Dynamic buffering of mitochondrial Ca\(^{2+}\) during Ca\(^{2+}\) uptake and Na\(^{+}\)-induced Ca\(^{2+}\) release

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Abstract In cardiac mitochondria, matrix free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{m}\)) is primarily regulated by Ca\(^{2+}\) uptake and release via the Ca\(^{2+}\) uniporter (CU) and Na\(^{+}/\)Ca\(^{2+}\) exchanger (NCE) as well as by Ca\(^{2+}\) buffering. Although experimental and computational studies on the CU and NCE dynamics exist, it is not well understood how matrix Ca\(^{2+}\) buffering affects these dynamics under various Ca\(^{2+}\) uptake and release conditions, and whether this influences the stoichiometry of the NCE. To elucidate the role of matrix Ca\(^{2+}\) buffering on the uptake and release of Ca\(^{2+}\), we monitored Ca\(^{2+}\) dynamics in isolated mitochondria by measuring both the extra-matrix free [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_{e}\)) and [Ca\(^{2+}\)]\(_{m}\). A detailed protocol was developed and freshly isolated mitochondria from guinea pig hearts were exposed to five different [CaCl\(_{2}\)] followed by ruthenium red and six different [NaCl]. By using the fluorescent probe indo-1, [Ca\(^{2+}\)]\(_{e}\) and [Ca\(^{2+}\)]\(_{m}\) were spectrofluorometrically quantified, and the stoichiometry of the NCE was determined. In addition, we measured NADH, membrane potential, matrix volume and matrix pH to monitor Ca\(^{2+}\)-induced changes in mitochondrial bioenergetics. Our [Ca\(^{2+}\)]\(_{e}\) and [Ca\(^{2+}\)]\(_{m}\) measurements demonstrate that Ca\(^{2+}\) uptake and release do not show reciprocal Ca\(^{2+}\) dynamics in the extra-matrix and matrix compartments. This salient finding is likely caused by a dynamic Ca\(^{2+}\) buffering system in the matrix compartment. The Na\(^{+}\)-induced Ca\(^{2+}\) release demonstrates an electrogenic exchange via the NCE by excluding an electroneutral exchange. Mitochondrial bioenergetics were only transiently affected by Ca\(^{2+}\) uptake in the presence of large amounts of CaCl\(_{2}\), but not by Na\(^{+}\)-induced Ca\(^{2+}\) release.

Keywords Mitochondria · Ca\(^{2+}\) uniporter · Na\(^{+}/\)Ca\(^{2+}\) exchanger · Ca\(^{2+}\) buffering · Bioenergetics

Introduction

Homeostasis of mitochondrial Ca\(^{2+}\) is well maintained by a balance of Ca\(^{2+}\) uptake, sequestration and release mechanisms (Santo-Domingo and Demaurex 2010; Gunter and Sheu 2009; Griffiths 2009). The main route for Ca\(^{2+}\) uptake is the ruthenium red-sensitive Ca\(^{2+}\) uniporter (CU) (Gunter and Pfeiffer 1990; Graier et al. 2007), which is in large part driven by the negative mitochondrial membrane potential (\(\Delta \Psi _{m}\)) and the Ca\(^{2+}\) gradient across the inner mitochondrial membrane (IMM) (Gunter and Sheu 2009; Saotome et al. 2005; Dash et al. 2009; Dedkova and Blatter 2008). The negative \(\Delta \Psi _{m}\) is generated by H\(^{+}\) pumping in the respiratory chain, which also creates a H\(^{+}\) gradient across the IMM resulting in an alkaline matrix pH (pH\(_{m}\)). The ability of the matrix compartment to sequester large amounts of Ca\(^{2+}\) is attributed to its Ca\(^{2+}\)-loading capacity with a strong buffering power (Olson et al. 2012). It is widely assumed that the formation of Ca\(^{2+}\)-phosphate precipitates inside the alkaline matrix plays an important role in the sequestration of large quantities of Ca\(^{2+}\) (Starkov 2010; Chalmers and Nicholls...
studies indicate a 3Na\(^+\):1Ca\(^{2+}\) (electrogenic) exchange (Cox and Matlib 1993) on the NCE kinetics were utilized to model extrudes Ca\(^{2+}\) in exchange for cytosolic Na\(^+\) (Pradhan et al. 2010a). In addition to Ca\(^{2+}\) transport via the CU and NCE, mitochondrial Ca\(^{2+}\) homeostasis in energized mitochondria may also be modulated by the $\Delta \Psi_m$, $\Delta pH$, Ca\(^{2+}/H^+\) exchanger (CHE) and Na\(^+\)/H\(^+\) exchanger (NHE).

It is noteworthy that the proteins forming the CU and NCE have been recently identified (Palty et al. 2010; Baughman et al. 2011; De Stefani et al. 2011). However, the precise stoichiometry of Na\(^+\)/Ca\(^{2+}\) exchange via the NCE remains unsettled. On the one hand, some experimental studies indicate a 2Na\(^+\):1Ca\(^{2+}\) (electroneutral) exchange (Brand 1985; Paucek and Jaburek 2004). On the other hand, other experimental studies indicate a 3Na\(^+\):1Ca\(^{2+}\) (electrogenic) exchange (Baysal et al. 1994; Jung et al. 1995; Crompton et al. 1976). An electrogenic exchange would be regulated by the $\Delta \Psi_m$, since additional positive charges would move into the matrix during the forward mode of the NCE (Crompton et al. 1976; Kim and Matsuoka 2008). Furthermore, biophysical computational approaches have been used to characterize the kinetics and stoichiometry of the NCE (Pradhan et al. 2010a; Dash and Beard 2008). In these computational studies, published data from previous experimental studies (Paucek and Jaburek 2004; Kim and Matsuoka 2008; Cox and Matlib 1993) on the NCE kinetics were utilized to predict the impact of extra-matrix [Ca\(^{2+}\)], [Na\(^+\)] and $\Delta \Psi_m$ on the NCE function. However, those approaches did not unambiguously identify the actual stoichiometry of the NCE (Pradhan et al. 2010a) due to limited available experimental data.

Previous experiments on Ca\(^{2+}\) regulation in heart mitochondria have provided insights into the CU and NCE dynamics by investigating the effects of adding either single bolus of CaCl\(_2\) and multiple boluses of NaCl, or multiple boluses of CaCl\(_2\) and one single bolus of NaCl to the extra-matrix compartment (Jung et al. 1995; Crompton et al. 1976; Kim and Matsuoka 2008; Cox and Matlib 1993; Wei et al. 2011). Furthermore, these studies measured changes only in either extra-matrix free [Ca\(^{2+}\)] (\([\text{Ca}^{2+}]_e\)) or matrix free [Ca\(^{2+}\)] (\([\text{Ca}^{2+}]_m\)). Recently, a study on mitochondrial Ca\(^{2+}\) uptake using repeated boluses of Ca\(^{2+}\) simultaneously measured changes in \([\text{Ca}^{2+}]_e\) and \([\text{Ca}^{2+}]_m\) (Wei et al. 2012). However, to date it has not been well elucidated how matrix Ca\(^{2+}\) buffering influences Ca\(^{2+}\) uptake via the CU under multiple Ca\(^{2+}\) loading conditions and how buffering affects Ca\(^{2+}\) release via the NCE by different Na\(^+\) perturbations.

In the present study, our aim was to examine the CU and NCE dynamics in isolated cardiac mitochondria under various Ca\(^{2+}\) uptake and release conditions by monitoring the effects of different amounts of CaCl\(_2\) and NaCl added to the extra-matrix compartment on changing both the \([\text{Ca}^{2+}]_e\) and \([\text{Ca}^{2+}]_m\). We postulated that Ca\(^{2+}\)- and Na\(^+\)-induced changes in \([\text{Ca}^{2+}]_e\) and \([\text{Ca}^{2+}]_m\) exhibit dissimilar dynamics that are due to a strong and dynamic Ca\(^{2+}\) buffering of the matrix compared to the extra-matrix compartment. To test this, we used established techniques to isolate mitochondria from guinea pig hearts and to measure \([\text{Ca}^{2+}]_e\) and \([\text{Ca}^{2+}]_m\) (Haumann et al. 2010; Heinen et al. 2007). A detailed protocol was developed to focus on the dynamics of Ca\(^{2+}\) transport via the CU and NCE. To better understand the dynamic modulation of mitochondrial Ca\(^{2+}\) homeostasis and its impact on mitochondrial bioenergetics, additional experiments were conducted to monitor changes in NADH (redox state), $\Delta \Psi_m$, pH\(_m\), and matrix volume.

Our data on \([\text{Ca}^{2+}]_e\) and \([\text{Ca}^{2+}]_m\) during Ca\(^{2+}\) uptake and Na\(^+\)-induced Ca\(^{2+}\) release were then utilized to quantify the mitochondrial Ca\(^{2+}\) buffering system using a mathematical model (see our companion paper (Bazil et al. 2012)). Indeed, findings derived from this computational study (Bazil et al. 2012) corroborate our experimental observations regarding the existence of a dynamic matrix Ca\(^{2+}\) buffering system during variations in Ca\(^{2+}\) uptake and release.

**Methods**

Mitochondrial isolation

All experiments conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee (IACUC). Mitochondria from guinea pig hearts were isolated as previously described (Haumann et al. 2010; Heinen et al. 2007). Guinea pigs (250–350 g) were anesthetized by intraperitoneal injection of 30 mg ketamine, and 700 units of heparin for anticoagulation. Hearts (n=64) were excised and minced to approximately 1 mm\(^3\) pieces in ice-cold isolation buffer containing 200 mM mannitol, 50 mM sucrose, 5 mM KH\(_2\)PO\(_4\), 5 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 1 mM EGTA and 0.1 % bovine serum albumin (BSA). Buffer pH was adjusted with KOH to 7.15. The minced pieces were suspended in 2.65 ml ice-cold buffer with 5U/ml protease (from Bacillus licheniformis), and homogenized at low speed for 30 s. Afterwards, 17 ml ice-cold isolation buffer was added and the