^{2+}-dependent physiological functions such as ATP production. At the same time, an important question is how the excessive cytosolic Ca^{2+} will be extruded if there is less Ca^{2+} uptake by mitochondria. In this case, additional Ca^{2+} buffering system in the cytoplasm would be needed. For the latter approach, it is crucial to ensure that the increased mitochondrial Ca^{2+} uptake will not exceed the threshold which triggers cell death pathways. In this case, neuroprotective agents that can increase or retain the activity of mitochondria will be useful to ensure normal mitochondrial

function. The excessive Ca^{2+} taken by mitochondria can then be used for metabolic activities of mitochondria.

In either case, we have to make sure that the normal Ca^{2+}-dependent mitochondrial functions such as ATP production and mitochondrial dynamics will not be affected while we are manipulating mitochondrial Ca^{2+} concentrations.

Heterogeneity of mitochondrial response

The microdomain hypothesis suggests that those mitochondria close to Ca^{2+} channels and ER stores are vulnerable to take up Ca^{2+} (Csordas et al., 2006; Rizzuto and Pozzan, 2006). It is interesting to study if the distance between the ER and mitochondria determines the vulnerability of mitochondria to Ca^{2+} overload? Moreover, how does the Ca^{2+} overload in one mitochondrion spread to other mitochondria? When considerable amount of mitochondria undergo membrane permeabilization, irreversible cell death mechanism is initiated. In this notion, would it be possible to attenuate Ca^{2+} overload among mitochondria to avoid cell death? Mitochondria have a quality control mechanism called mitophagy in which damaged mitochondria are selectively eliminated by autophagy (Lemasters, 2005). Recent work has demonstrated that NIX, ULK1 and Parkin are involved in regulation of mitophagy in mammalian cells (Tolkovsky, 2009).

However the exact molecular mechanism and how mitophagy is initiated remains unclear. It is important to understand whether mitophagy can serve as a protective mechanism prior initiation of apoptosis.

8. Conclusions

At this point, there is still no single drug that can provide a cure for AD. Although there is evidence supporting the role of modulating mitochondria Ca^{2+} in neuroprotection, whether this approach can be an effective treatment for AD remains obscure. A combination with other drugs which aim to increase the ability of neurons for synaptic transmission and modulate the cytosolic calcium homeostasis may be beneficial in treating AD. For future development of drugs targeting mitochondrial Ca^{2+}, agents that can enhance the activity of mitochondria should also be applied to increase the ability of mitochondria to buffer the excessive Ca^{2+}.

AGENT/DRUG	SITE OF ACTION	EFFECT	MODEL	NEUROTOXICITY MODEL	REFERENCE
FCCP	ΔΨ	Depolarization Reduce Ca^{2+} uptake	Rat cerebellar granule neurons Rat cortical neurons	$A\beta_{1-42}$ oligomer	Sanz-Blasco et al. (2008)
NSAIDS	ΔΨ	Depolarization Reduce Ca^{2+} uptake	Rat cerebellar granule neurons	$A\beta_{1-42}$ oligomer	Sanz-Blasco et al. (2008)
Minocycline	VDAC ΔΨ	Depolarization Reduce Ca^{2+} uptake	Rat cerebellar granule neurons	NMDA	Garcia-Martinez et al. (2010)
KB-R7943	Na^{+}/Ca^{2+} exchanger	Reduce Ca^{2+} uptake	Rat cerebellar granule neurons	Glutamate	Storozhevykh et al. (2009)
TAB	Unknown	Reduce Ca^{2+} uptake	Rat cortical neurons	$A\beta_{25-35}$	Chi et al. (2008)
Dimebon	mPTP	Inhibit mPTP opening	Rat liver mitochondria	$A\beta_{25-35}$	Bachurin et al. (2003)
Cyclosporin A	Cyclophilin D	Inhibit mPTP opening Increase Ca^{2+} buffering capacity	Mouse cortical mitochondria	mAPP Trangenic mice	Du et al. (2008)

Table 1. Current agents showing neuroprotective effect via modulation of mitochondrial Ca^{2+} concentrations. ΔΨ (mitochondrial membrane potential); Ca^{2+} (calcium ions); FCCP [carbonyl cyanide-p-(trifluoromethoxy) phenylhydrazone]; mAPP (mutant amyloid precursor protein); mPTP (mitochondrial permeability transition pore); NMDA (N-methyl D-aspartate); NSAIDs (non-steroid anti-inflammatory drugs), TAB (Tournefolic acid B); VDAC (voltage-dependent anion channel).

Fig. 1. Life and death pathways of mitochondrial Ca^{2+} accumulation. Left: Under normal conditions ,Ca^{2+} influx from extracellular matrix or Ca^{2+} release from the ER causes increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) . Mitochondria rapidly take up cytosolic Ca^{2+}, which is crucial for life processes such as mitochondrial movement, Ca^{2+} homeostasis and bioenergetic metabolism. Right: When mitochondria are overloaded with Ca^{2+}, mitochondrial permeability transition pores will be triggered to open. Several pro-apoptotic factors will be released to the cytosol, thereby inducing apoptosis.

Fig. 2. Mitochondrial Ca^{2+} signaling pathways. $\Delta\Psi_m$ (mitochondrial membrane potential); $[Ca^{2+}]_m$ (mitochondrial Ca^{2+} concentration); $[Ca^{2+}]_c$, (cytosolic Ca^{2+} concentration); H^+ (hydrogen ions); PTP (mitochondria permeability transition pore); Na^+ (sodium ions), VDAC (voltage-dependent anion channel); CypD (cyclophilin D); ANT (adenine nucleotide translocase)

Acknowledgements:

The work in this laboratory is supported by GRF (755206M & 761609M) and NSFC/RGC Joint Research Scheme (N_HKU 707107M), HKU Seed Funding for Basic Science Research (200911159082), HKU Alzheimer's Disease Research Network under Strategic Theme Research for Healthy Aging, and Strategic Theme Research for Drug Discovery.

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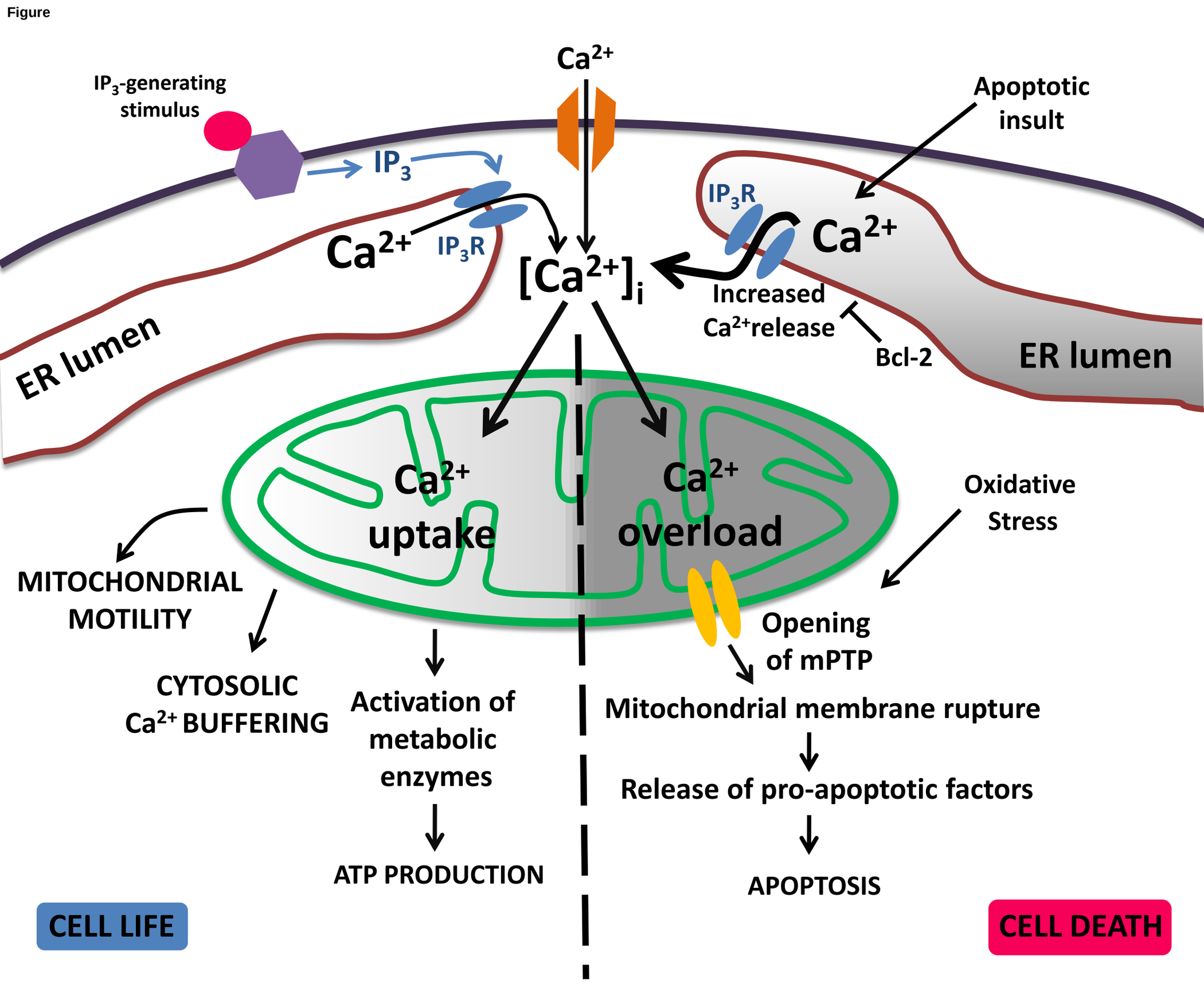
Figure


Ca2+
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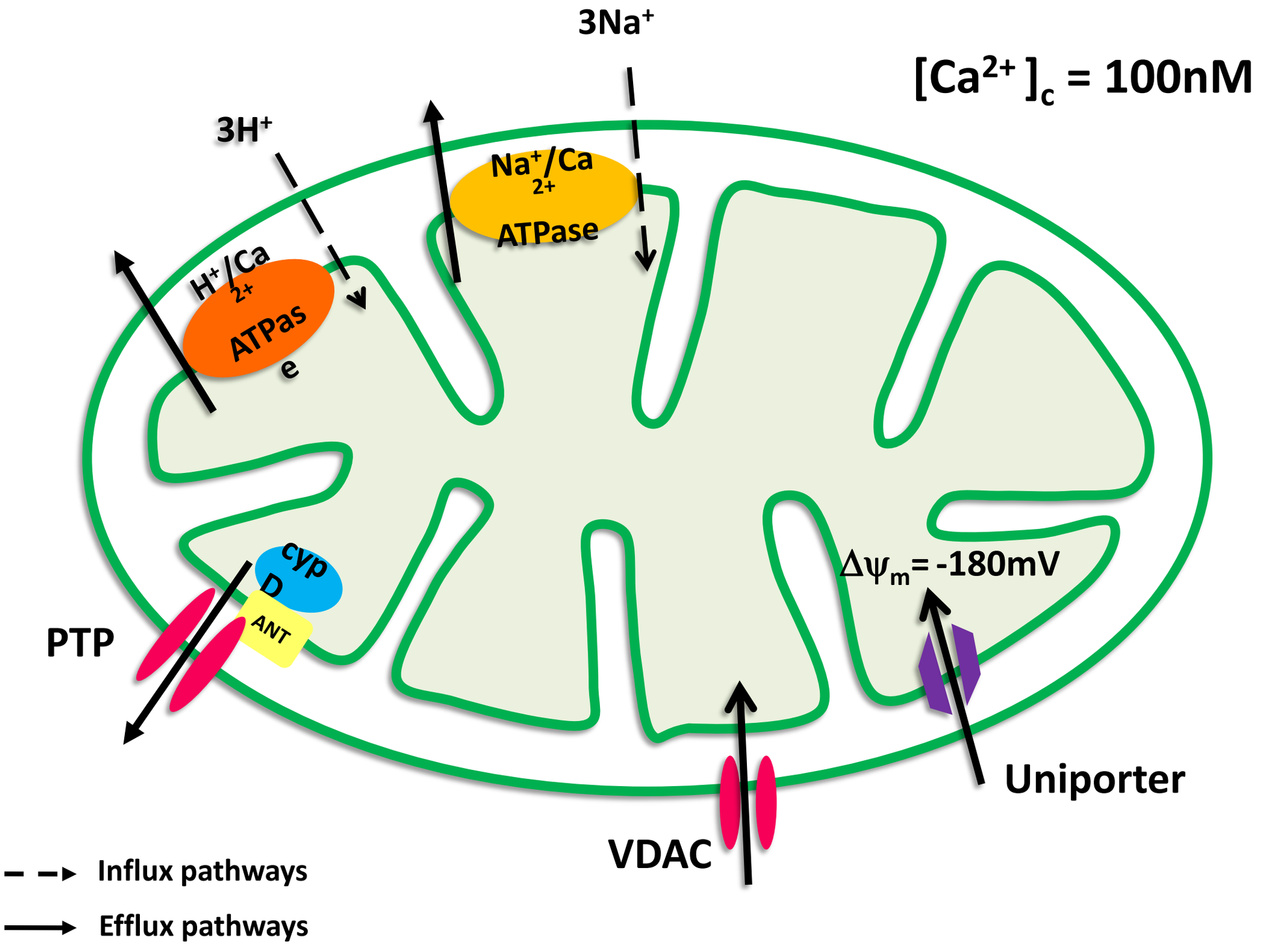
Bcl-2
ER lumen
ER lumen
Ca2+ uptake
Ca2+ overload
Oxidative Stress
MITOCHONDRIAL MOTILITY
CYTOSOLIC Ca2+ BUFFERING
Activation of metabolic enzymes
ATP PRODUCTION
Opening of mPTP
Mitochondrial membrane rupture
Release of pro-apoptotic factors
APOPTOSIS
CELL LIFE
CELL DEATH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ANT
PTP
Δψm= -180mV
Uniporter
VDAC
Influx pathways
Efflux pathways