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REVIEW

How does mitochondrial Ca^{2+} change during ischemia and reperfusion? Implications for activation of the permeability transition pore

Elizabeth Murphy[1] and David A. Eisner[2]

Cardiac ischemia followed by reperfusion results in cardiac cell death, which has been attributed to an increase of mitochondrial Ca^{2+} concentration, resulting in activation of the mitochondrial permeability transition pore (PTP). Evaluating this hypothesis requires understanding of the mechanisms responsible for control of mitochondrial Ca^{2+} in physiological conditions and how they are altered during both ischemia and reperfusion. Ca^{2+} influx is thought to occur through the mitochondrial Ca^{2+} uniporter (MCU). However, with deletion of the MCU, an increase in mitochondrial Ca^{2+} still occurs, suggesting an alternative Ca^{2+} influx mechanism during ischemia. There is less certainty about the mechanisms responsible for Ca^{2+} efflux, with contributions from both Ca^{2+}/H^{+} exchange and a Na^{+}-dependent Ca^{2+} efflux pathway. The molecular details of both mechanisms are not fully resolved. We discuss this and the contributions of both pathways to the accumulation of mitochondrial Ca^{2+} during ischemia and reperfusion. We further discuss the role of mitochondrial Ca^{2+} in activation of the PTP.

Introduction

There is great interest in the control of the calcium (Ca^{2+}) concentration in the mitochondrial matrix. An increase of mitochondrial Ca^{2+}, as often occurs with cellular activity, has been shown to activate enzymes of the tricarboxylic acid cycle and thereby ensure that ATP production is matched to the increased metabolic demand. On the other hand, an excessive increase of mitochondrial Ca^{2+} has been linked to cell death via activation of the mitochondrial permeability transition pore (PTP) (Murphy and Steenbergen, 2021). The aim of this review is to critically evaluate the hypothesis that an increase in mitochondrial Ca^{2+} during ischemia and reperfusion is responsible for initiating cell death via activation of the PTP and that inhibition of this increase in mitochondrial Ca^{2+} might be a cardioprotective strategy. Mitochondrial Ca^{2+} concentration is determined by a balance between influx and efflux, so the next section will consider the possible transport mechanisms involved.

Regulation of mitochondrial Ca^{2+}

Mitochondrial Ca^{2+} uniporter complex

Ca^{2+} enters the mitochondria via the mitochondrial Ca^{2+} uniporter (MCU) (Baughman et al., 2011; De Stefani et al., 2011). The driving force for Ca^{2+} entry by MCU is provided by the mitochondrial membrane potential ($\Delta\psi$), with the mitochondrial matrix negative by ~180 mV, which is generated by electron transport. The MCU exists in a complex (MCUC) that contains MCU, essential MCU regulator (EMRE) (Sancak et al., 2013), and three mitochondrial Ca^{2+} uptake proteins (MICU1, -2, and -3); EMRE is required for MCU activity in metazoans. MICU1 binds directly to MCU and EMRE (Paillard et al., 2018). In addition to binding to MCU and EMRE, MICU1 can either form homodimers with MICU1 or heterodimers with MICU2 or MICU3. The apparent threshold for Ca^{2+} uptake into the mitochondria is set by which MICUs compose the dimer (Csordas et al., 2013; Liu et al., 2016; Kamer et al., 2017). With homodimers of MICU1, the threshold for Ca^{2+} uptake is ~300 nM, whereas the threshold is ~600 nM for a complex with MICU1/2 heterodimers (Kamer et al., 2017). The ratio of MICU1 to MCU varies among tissues, and the dimer composition is also tissue dependent (Paillard et al., 2017). The exact mechanisms that regulate the composition of the MCUC are not well established and require further study (Csordas et al., 2013; Plovanich et al., 2013; Patron et al., 2014; Kamer et al., 2017). MCUC and its regulation are discussed in detail elsewhere (Boyman et al., 2021; Murphy and Steenbergen, 2021; Garbincius and Elrod, 2022; Rodríguez-Prados et al., 2023; Vecellio Reane et al., 2024) and are not the focus of this review.

[1]Cardiac Physiology Section, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA; [2]Unit of Cardiac Physiology, Division of Cardiovascular Sciences, University of Manchester, Manchester, UK.

Correspondence to Elizabeth Murphy: murphy1@nhlbi.nih.gov.

Efflux mechanisms

At steady state, any Ca^{2+} that enters the mitochondria on MCU must be extruded. Two mitochondrial Ca^{2+} efflux pathways have been described: a Na^+-independent mechanism generally thought to be a Ca^{2+}/H^+ exchange (CHE) and a Na^+ dependent Ca^{2+}-efflux pathway (Crompton et al., 1977; Nicholls and Crompton, 1980; Brand, 1985). The relative role of each pathway is thought to be tissue specific and the Na^+-dependent Ca^{2+} efflux mechanism is generally thought to be the primary mitochondrial Ca^{2+} efflux mechanism in cardiac cells (Wei et al., 2011; Garbincius and Elrod, 2022).

CHE

The identity of the Ca^{2+}/H^+ exchanger has been debated, and roles for both leucine zipper-EF-hand–containing transmembrane protein 1 (LETM1) and transmembrane BAX inhibitor motif protein 5 (TMBIM5) have been proposed. LETM1 was originally proposed to be a $K+/H^+$ exchanger, although later studies suggested it exchanged Ca^{2+} for H^+ (Jiang et al., 2009). It was initially reported to be electrogenic, exchanging $1H^+$ for $1Ca^{2+}$ (Jiang et al., 2009). This and many studies of pH-dependent Ca^{2+} exchange rely on studies which involve altering pH. One needs to be cautious in interpreting these studies as change in pH affect many transporters as well as the charge state of inorganic phosphate. Previous studies have shown that Ca^{2+} uptake into the mitochondria is very small in the absence of permeant anions (Wei et al., 2012). Recent studies with purified LETM1 reconstituted into liposomes suggest that LETM1 exchanges $2H^+$ for $1Ca^{2+}$ and is therefore electroneutral (Tsai et al., 2014).

Recently, three publications have suggested a different molecular identity for mitochondrial CHE (TMBIM5); although they differ in detail regarding the direction of exchange (Austin et al., 2022; Patron et al., 2022; Zhang et al., 2022). Zhang et al. (2022) examined the effect of TMBIM5 overexpression in HEK293 cells. ATP was added to release ER Ca^{2+}, and mitochondrial Ca^{2+} was measured using a mitochondrial-targeted genetically encoded Ca^{2+} indicator. Overexpression of TMBIM5 enhanced mitochondria Ca^{2+} uptake and deletion decreased mitochondrial H^+. They also generated a mouse with a mutation in a negatively charged aspartate in the pore domain (D326R), which had been shown previously to block activity. This mutation led to the degradation of the protein. Homozygous mice were born at less than Mendelian ratios and developed a skeletal muscle myopathy. In another study, Patron et al. (2022) reported that TMBIM5 transports protons into the matrix in exchange for Ca^{2+} efflux, and that with loss of TMBIM5, there is an increase in matrix pH from 7.25 in WT to 7.6, consistent with reduced exchange of matrix Ca^{2+} for cytosolic H^+. As pointed out below, this direction of net flux would be consistent with an electroneutral exchanger but not with a $1Ca^{2+}$:$1H^+$ exchanger. TMBIM5 was also shown to interact with the m-AAA protease AFG3L2 and inhibit its activity. Austin et al. (2022) also reported data showing that TMBIM5 is a mitochondrial Ca^{2+}/H^+ exchanger. In studies in which the Na^+-Ca^{2+}-Li^+ exchanger (NCLX) was either depleted or inhibited, they showed that compared with WT cells, cells with loss of TMBIM5 had reduced mitochondrial Ca^{2+} efflux measured in permeabilized HEK293 cells following the addition of Ru360 to inhibit MCU-mediated Ca^{2+} uptake and thereby reveal mitochondrial Ca^{2+} efflux. They further showed that knocking down LETM1 did not reduce mitochondrial Ca^{2+} efflux and concluded that LETM1 is not responsible for CHE.

Stoichiometry is crucial for the direction of ion flux, and we will illustrate this in detail for CHE. The electron transport chain extrudes protons, thereby generating a large negative $\Delta\psi$ and a pH gradient (ΔpH) with an alkaline matrix pH. A stoichiometry of $1H^+$ for $1Ca^{2+}$ means that Ca^{2+} efflux is associated with net movement of positive charge out of the mitochondrion, which will be opposed by the matrix-negative $\Delta\psi$. In contrast, the alkaline matrix pH would increase the driving force for Ca^{2+} transport out of the matrix in exchange for H^+ entry. The net direction of flux would therefore depend on the relative magnitudes of the pH gradient and membrane potential. In general, for a transporter that exchanges n protons for one Ca^{2+} ion, net Ca^{2+} efflux will occur when the Ca^{2+} concentration gradient is given by Eq. 1:

$$\frac{[Ca^{2+}]_{mito}}{[Ca^{2+}]_{cyto}} > \left\{\frac{[H^+]_{mito}}{[H^+]_{cyto}}\right\}^n \bullet e^{\left\{[n-2]\cdot\frac{V_F}{RT}\right\}} \quad (1)$$

Therefore, the more acidic the matrix pH, the higher the matrix Ca^{2+} must be to obtain net Ca^{2+} efflux. If matrix pH is 0.5 pH units more alkaline than the cytoplasm, $[H^+]_{mito}/[H^+]_{cyto}$ = 0.31. The effects of membrane potential depend on the stoichiometry. If n = 1, then the more the negative is $\Delta\psi$, the higher the Ca^{2+} required for net Ca^{2+} efflux. With this stoichiometry, Eq. 1 reduces to:

$$\frac{[Ca^{2+}]_{mito}}{[Ca^{2+}]_{cyto}} > 0.31 \bullet e^{\left\{-\frac{V_F}{RT}\right\}} \quad (2)$$

Under physiological conditions, with $\Delta\psi$ of about −180 mV and RT/F = 25.2 mV at body temperature, where R, universal gas constant; T, temperature; F, Faraday constant, Eq. 2 predicts that with n = 1, net efflux will only occur if $[Ca^{2+}]_{mito}$ is 392 times greater than $[Ca^{2+}]_{cyto}$. However, under physiological conditions, mitochondrial Ca^{2+} is 100–200 nM and cytoplasmic Ca^{2+} ranges from about 0.1 µM in diastole to 1 µM in systole, meaning that mitochondrial Ca^{2+} varies from 2 to 0.1 × cytoplasmic, much less than that required for net Ca^{2+} efflux. Peak Ca^{2+} in the space between the sarcoplasmic reticulum and mitochondria is likely much higher (Lu et al., 2013). Therefore with exchange of $1H^+$ for 1 Ca^{2+}, the expected flux would be net Ca^{2+} entry and H^+ efflux. With loss or reduction in $\Delta\psi$, especially with cytosolic acidification, it has been suggested that LETM1 can function to extrude matrix Ca^{2+} in exchange for cytosolic H^+ (Jiang et al., 2009; Waldeck-Weiermair et al., 2011). It is worth noting that with a 0.5 pH unit gradient and mitochondrial Ca^{2+} twice cytoplasmic, $\Delta\psi$ would have to be reduced to about −47 mV for net Ca efflux to occur. If mitochondrial and cytoplasmic Ca^{2+} are equal, then net efflux will only occur if $\Delta\psi$ is decreased to −30 mV. Finally, if the pH gradient is entirely dissipated, then with mitochondrial and cytoplasmic Ca^{2+} equal, net efflux can only occur with the unlikely case of a positive $\Delta\psi$.

In contrast, if the exchanger is electroneutral ($2H^+$ for $1Ca^{2+}$), then Eq. 1 reduces to Eq. 3.

$$\frac{[Ca^{2+}]_{mito}}{[Ca^{2+}]_{cyto}} > \left\{\frac{[H^+]_{mito}}{[H^+]_{cyto}}\right\}^2 \quad (3)$$

Net Ca^{2+} efflux will occur if $[Ca^{2+}]_{mito}/[Ca^{2+}]_{cyto} > 0.31^2$ (~0.1). In other words, an electroneutral exchanger can pump Ca^{2+} against a 10-fold gradient. It is clear that the stoichiometry will have important implications for Ca^{2+} regulation and it is therefore important to resolve the stoichiometry of the exchanger.

Interestingly, the stoichiometry of TMBIM5-mediated CHE has not been reported. Another question is whether both TMBIM5 and LETM1 exchange Ca^{2+} for H^+ and if they do so with different stoichiometries? Austin et al. (2022) reported that knocking down LETM1 did not block Na^+-independent mitochondrial Ca^{2+} efflux, but further work will be needed to resolve the relative function of TMBIM5 and LETM1. Assuming that LETM1 is electrogenic, in the presence of a large negative $\Delta\psi$, it is likely to function as a Ca^{2+} influx mechanism and thus Ca^{2+} efflux might not be observed under these conditions. It is also curious that cardiac cells have high expression of TMBIM5 but very little Na^+-independent Ca^{2+} efflux. This would be consistent with TMBIM5 acting as a Ca^{2+} influx pathway. Future studies establishing the stoichiometry will be key to addressing this issue. Alternatively, TMBIM5 might require additional cofactors for activation which are not present in heart under basal conditions, although it is curious that the cardiac cell has such high levels of TMBIM5 at baseline.

Na^+-dependent Ca^{2+} efflux

Palty et al. (2010) reported that a protein encoded by *slc8b1* (subsequently referred to as NCLX) was responsible for Na^+-dependent Ca^{2+} efflux. A Ca^{2+}-sensitive mitochondrial-targeted Ca^{2+} indicator was loaded into SHSY-5Y cells and mitochondrial Ca^{2+} efflux was monitored. Mitochondria were loaded with Ca^{2+} using ATP to release it from the ER, and data showed that overexpression of NCLX enhanced and knockdown of NCLX reduced mitochondrial Ca^{2+} efflux. Luongo et al. (2017) generated a tamoxifen-inducible, cardiac-specific NCLX knockout (KO) mouse. After tamoxifen treatment, 87% of the mice died and this did not occur if the mice were crossed with mice in which cyclophilin D (an activator of the PTP) had been ablated, suggesting that the death was due to Ca^{2+} overload activation of PTP. In additional studies on digitonin-permeabilized adult cardiomyocytes, mitochondrial Ca^{2+} efflux was reduced by knocking out NCLX (Luongo et al., 2017).

However, the regulation of Na^+-dependent Ca^{2+} efflux appears to be complicated. Three recent studies have shown that Na^+-dependent Ca^{2+} efflux is blocked by the deletion of transmembrane protein 65 (TMEM65) (Garbincius et al., 2023, *Preprint*; Vetralla et al., 2023, *Preprint*; Zhang et al., 2023, *Preprint*). Vetralla et al. (2023, *Preprint*) measured mitochondrial Ca^{2+} efflux in permeabilized HeLa cells in which mitochondria were loaded with an aequorin-based Ca^{2+}-sensitive indicator. These measurements were made in the presence and absence of Na^+ to follow Na^+-dependent and Na^+-independent Ca^{2+} efflux. In the presence of Na^+, mitochondrial Ca^{2+} efflux was enhanced by overexpression of TMEM65 and reduced by TMEM65 downregulation. In another study, Garbincius et al. (2023, *Preprint*) identified TMEM65 as interacting with NCLX. They overexpressed TMEM65 in AC16 cells and found an increase in the rate of Na^+-dependent Ca^{2+} efflux measured using FuraFF to monitor extramitochondrial Ca^{2+} in digitonin-permeabilized cells. They further showed that CGP37157, an inhibitor of Na^+-dependent Ca^{2+} exchange, blocked this Na^+-dependent mitochondrial Ca^{2+} efflux. Knockdown of TMEM65 in mice using shRNA led to a skeletal muscle myopathy and decreased cardiac contractility. In another study, Zhang et al. (2023, *Preprint*) generated a global (whole body) TMEM65-KO mouse, which exhibited retarded growth and developed brain seizures leading to death at ~3 wk of age. Similar effects were seen with a neuronal-specific deletion of TMEM65. A skeletal muscle–specific TMEM65-KO exhibited no defects at 2 mo of age but showed growth delay and reduced muscle mass at 3 mo. Mitochondrial Ca^{2+}, measured using Rhod-2, was significantly higher in TMEM65-KO fibers compared with the floxed control. Furthermore, Na^+-dependent mitochondrial Ca^{2+} efflux measured in isolated mitochondria was present in the mitochondria from the floxed control but was absent in TMEM65-KO mitochondria. Interestingly, crossing the global TMEM65-KO mouse with the global MCU-KO mouse rescued the development of seizure and death that occurred in the global TMEM65-KO mouse. Taken together, these three studies agree that Na^+-dependent Ca^{2+} efflux requires TMEM65.

The exact details of how TMEM65 and NCLX interact to regulate mitochondrial Na^+-dependent Ca^{2+} efflux is unclear, but perhaps, like the relationship between EMRE and MCU, both are needed for the exchanger to function. It is also possible that TMEM65 and NCLX function independently as Na^+-dependent Ca^{2+} exchangers. For this reason, we use NCLX to refer to transport regulated by this protein and Na-dependent Ca^{2+} efflux to refer to overall transport (which may or may not be entirely mediated by NCLX). Another important issue is the stoichiometry. Studies have reported both electrogenic exchange of $3Na^+$ for $1Ca^{2+}$ and electroneutral $2Na^+$ for $1Ca^{2+}$ (Affolter and Carafoli, 1980; Brand, 1985; Jung et al., 1995; Dash and Beard, 2008; Kim and Matsuoka, 2008; Giladi et al., 2022). For the former, net Ca^{2+} efflux is associated with the entry of positive charge and (in contrast to the $1Ca^{2+}$:$1H^+$ CHE discussed above) the matrix- negative $\Delta\psi$ will facilitate Ca^{2+} efflux. The electroneutral case will only give net Ca^{2+} efflux if $([Na^+]_{cyto}/[Na^+]_{matrix})^2 > ([Ca^{2+}]_{cyto}/[Ca^{2+}]_{matrix})$. Measurement of mitochondrial Na^+ would provide some insight into the regulation of mitochondrial Na^+/Ca^{2+} exchange. There are only a few measurements of mitochondrial Na^+, and the studies suggest that basal mitochondrial Na^+ is ~5 mM, which is lower than cytosolic Na^+, and that mitochondrial Na^+ increases with addition of cyanide or mitochondrial uncoupler (Donoso et al., 1992). Additional studies are needed to clarify the role of TMEM65 and NCLX in Na^+-dependent Ca efflux. Development of mitochondrial targeted Na^+ indicators would also help address these questions.

Mitochondrial Ca^{2+} during ischemia and reperfusion

It has been proposed that an increase in mitochondrial Ca^{2+} during ischemia and reperfusion is responsible for initiating cell death via activation of the mitochondrial PTP and that inhibition of the increase in mitochondrial Ca^{2+} might be cardioprotective (Bauer and Murphy, 2020; Bround et al., 2020; Marta et al., 2021; Bernardi et al., 2023; Murphy and Liu, 2023; Robichaux et al., 2023). Such a strategy would require knowledge of the mechanisms responsible for the increase in mitochondrial Ca^{2+}. Furthermore, for inhibition of mitochondrial Ca^{2+} uptake to be protective, ideally the increase in mitochondrial Ca^{2+} would need to occur on reperfusion so that drugs could be administered before the increase occurred. To address these issues, one needs to have reliable methods to measure mitochondrial Ca^{2+} in a model that is suitable for inducing ischemia and reperfusion. Ideally, this requires an intact heart. Many studies using isolated myocytes have used "simulated ischemia" by applying solutions to attempt to mimic some of the changes occurring in ischemia and measured mitochondrial-free Ca^{2+} with a mitochondrial-targeted indicator. However, ischemia is defined as lack of blood flow to an organ which reduces oxygen and nutrient delivery to the organ and reduces washout of end products of metabolism; thus, it is difficult to exactly simulate these conditions with isolated cells. Furthermore, the extracellular volume is frequently very different between an intact heart and isolated cardiomyocytes and this can alter the ion gradients.

In studies carried out >20 years ago, mitochondrial Ca^{2+} was measured in isolated adult rat cardiomyocytes loaded with the Ca^{2+}-sensitive indicator indo-1 during hypoxia and reoxygenation (Miyata et al., 1992; Griffiths et al., 1998). Manganese was added to the cells to quench any indo-1 in the cytosol. It should be noted that the addition of manganese can have effects on Ca^{2+} transporters and can slowly enter the mitochondria (Gavin et al., 1999). An increase in mitochondrial Ca^{2+} occurred at the onset of rigor. Approximately 50% of the cardiomyocytes hypercontracted on reoxygenation. At 40 min of hypoxia following the onset of rigor, mitochondrial Ca^{2+} increased from basal levels (~100 nM) to 280 nM in the cells that subsequently recovered and 743 nM in those that hypercontracted (Griffiths et al., 1998). Studies using clonazepam to inhibit the mitochondrial Na^+/Ca^{2+} exchange showed that none of the cells hypercontracted on reoxygenation. Clonazepam also blocked the increase in mitochondrial Ca^{2+} during the 40 min of hypoxia following rigor. However, on reoxygenation, clonazepam caused an increase in mitochondrial Ca^{2+}, with mitochondrial Ca^{2+} rising to 465 nM by 15 min of reoxygenation (Griffiths et al., 1998). These data suggest that the increase in mitochondrial Ca^{2+} during hypoxia is via the reverse mode of Na^+/Ca^{2+} exchange, but that forward Na^+/Ca^{2+} exchange decreases mitochondrial Ca^{2+} on reoxygenation.

Studies in HL-1 cells (an immortalized cardiomyocyte line) subjected to simulated ischemia and reperfusion reported that addition of 25 μM ruthenium red (to inhibit MCU) reduced the rise in mitochondrial Ca^{2+} during simulated ischemia; however, this reduction in mitochondrial Ca^{2+} was associated with an increase in lactate dehydrogenase (LDH) release, which was attributed to an increase in cytosolic Ca^{2+} in the presence of ruthenium red (Ruiz-Meana et al., 2006). Ashok et al. also tested a role for MCU in mitochondrial Ca^{2+} uptake during 60 min of coverslip-simulated ischemia in neonatal mouse ventricular cardiomyocytes from WT and MCU-KO mice (Ashok et al., 2023). In this study, MCU floxed cells were transfected with a Cre virus to knockdown MCU. Mitochondrial Ca^{2+} increased during the first 25 min of simulated ischemia. This was, however, followed by a decline in mitochondrial Ca^{2+} to basal levels occurring at the time of loss of $\Delta\psi$. The mechanism responsible for the decline is unclear. There was no difference in mitochondrial Ca^{2+} levels during simulated ischemia or reperfusion between the WT and the MCU-KO cardiomyocytes, suggesting that the increase in mitochondrial Ca^{2+} observed during I/R is not due to Ca^{2+} entry via MCU. Furthermore, there was no difference in LDH release measured following simulated reperfusion, although the levels of LDH released were low. Interestingly, it was observed that addition of CGP37157, an inhibitor of mitochondrial Na^+-dependent Ca^{2+} exchanger, blocked the increase in mitochondrial Ca^{2+} during simulated ischemia, suggesting that Ca^{2+} entry during ischemia is due to mitochondrial Na^+/Ca^{2+} exchange operating in the direction to transport Ca^{2+} into the mitochondria. This finding is consistent with previous studies mentioned above (Griffiths et al., 1998), which showed in adult cardiomyocytes that inhibition of mitochondrial Na^+/Ca^{2+} exchange blocked the ischemic-induced increase in mitochondrial Ca^2.

The ability of mitochondrial Na^+/Ca^{2+} exchange to operate in the Ca^{2+} influx mode will depend on the stoichiometry of the exchanger. If Na^+/Ca^{2+} is electroneutral, exchanging 2 Na^+ for 1 Ca^{2+}, then at equilibrium, the direction of the exchanger would depend only on the gradients for Na^+ and Ca^2. With the large rise in cytosolic Ca^{2+} that occurs during ischemia with electroneutral Na^+/Ca^{2+}, the exchanger could mediate Ca^{2+} entry into the mitochondria. However, if the exchanger is electrogenic ($3Na^+$ exchanging with 1 Ca^2), then Ca^{2+} entry will be decreased by the negative $\Delta\psi$. Rewriting Eq. 1 for Na^+, with $n = 3$ (and assuming that mitochondrial and cytoplasmic Na^+ concentrations are equal), we can predict that with a $\Delta\psi$ of −180 mV, net Ca^{2+} uptake (and Na^+ efflux) will only occur if cytoplasmic Ca^{2+} is >1,000 times greater than mitochondrial Ca^{2+}. Reducing $\Delta\psi$ to −120 or −60 means that net Ca^{2+} influx requires a concentration gradient of 100 or 10 times, respectively. Thus, it is important to determine both $\Delta\psi$ during ischemia and the stoichiometry of Na^+/Ca^{2+}. As mentioned above, there are reports suggesting both electroneutral and electrogenic mitochondrial Na^+/Ca^{2+} exchange (Affolter and Carafoli, 1980; Brand, 1985; Jung et al., 1995; Dash and Beard, 2008; Kim and Matsuoka, 2008). Interestingly, a recent study utilizing NCLX mutations, the voltage dependence of transport, and molecular dynamic simulation (Giladi et al., 2022) reported that NCLX is electroneutral. Further studies are needed to establish the stoichiometry of the exchanger.

Mastoor et al. (2024) also used neonatal mouse ventricular cardiomyocytes to examine alterations in mitochondria Ca^{2+} during coverslip ischemia and reperfusion. One problem is that simulated ischemia causes a large intracellular acidification, which can alter the K_d for Ca^{2+} binding and fluorescent

properties of Ca^{2+} indicators. To address this concern, a pH-insensitive, genetically encoded, mitochondrial-targeted Ca indicator, TqFLITS, was employed. TqFLITS monitors Ca^{2+} via changes in fluorescent lifetime, which allows measurement of Ca^{2+} that is independent of the indicator concentration, thus allowing one to calculate free Ca^{2+} rather than just changes of fluorescence. In WT cells, after 60 min of simulated ischemia mitochondrial Ca^{2+} increased 2.8-fold, whereas in the MCU-KO the increase was only 1.7-fold, suggesting that a pathway in addition to the MCU contributes to Ca^{2+} entry. Cell death was also monitored during coverslip ischemia and reperfusion using propidium iodide staining. There was no difference in cell death, after 60 min of coverslip ischemia between WT and MCU-KO, again suggesting a role for something other than the MCU.

Given the problems of mimicking ischemia in isolated cells, it is important to measure mitochondrial Ca^{2+} in an intact beating heart preparation. Miyamae et al. (1996) employed the Ca^{2+} indicator indo-1 to measure mitochondria Ca^{2+} was not measured during ischemia. We have recently developed a new method to measure mitochondrial Ca^{2+} transmurally in a beating perfused heart (Petersen et al., 2023). A genetically encoded mitochondrial-targeted Ca^{2+} indicator, red genetically encoded calcium indicator for optical imaging (R-GECO), is directed to the heart by injecting AAV9-encoding R-GECO into 3–5-day-old mouse pups. At 10–12 wk of age, the heart is perfused in Langendorff mode with a laser inserted into the left ventricle to excite the R-GECO in the heart mitochondria. The heart is placed in an integrating sphere to minimize motion artifacts and the emitted light collected by a spectrometer and analyzed for changes in emission fluorescence. Using this method, a two- to threefold increase in mitochondria Ca^{2+} was observed during ischemia, followed by a slow recovery of mitochondrial Ca^{2+} toward baseline during reperfusion. The fact that the increase in mitochondrial Ca^{2+} occurs during ischemia limits the ability to target it on reperfusion. However, it is still of interest to understand the mechanisms responsible for the increase in mitochondrial Ca^{2+} during ischemia as this could potentially be useful to develop cardioprotective approaches for use in cardiac surgery.

To test whether MCU is responsible for the increase in mitochondrial Ca^{2+} during I/R, mitochondrial Ca^{2+} was measured in MCU-KO and WT hearts using mitochondrial-targeted R-GECO (Petersen et al., 2023). In WT hearts, mitochondrial Ca^{2+} increased by 2.4-fold during ischemia compared with a 1.7-fold increase in MCU-KO hearts. Thus, the deletion of MCU significantly reduced but did not eliminate the rise in mitochondrial Ca^{2+} during ischemia, suggesting that, as was seen in isolated cardiomyocytes (Mastoor et al., 2024), mechanisms other than MCU contribute to the increase in mitochondrial Ca^{2+} observed during ischemia. It will be interesting to test whether inhibition of pathways such as Na^+/Ca^{2+} can block the rise in mitochondrial Ca^{2+} during ischemia, similar to what has been reported in isolated cardiomyocytes (Griffiths et al., 1998; Ashok et al., 2023).

It is possible that the rise in mitochondrial-free Ca^{2+} may not simply result from altered fluxes across the inner mitochondrial membrane; alterations in Ca^{2+} buffering could also contribute (Hernansanz-Agustín et al., 2020). Depending on the conditions, it has been reported that >95% of the Ca^{2+} in the mitochondria is bound, much of it in Ca-P granules (reviewed in Eisner et al. [2023]). Acidic conditions, as occur during ischemia, have been shown to promote dissociation of the Ca-P granules (Chalmers and Nicholls, 2003), providing another mechanism to increase mitochondrial-ionized Ca^{2+} that is independent of Ca^{2+} uptake into the mitochondria.

As mentioned previously, it is generally thought that an increase in mitochondrial Ca^{2+} during ischemia or reperfusion activates the PTP leading to cell death. Studies find that mitochondrial Ca^{2+} rises approximately two- to threefold during simulated ischemia or ischemia (Ashok et al., 2023; Petersen et al., 2023). Basal Ca^{2+} is typically reported to be in the range of ~100 nM; therefore, during ischemia and reperfusion, the rise in mitochondrial Ca^{2+} would be in the range of 200–300 nM. It is important to consider whether this modest increase in mitochondrial Ca^{2+} is sufficient to activate PTP. PTP is typically assayed in isolated mitochondria by measuring the Ca^{2+} retention capacity using an extramitochondrial Ca^{2+} indicator to determine how much total Ca^{2+} uptake is needed to trigger PTP opening. When total mitochondrial Ca^{2+} is low (<10 nmol/mg protein) and phosphate is present, there is a more or less linear relationship between Ca^{2+} uptake and free mitochondrial Ca^{2+} levels (Nicholls, 1978). However, as the total mitochondrial Ca^{2+} increases above 10 nmol/mg protein, the added Ca^{2+} is largely buffered and with additional Ca^{2+} uptake, there is little further increase in free mitochondrial Ca^{2+}. In fact, it has been reported that activation of PTP is linked to total mitochondrial Ca^{2+} rather than free Ca^{2+} (Chalmers and Nicholls, 2003; Wei et al., 2012). Thus in isolated mitochondria, under conditions that activate PTP, the free matrix Ca^{2+} is highly buffered. This buffering might explain, in part, why the rise in Ca^{2+} during ischemia is low. Another point to consider is that during ischemia, the matrix would be expected to become more acidic and this would alter the Ca^{2+} buffering. The studies examining free to bound Ca^{2+} were typically performed at pH 7.2–7.4. The observation that total rather than free mitochondrial Ca^{2+} activates PTP raises the question of whether inhibition of MCU or Ca^{2+} efflux mechanisms alters total as well as free mitochondrial Ca^{2+}. It is also important to understand how a change in total mitochondrial Ca^{2+} might lead to activation of PTP. One possibility is that the PTP interacts with the major buffer and therefore senses total Ca^{2+}. Interestingly, polyphosphate has been suggested to regulate PTP and perhaps polyphosphate is involved in sensing total Ca^{2+} (Seidlmayer et al., 2012). Future studies need to examine mechanisms by which total Ca^{2+} might regulate PTP.

Role of mitochondrial Ca^{2+} transporters in I/R injury

Regardless of whether it is total or free Ca^{2+} that activates PTP and initiates cell death, the question remains as to whether inhibiting mitochondrial Ca^{2+} uptake (which should inhibit the increase in total mitochondrial Ca^{2+}) is cardioprotective. Several studies, using either pharmacological inhibitors or genetic mouse models, have examined the effects of inhibiting the MCU. Studies in perfused rat heart have found that addition of

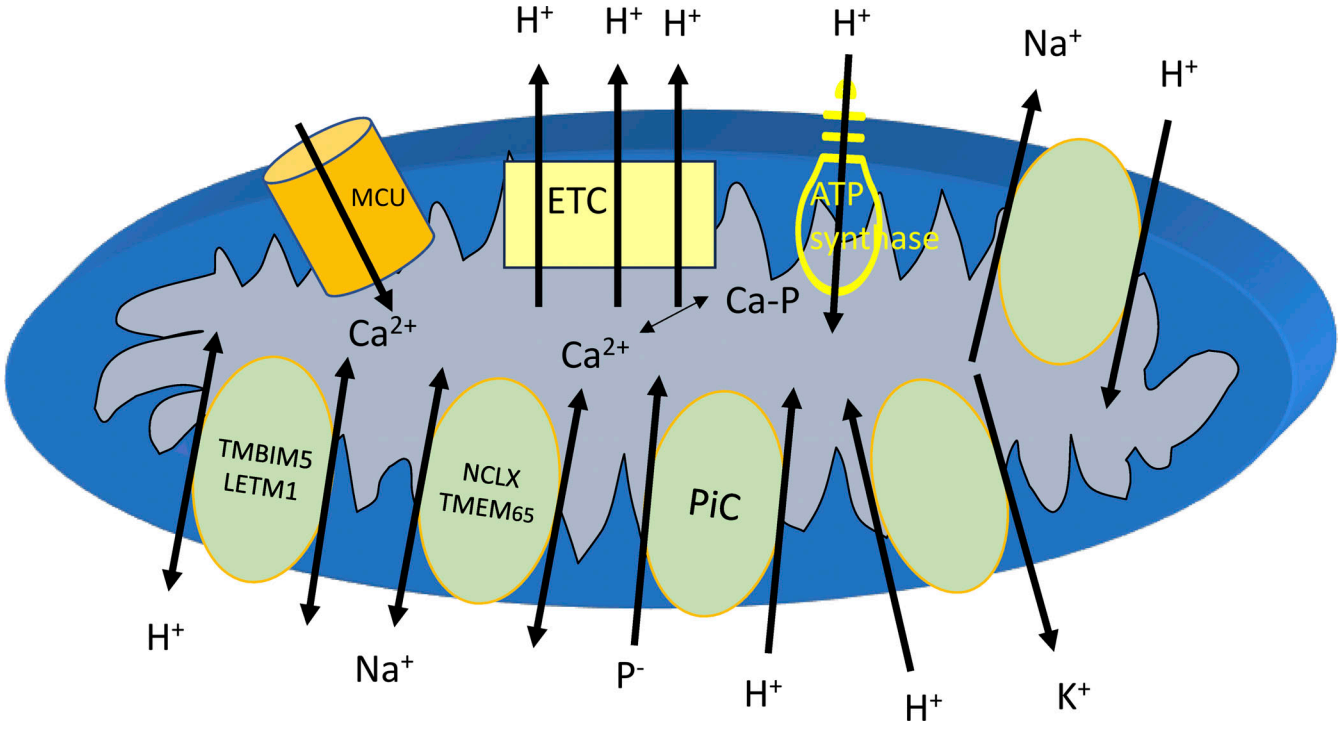

Figure 1. **Mechanisms regulating mitochondrial Ca^{2+}.** The MCU is the primary protein responsible for Ca^{2+} uptake into the mitochondria. The energy for Ca^{2+} uptake is provided by the large negative membrane potential that is generated by the electron transport chain (ETC). The ETC is also responsible for generating an alkaline matrix pH. The inwardly directed proton gradient drives the efflux of a number of ions including Na^+, K^+, and Ca^{2+}. In addition to CHE, Ca^{2+} efflux from the matrix occurs via a CHE. There are also data suggesting a role for Ca^{2+} entry via an electrogenic Ca^{2+}/H^+ exchanger, although this needs confirmation. Much of the mitochondrial Ca^{2+} is buffered within Ca-P granules. The phosphate enters the mitochondria on the phosphate carrier (PiC) in exchange for proton.

ruthenium red improved after ischemic contractile function (Grover et al., 1990; Benzi and Lerch, 1992; Zhang et al., 2006) and reduced infarct size or enzyme release (Benzi and Lerch, 1992; Zhang et al., 2006). One issue regarding ruthenium red is that it may affect other Ca^{2+} transporters. Ru360 has been isolated as the active compound in ruthenium red that is responsible for inhibition of MCU, and a study with this more specific compound also found it to improve ischemic contractile function (de Jesus Garcia-Rivas et al., 2005). A potential limitation of Ru360 is that it is reported to poorly permeate the plasma membrane (Hajnoczky et al., 2006). However, arguing against this concern, a number of studies have reported Ru360 inhibition of mitochondrial Ca^{2+} uptake in cardiac cells and tissue (Unitt et al., 1989; Matlib et al., 1998; Kosmach et al., 2021). As discussed, the ability of Ru360 to accumulate in cells at a level sufficient to inhibit MCU may depend on the cell type as Ru360 and MICU1 compete for binding to MCU (Rodríguez-Prados et al., 2023). Therefore, the ability of Ru360 to inhibit MCU is dependent on the tissue levels of MICU1. The heart has a very low ratio of MICU1 to MCU, perhaps explaining why Ru360 is effective in blocking MCU in the heart. A more potent inhibitor of MCU, Ru265, has also been developed (Woods et al., 2019). Interestingly, Cys 97 of MCU appears to be required for inhibition by Ru265, suggesting that Ru265 might inhibit by a different mechanism than Ru360. Experiments on mice with genetic deletion of MCU have yielded mixed results. Perfused hearts from mice with germline deletion of MCU subjected to I/R did not show protection; infarct size was not different between WT and MCU-KO (Pan et al., 2013). Similarly, perfused hearts from a mouse model in which a dominant negative MCU was overexpressed in cardiac cells using the α-myosin heavy chain promoter were not protected from I/R injury (Rasmussen et al., 2015). In contrast, mice with a tamoxifen-inducible cardiac-specific loss of MCU in adults showed reduced infarct size in an in vivo I/R model (Kwong et al., 2015; Luongo et al., 2015). These differences have typically been attributed to adaptations that occur in mice when MCU is deleted prior to birth. However, recent studies in neonatal mouse ventricular cardiomyocytes, in which MCU is acutely deleted, did not show a reduction in death following simulated ischemia (Ashok et al., 2023; Mastoor et al., 2024). Additional studies will be needed to address these discrepancies.

Very few studies have been performed to test whether the mitochondria Na^+/Ca^{2+} exchange inhibitor, CGP37157, is cardioprotective when given to hearts before I/R. Griffiths et al. reported that addition of clonazepam blocked the development of hypercontracture on reoxygenation (Griffiths et al., 1998). However, even though Ashok et al. found that addition of CGP37157 reduced the rise in Ca^{2+} during ischemia, there was no reduction in LDH release or alteration in $\Delta\psi$ recovery (Ashok et al., 2023). One issue is that blocking Ca^{2+} efflux at the beginning of reperfusion would be detrimental and it is difficult to remove the inhibitor at the start of reperfusion. Somewhat in contrast to the reduction in mitochondrial Ca^{2+} observed with

addition of CGP37157, when a mouse with cardiac-restricted overexpression of NCLX was subjected to an in vivo model of coronary ligation for 40 min followed by 24 h of reperfusion, the infarct size was less than that in WT (Luongo et al., 2017). Thus, the role of Na^+-dependent Ca^{2+} exchange in regulating mitochondrial Ca^{2+} during I/R is unclear. There is much we need to better understand about Na^+-dependent Ca^{2+} exchange, including the stoichiometry and the relationship of TMEM65 and NCLX. We also need a better understanding of the regulation of mitochondrial Na^+.

Conclusion

Taken together, data in the literature suggest that a rise in mitochondrial Ca^{2+} occurs during ischemia. However, the mechanisms responsible for this increase are not totally defined. Inhibition of MCU only partially blocks the increase in mitochondrial Ca^{2+} during ischemia. Studies using inhibitors of mitochondrial Na^+-dependent Ca^{2+} exchange have suggested that during ischemia, Ca^{2+} may enter the mitochondria in exchange for Na^+. The contribution of CHE to the control of mitochondrial Ca^{2+} is also unclear. As discussed, the roles of these exchangers will depend on their stoichiometry and further work is needed. Release of Ca^{2+} from Ca-P could also provide a source for the rise in mitochondrial Ca^{2+} during ischemia. A major question that needs to be addressed is the mechanism by which increased uptake of Ca^{2+} into the mitochondria actives PTP. It appears that activation of PTP correlates better with an increase in total rather than free Ca^{2+}. Despite the fact that mitochondrial Ca^{2+} has been studied for over 60 years, the details of mechanisms regulating mitochondrial Ca^{2+} uptake and release, especially the stoichiometry of the efflux pathways is still unclear (Fig. 1). Furthermore, the role of Ca^{2+} buffering in mitochondrial Ca^{2+} homeostasis and in activation of the PTP also needs additional study.

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