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NCLX: The Mitochondrial Sodium Calcium Exchanger

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Abstract

The free Ca^{2+} concentration within the mitochondrial matrix ($[Ca^{2+}]_m$) regulates the rate of ATP production and other $[Ca^{2+}]_m$ sensitive processes. It is set by the balance between total Ca^{2+} influx (through the mitochondrial Ca^{2+} uniporter (MCU) and any other influx pathways) and the total Ca^{2+} efflux (by the mitochondrial Na^+/Ca^{2+} exchanger and any other efflux pathways). Here we review and analyze the experimental evidence reported over the past 40 years which suggest that in the heart and many other mammalian tissues a putative Na^+/Ca^{2+} exchanger is the major pathway for Ca^{2+} efflux from the mitochondrial matrix. We discuss those reports with respect to a recent discovery that the protein product of the human FLJ22233 gene mediates such Na^+/Ca^{2+} exchange across the mitochondrial inner membrane. Among its many functional similarities to other Na^+/Ca^{2+} exchanger proteins is a unique feature: it efficiently mediates Li^+/Ca^{2+} exchange (as well as Na^+/Ca^{2+} exchange) and was therefore named NCLX. The discovery of NCLX provides both the identity of a novel protein and new molecular means of studying various unresolved quantitative aspects of mitochondrial Ca^{2+} movement out of the matrix. Quantitative and qualitative features of NCLX are discussed as is the controversy regarding the stoichiometry of the NCLX Na^+/Ca^{2+} exchange, the electrogenicity of NCLX, the $[Na^+]_i$ dependency of NCLX and the magnitude of NCLX Ca^{2+} efflux. Metabolic features attributable to NCLX and the physiological implication of the Ca^{2+} efflux rate via NCLX during systole and diastole are also briefly discussed.

Keywords

Mitochondria; Calcium transport; Cardiac myocytes; Sodium/calcium exchange; NCLX; Computational model

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None.

1. Introduction

Considerable evidence suggests that the free $[Ca^{2+}]$ in the mitochondrial matrix ($[Ca^{2+}]_m$) is important in the regulation of mitochondrial metabolism. This is due largely to the $[Ca^{2+}]_m$ sensitivity of key steps in energy production [1,2]. Such $[Ca^{2+}]_m$ dependent components include the Krebs cycle dehydrogenases that supply substrate to the electron transport chain (ETC) [1–5], F_1F_0-ATPase (Complex V) [6] and additional components of the ETC [7], the uncoupling proteins [8–11], the putative permeability transition pore (PTP) [12–14] and other proteins [15,16]. This review seeks to present a current view of the molecular and biophysical properties of NCLX and its role in regulating $[Ca^{2+}]_m$.

$[Ca^{2+}]_m$ is set in the steady state by the balance of Ca^{2+} leak into the matrix by any Ca^{2+} entry pathway and the efflux by any Ca^{2+} extrusion/pump mechanism. Passive Ca^{2+} entry is favored by the very large potential across the inner mitochondrial member, $\Delta\Psi_m$ (−150 to −200 mV). The proton-motive potential powers the ATP synthase so that it can make ATP and is a combination of the inner membrane potential ($\Delta\Psi_m$) and the pH gradient across the inner membrane (the matrix is more alkaline, a pH of 7.8 compared to 7.2 in the cytosol). The large $\Delta\Psi_m$ also favors entry of Ca^{2+} down its electrochemical gradient. The primary Ca^{2+} entry pathway is the mitochondrial Ca^{2+} uniporter (MCU), a channel thought to be highly selective for Ca^{2+} [17,18]. Other features related to the MCU are now in dispute and will be presented here only briefly. These features that are currently the topic of active investigation by us and others relate to the abundance of the MCU in the inner membrane, its open probability, its conductance, its dependence on cytosolic and matrix regulators and its gating [19–25]. Nevertheless, two groups identified a compelling candidate protein that appears to have all of the properties consistent with an MCU [26,27] and appears to be the same protein. The Ca^{2+} efflux from the mitochondrial matrix in excitable-cells appears to be mediated by the recently identified mitochondrial Na^+/Ca^{2+} exchanger (NCLX) [28]. This Ca^{2+} extrusion from the matrix is powered by the electrochemical gradient for Na^+ entry into the mitochondrial matrix from the cytosol. The energy available to NCLX for mitochondrial Ca^{2+} extrusion thus depends on the concentrations of Na^+ in the cytosol ($[Na^+]_i$) and matrix ($[Na^+]_m$), on $\Delta\Psi_m$ and also on the stoichiometry of NCLX. In non-excitable cells (e.g., liver cells) Ca^{2+} efflux is also mediated by a H^+/Ca^{2+} exchanger of unknown molecular identity [29].

The mitochondrial Na^+/Ca^{2+} exchanger gene was identified by Cai *et al*., 2003 [30] and Palty *et al*., 2004 [31] and its initial function was characterized as described below. It is called NCLX an abbreviate term for (Na/Li/Ca exchanger) because Palty *et al*., 2004 found that it can transport either lithium (Li^+) or sodium (Na^+) in exchange for Ca^{2+} while the plasma membrane exchangers NCX and NCKX do not transport Li^+. Two important issues that motivate current mitochondrial research but are still poorly understood are; (1) the amount of Ca^{2+} that can be transported by NCLX when it is fully activated and (2) the extent and kinetics of NCLX transport rate and how transport is influenced by cytosolic and matrix regulatory factors. Here we present an overview of the molecular and physiologic function of NCLX. Since mitochondria from different tissues exhibit great differences in their permeability to Ca^{2+} [23], unless stated otherwise we focused here on the quantitative information from investigations of cardiac cells or isolated cardiac mitochondria.

2. Critical Features of NCLX

2.1. The Movement of Ca^{2+} Across the Inner Mitochondrial Membrane

The $[Ca^{2+}]_i$ transient, activated by the cardiac action potential (AP), underlies the cardiac contraction. Ca^{2+} enters the cell primarily through voltage-gated L-type Ca^{2+} channels, triggers and synchronizes Ca^{2+} sparks to produce the global or cell-wide $[Ca^{2+}]_i$ transient

(70–80 μmoles of Ca^{2+} per liter of cytosol) during the "systolic period". The elevated $[Ca^{2+}]_i$ is reduced as Ca^{2+} is reacquired by the sarcoplasmic reticulum (SR) or extruded from the cell and this reduction of $[Ca^{2+}]_i$ underlies the relaxation phase of the cardiac contraction; the "diastolic period" [32]. One of the potential sources and sinks for Ca^{2+} is the large array of mitochondria in the cytosol. During systole, cytosolic $[Ca^{2+}]_i$ increases cell-wide from about 100 nM to approximately 0.5 to 1.0 μM. A fraction of the elevated $[Ca^{2+}]_i$ enters the mitochondrial matrix where it activates ATP generation [3,33]. The exact amount of Ca^{2+} that enters the mitochondria is a highly debated topic (see below). However, in the steady state this Ca^{2+} influx must be balanced by Ca^{2+} efflux. Or put another way, under steady-state conditions the same amount of Ca^{2+} that enters each mitochondrion during a $[Ca^{2+}]_i$ transient must be extruded from it before the next $[Ca^{2+}]_i$ transient. Therefore, if the mitochondrial Ca^{2+} uniporter (MCU) is the only pathway for Ca^{2+} to enter the mitochondrial matrix and the mitochondrial Na^+/Ca^{2+} exchanger (NCLX) is the only pathway for mitochondrial Ca^{2+} extrusion, then MCU influx must equal the NCLX efflux. In reviewing this topic two central questions will be discussed: (1) what is the magnitude of this Ca^{2+} efflux and (2) what are its kinetics. Exploring these questions leads us to a quantitative analysis of the influence of NCLX stoichiometry and of $[Na^+]_i$ on the magnitude and kinetics of NCLX Ca^{2+} efflux. See below.

To investigate the magnitude and kinetics of mitochondrial Ca^{2+} fluxes, two approaches have been used by investigators. The first [34–37] seeks primarily to measure and calibrate $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ in cardiac cells. The second seeks to examine cellular and mitochondrial dynamics in diverse cell types under a much wider range of conditions [5,38–42]. These two approaches come to very different conclusion regarding Ca^{2+} uptake by mitochondria. They either suggest that very little Ca^{2+} enters the mitochondria under physiological conditions or that a great deal of Ca^{2+} enters mitochondria, respectively.

The first group of studies (see [35–37,43]) finds that 99% of the Ca^{2+} that enters the cytosol from the extracellular space or from the SR during systole is removed by the joint action of the Na^+/Ca^{2+} exchanger (NCX) located on the sarcolemma (SL) and the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) located on the sarcoplasmic reticulum (SR) membrane. The remaining 1% is removed equally by the mitochondria (entry into the matrix through MCU channels) and the plasmalemmal/sarcolemmal Ca^{2+}-ATPase pump, PMCA [34,35]. If true, under physiological conditions the amounts of Ca^{2+} that are transiently removed from the cytosol during systole by entering the mitochondrial matrix is inconsequential with respect to excitation-contraction coupling in the heart [44]. This would suggest that the highest measured mitochondrial $[Ca^{2+}]_m$ transient will be small (i.e., 10 to 30 nM [36,37]).

The second group indicates that much higher mitochondrial Ca^{2+} fluxes and changes in $[Ca^{2+}]_m$ occur during the cytosolic $[Ca^{2+}]_i$ transient. Results from these studies suggest large changes in $[Ca^{2+}]_m$ which parallel changes in $[Ca^{2+}]_i$. These results rely in part on the loading of inorganic dyes (e.g., Fluo-3 AM and Rhod-2 AM) into the mitochondrial matrix. Since application of the known MCU inhibitor, RU-360, prevents changes in the mitochondrially localized $[Ca^{2+}]$ signal, it is argued that this signal is now reporting changes in $[Ca^{2+}]_m$. Results from these studies suggest that the steady-state $[Ca^{2+}]_i$ transient is reduced by as much as 36% due to the uptake of Ca^{2+} by the mitochondria [5]. In support of these early studies are the findings of a more recent investigation in which genetically encoded Ca^{2+} indicators and additional molecular tools are used [41]. It is argued that these findings are all consistent with the mitochondria functioning as a dynamic buffer of $[Ca^{2+}]_i$ due to the dramatic increase in the $[Ca^{2+}]_i$ transient that has been reported following silencing of the expression of MCU by siRNAs by Drago n *et al*., 2012 [41].

The question that arises as a result of these contrary reports is how much Ca^{2+} actually moves across the mitochondrial inner membrane under physiological conditions. Quantitative estimates of Ca^{2+} influx into the mitochondrial matrix through MCU's and efflux through NCLX are the topics of active investigation by us and others. The investigations can benefit by additional state-of-the-art quantitative experiments that can be used to constrain computational estimates with all known fluxes, channels and transport processes included (see below).

2.2. The Molecular Identity of the mitochondrial Na^+/Ca^{2+} Exchanger

The suggestion that a putative mitochondrial Na^+/Ca^{2+} exchanger type carrier exists was initially based on the observation that addition of Na^+ to the solution outside isolated mitochondria causes a release of Ca^{2+} from the mitochondria. All other tested cations with the exception of Li^+ were without effect on this efflux and the amount of [Na^+]-dependent Ca^{2+} released was found to depend on the Na^+ concentrations (Fig.1A). Ruthenium red, an inhibitor of the Ca^{2+} uptake pathway (i.e., MCU) did not block the release but rather amplified the effect of Na^+ on Ca^{2+} release. This finding was taken to mean that the efflux of Ca^{2+} from the mitochondria was mediated by a pathway that was distinct from the influx [45]. While mitochondrial Na^+/Ca^{2+} exchange was first reported in studies carried out with a suspension of mitochondria isolated from the heart [45,46], similar results were obtained in mitochondria isolated from other tissues including brain, skeletal muscle, parotid gland, adrenal cortex [8–11,47] and liver [48,49]. In a subsequent study a ~110-kDa polypeptide was purified from bovine heart mitochondria. This polypeptide, when reconstituted in liposomes, catalyzed Na^+/Ca^{2+} and Na^+/Li^+ exchange [50] In 2004 a new member of the Na^+/Ca^{2+} exchanger superfamily [30,31] was cloned and found to catalyze both Na^+/Ca^{2+} and Li^+/Ca^{2+} exchange (i.e., NCLX) [17,18,31]. It was then found that silencing NCLX expression by siRNA's inhibited mitochondrial Ca^{2+} transport while over expression of NCLX enhanced mitochondrial Ca^{2+} efflux [28] (Fig. 1B). Immunoblot analysis and immuno-electron microscopy analyses revealed that NCLX localizes to the mitochondrial inner membrane (Fig. 1C). The biochemical and pharmacological properties of NCLX [51,52] are thus appropriate for the putative mitochondrial Na^+/Ca^{2+} exchanger [53] hypothesized to underlie Ca^{2+} efflux from mitochondria. The details of structure and function and recent findings are described below and this is followed by a provocative and quantitative discussion of current issues related to NCLX stoichiometry and quantitative Ca^{2+} fluxes.

2.3. Mitochondrial Na^+/Ca^{2+} Exchanger Structure

The protein coded by the full open-reading frame of the mouse FLJ22233 gene was first reported to be a new, sixth member of the K^+-dependent Na^+/Ca^{2+} exchanger family. It was initially termed NCKX6 and it was found that only a spliced isoform of this protein could reach the plasma membrane [30]. Yet, parallel and subsequent investigations cloned the protein product of the human full open-reading frame FLJ22233 gene and showed that it is K^+ independent [31,54]. Furthermore, it was found to be endogenously expressed in the mitochondrial inner membrane (IMM) where it appeared to mediate Na^+/Ca^{2+} or Li^+/Ca^{2+} exchange [28] (i.e., NCLX). In immunoblot analysis of mitochondrial enriched fractions from heart and brain, NCLX protein appeared as a 50 kDa monomer, a 70 kDa monomer and also was found to form 100-kDa dimers This size was similar to that in previous reports of an unidentified purified mitochondrial polypeptide that exhibits Na^+/Ca^{2+} and also Li^+/Na^+ exchange (i.e., ~110-kDa) [50,55]. NCLX shares similar structural motifs with the other members of the Na^+/Ca^{2+} exchanger superfamily (NCX1–3 and NCKX1–4). It has two transmembrane domains termed α1 and α2 which form the sites where ions are thought to bind and from which the ions are subsequently translocated across the membrane [56,57]. NCLX shares about 62% sequence similarity with the other exchangers in the α-repeat

domains. Outside these regions there is sequence similarity [5,30,38–40] but less than is found the α-repeat domains. Phylogenetic analysis revealed that the NCLX gene diverged earlier in evolution to form a subfamily with a single mammalian member that is distinct from the subfamilies of NCX1–3 or NCKX1–4 [30,58].

2.4. The Biochemical and Biophysical Features of the Mitochondrial Na^+/Ca^{2+} Exchanger

Transport mechanism—Much of what is known about mitochondrial Na^+/Ca^{2+} exchange is from investigations carried out with suspensions of isolated mitochondria from various tissues. In these experiments, efflux of Ca^{2+} from the matrix of heart mitochondria in the absence of extra-mitochondrial $[Na^+]$ is minimal or hardly detected. (Extra-mitochondrial $[Na^+]$ in these experiments is equivalent to cytosolic $[Na^+]_i$ within a cell and these terms are used interchangeably in this review for the sake of consistency and simplicity.) The mitochondrial efflux is significantly enhanced by increasing $[Na^+]_i$ [45,46] (also see Fig. 1A). The apparent mechanism of ion translocation by NCLX is thought to be similar to that of the sarcolemmal NCX [56,57,59] where sequential transport of the Na^+ and Ca^{2+} is thought to occur. If true, translocation of Na^+ or Ca^{2+} across the mitochondrial inner membrane are separate (sequential) stochastically occurring steps, and, in such a case, higher $[Na^+]_i$ increases the probability that Na^+ will be translocated into the matrix and Ca^{2+} extruded [60]. However, while our understanding of NCX is relatively robust, many of the biophysical features of NCLX remain either unknown or controversial. For example, a recent investigation [61] provided insights into Na^+/Ca^{2+} exchange mechanism by solving the crystal structure of an NCX homolog from *Methanococcus jannaschii*. Consistent with prior conceptions, four ion-binding sites were identified, three for Na^+ and one specific for Ca^{2+} within the α1 and α2 translocation regions. Note that these regions are highly conserved among the Na^+/Ca^{2+} exchanger superfamily (e.g., NCX 1–3, NCKX 1–4, and importantly, NCLX). This new structural information provides insight into the mechanism and stoichiometry of Na^+/Ca^{2+} exchange. However, NCLX is the only known member of the Na^+/Ca^{2+} exchanger superfamily that can also transport Li^+, suggesting that the mechanism for ion selectivity may differ in NCLX. Although sequential transport by NCLX seems likely, many of the biophysical features of NCLX (as opposed those of NCX) remain unknown, murky or controversial. Such uncertainty is not surprising since NCLX was only recently identified and because the mitochondrial inner membrane is not readily accessible experimentally. Importantly too is that isolated mitochondrial preparations change the gross appearance of the mitochondria where they change from the native shapes to spherical elements. What the isolation process does to ultrastructure and function remains an open question. Nevertheless, many features of mitochondrial biology appear crudely similar in intact cells and in isolated preparations. Quantitative biophysical, biochemical, and ultrastructure investigations now in progress by several laboratories should address these important open questions.

Magnitude of ionic fluxes and ionic selectivity—When experimental conditions push $[Na^+]_i$ beyond the physiological range (to over 15 mM) the measured Ca^{2+} efflux via NCLX does not continue to increase indefinitely; instead it saturates [46,47,60,62] or decreases [63]. A large influx of Na^+ into the mitochondrial matrix via NCLX appears to underlie a substantial degradation of the voltage gradient across the mitochondrial inner membrane (IMM) ($\Delta\Psi_m$) [47]. The reduced magnitude of $\Delta\Psi_m$ lowers the driving force for Na^+ influx into the mitochondrial matrix. It has been argued that this may arise, at least in part, to the extrusion of Na^+ and influx of protons (H^+) due to the mitochondrial Na^+/H^+ exchanger (NHE) and a reduction of the proton gradient (i.e., $\Delta\Psi_m$) [64]. Some reduction of $\Delta\Psi_m$ could also be attributed to Na^+ (and charge) influx via the NCLX if NCLX is electrogenic (see discussion below). Also if Ca^{2+} influx through the MCU were to be large, it too would degrade $\Delta\Psi_m$ through the entry of positive charge. $\Delta\Psi_m$ arises in respiring mitochondria

primarily through proton (and charge) extrusion ("pumping") by the molecular components of the electron transport chain (ETC) (see Fig. 2) [65] to establish the proton-motive force across the mitochondrial inner membrane. Using isolated mitochondrial inner membranes from individual mitochondria (called "mitoplasts") Kirichok *et al*., 2004 introduced a new experimental approach where $\Delta\Psi_m$ is held constant by patch-clamping the mitoplast (2–5 μm vesicles) [17,18,22]. In studies such as these, where both the voltage and the ionic gradients are controlled, novel properties of MCU were revealed. In particular, the K_m (19 mM) for Ca^{2+} entry through the MCU influx was ~2000 times higher [17] than the K_m (9 μM) determined from experiments using suspensions of respiring, isolated mitochondria [53]. This quantitative result suggests that, under the "right" conditions the MCUs have enormous influx capacity. These results also point out that the uncertainty of $\Delta\Psi_m$ during diverse experiments may have "contaminated" results obtained by many other investigators.

Beginning in the 1970s, several elegant experiments used suspensions of isolated mitochondria to reveal the biochemical properties of a mitochondrial Ca^{2+} extrusion process (that has recently been identified as NCLX) [46,47]. These studies determined the maximal NCLX Ca^{2+} flux (18 nmol•mg^{-1}•min^{-1}) and the K_m values for $[Na^+]_i$ and $[Ca^{2+}]_m$ (~8 mM and 13 μM, respectively). Future studies will be required to examine if and how these K_m values are influenced by fluctuations in $\Delta\Psi_m$ in the same manner as MCU (See discussion on NCLX stoichiometry below). Additionally, future experiments that simultaneously measure concentration gradients (for both Na^+ and Ca^{2+}) and NCLX flux would provide further insight into the thermodynamic conditions facing Na^+/Ca^{2+} exchange across the mitochondrial inner membrane versus Na^+/Ca^{2+} exchange across the plasma/sarcolemmal membrane [5,34,35,38–41,57,66–68]. Such work would refine existing kinetic features of NCLX which now is reported to have a sigmoidal dependency on $[Na^+]_i$ with a hill coefficient between 2 [47,60] and 3 [46] or a hyperbolic dependency [62] or even a linear relationship [63] and thus is not fully resolved

Stoichiometry—Another critically important aspect of NCLX function is the stoichiometry of the Na^+/Ca^{2+} exchange as noted above. If 3 Na^+ ions are transported for every 1 Ca^{2+} ion, NCLX would have a stoichiometry of 3:1, and it would be electrogenic in a manner similar to NCX on the plasma/sarcolemmal membrane. If, however, the exchange ratio is 2:1 then NCLX would be electroneutral.

Three approaches have been used to investigate electrogenicity versus electroneutrality in Na^+ dependent Ca^{2+} extrusion from the mitochondria. First, a thermodynamic analysis can be used. By this approach some investigators suggest a 2:1 stoichiometry [69] while others suggest it is 3:1 or greater [70,71]. However, the presence of other mitochondrial ionic transporters can also influence the Na^+ and Ca^{2+} gradients at steady-state [29] and their presence complicates the interpretation of simple thermodynamic studies. A second approach examines how $\Delta\Psi_m$ influences transport by NCLX and also determines if NCLX can affect $\Delta\Psi_m$. These studies are controversial since some studies have shown that $\Delta\Psi_m$ decreases during this Ca^{2+} efflux [72] while others have shown no apparent change in $\Delta\Psi_m$ [73]. Regardless of NCLX electrogenicity, the activity of NCLX results in a net influx of protons into the matrix (via the NHE, see Fig. 2 and discussion above), which can subtly alter the interpretation of some experiments. Thirdly, direct electrophysiological experiments can be carried out. This is now possible because of the NCLX molecular identity is known and function can be measured. Overexpressing NCLX in HEK-293 cells results in ectopic expression of NCLX on the plasma membrane [31,54]. This allowed the measurement of a 2 pA/pF current following a step increase (0 to 200 μM) in the extracellular $[Ca^{2+}]$ that did not occur in control HEK-293 cells [54]. (See also Section 2.4 above.) While this result strongly supports electrogenic NCLX function, there is some concern about the interpretation of the experiments because of the mis-targeting of the

NCLX proteins. Consequently, additional experiments should be conducted so that NCLX function can be examined in its native environment (i.e., in mitochondria). Additionally, quantitative measurements of net ion movement and the associated charge transfer across the mitochondrial inner membrane would provide conclusive evidence regarding NCLX stochiometry. However, while valuable, these experiments would be very challenging to perform in the mitochondria.

2.5. Dynamic Mitochondrial Na^+/Ca^{2+} Exchange During EC Coupling in the Heart

Both Na^+ and Ca^{2+} have an immense electrochemical driving force across the mitochondrial inner membrane that favors entry of these cations into the mitochondrial matrix. This is due largely to the very large $\Delta\Psi_m$ (approximately −180 mV) which establishes the equilibrium concentrations due to $\Delta\Psi_m$ alone for $[Na^+]_m$ and $[Ca^{2+}]_m$ as about 1000-fold and 1,000,000-fold greater in the mitochondrial matrix than in the cytosol. While physiologic diastolic $[Ca^{2+}]_i$ is about 100 nM, physiological cytosolic Na^+ ($[Na^+]_i$) is about 10 mM (or possibly somewhat less). Under steady-state diastolic conditions mitochondrial matrix calcium $[Ca^{2+}]_m$ is about 200 nM [37,74] while sodium $[Na^+]_m$ is about 5 mM [75]. Nevertheless, NCLX extrudes Ca^{2+} from the matrix against its electrochemical gradient using the electrochemical potential of sodium as the thermodynamic driving force for the ion exchange.

The dynamic effects of NCLX stoichiometry on $[Ca^{2+}]_m$ during the $[Ca^{2+}]_i$ transient are not intuitively obvious yet their actions are likely to be important. They clearly depend on many non-linear factors, each of which is likely to change with time. Thus a simple computational estimate of the effects of both NCLX stoichiometry and of cytosolic sodium, $[Na^+]_i$, is presented. To illustrate the dynamics of these fluxes during EC coupling we formulated a minimal, yet thermodynamically balanced, model of mitochondrial Ca^{2+} dynamics. In this model, $[Ca^{2+}]_m$ is determined by the balance between Ca^{2+} entry via the MCU and extrusion via either an electroneutral NCLX or electrogenic NCLX. The NCLX model formulation was taken from Dash *et al*., 2008 [76]. Mitochondrial $[Na^+]$ was assumed to be constant at 5 mM and cytosolic Na^+ was varied between simulations in increments of 5 mM (see colored lines in Figs. 3 and 4). Each simulation in Fig. 3 began with the cell in a quiescent (prolonged diastolic) state where $[Ca^{2+}]_i$ was 100 nM and $[Ca^{2+}]_m$ was approximately 200 nM (consistent with [37]). The cell was then stimulated at 0.5 Hz for 200 seconds.

Note, the peak of the $[Ca^{2+}]_i$ transient was artificially increased to 2.5 μM (from the typical range of 0.5 – 1 μM) in order to account for influence of elevated $[Ca^{2+}]$ in micro domains which bathe the ends of intermyofibrillar mitochondria during a $[Ca^{2+}]_i$ release event. Figure 3 (left) shows NCLX and $[Ca^{2+}]_m$ dynamics during the initial transient and Fig. 3 (right) shows steady-state transients after 200 seconds. In Fig. 3, the total amount of Ca^{2+} that entered the matrix during the initial $[Ca^{2+}]_i$ transient was 0.7 μmoles per liter of cytosol (consistent with [32,34,77]). Figure 3B shows the NCLX fluxes which govern the changes in $[Ca^{2+}]_m$ induced by the simulated $[Ca^{2+}]_i$ transients shown in Fig. 3A. Not surprisingly, the electrogenic NCLX flux was less sensitive to changes in $[Na^+]_i$ or $[Ca^{2+}]_i$ since this exchange mechanism is dominated by $\Delta\Psi_m$. Interestingly, the electroneutral NCLX flux was extremely sensitive to $[Ca^{2+}]_i$ and appeared to even reverse when $[Ca^{2+}]_i$ was high or $[Na^+]_i$ was low (see Fig. 3B). However, both forms of NCLX exchange are capable of reproducing physiological mitochondrial Ca^{2+} dynamics when scaled appropriately. See discussion of Dash & Beard (2008) below.

Figure 4 shows NCLX and $[Ca^{2+}]_m$ dynamics when mitochondrial Ca^{2+} fluxes (MCU and NCLX) were increased 36 fold when compared to those shown in Fig. 3. This high flux is required to reproduce the findings of Maack *et al*., 2006, Gauthier *et al*., 2012, and Drago *et al*., 2012 [5,41,78] where mitochondria function as dynamic buffers of $[Ca^{2+}]_i$. Note that the

low flux conditions (i.e., Fig. 3), are not able to reproduce the $[Ca^{2+}]_m$ dynamics seen in Fig. 4 by merely changing the pacing frequency or $[Ca^{2+}]$ transient levels (see Figs. 1S and 2S, respectively). Under high Ca^{2+} flux conditions, significant $[Ca^{2+}]_m$ transients are observed and the electrogenic NCLX flux is more dynamic (compare Fig. 3B and Fig. 4B with respect to both scale and time-course) but still less sensitive to $[Na^+]_i$. Note, however, the mitochondria are now also accumulating larger amounts of Na^+ since 50 to 75 μmoles of Na^+ enter the mitochondria during each Ca^{2+} transient (for electroneutral and electrogenic NCLX, respectively). This Na^+ will then need to be removed by the Na^+/H^+ exchanger and this process must consume some of the proton electromotive reserve.

Some key biophysical details of NCLX function are still unclear; this is primarily due to the technical limitations regarding the accessibility of the inner mitochondrial membrane. Given the current information, distinguishing between possible modes of NCLX function is challenging. A careful examination of NCLX electrogenicity by Dash & Beard (2008) suggested that a $3Na^+$:$1Ca^{2+}$ exchange ratio (electrogenic) is more likely. However, when $[Ca^{2+}]_i$ is assumed to be low, electroneutral exchange can also satisfactorily reproduce the experimental observations [79] utilized by Dash & Beard (2008) [76]. This does focus our attention on two important features. First, the need for more and better quantitative information on $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$. Second, for the adoption and extension of robust computational models that are constrained and informed by the biology.

Insights on $[Na^+]_i$ and NCLX stochiometry—Electroneutral NCLX appears to be very sensitive to $[Na^+]_i$ (see Figs 3 and 4). If NCLX exchange is electroneutral, then its activity, and consequently $[Ca^{2+}]_m$ dynamics should be particularly sensitive to changes in $[Na^+]_i$. Note that electrogenic NCLX will still be influenced by $[Na^+]_i$ but to a lesser degree because it is dominated by $\Delta\Psi_m$. It is known that in some cardiac pathologies $[Na^+]_i$ is elevated (see [75,80–82]). It is suggested that this elevated $[Na^+]_i$ could adversely affect mitochondrial function. For example, when $[Na^+]_i$ was elevated experimentally from 5 mM to 15 mM the "energetic adaptation" of the mitochondria to the work load was impaired according to Maack *et al*., 2006. In these conditions when the work load increases the levels of unoxidized NADH may decline substantially (see [5] and [81]). This was attributed to NCLX extruding more $[Ca^{2+}]_m$ and thereby reducing the activity of the Ca^{2+}-sensitive TCA cycle dehydrogenases. The model results (see Figs. 3 and 4) suggest that perhaps an electroneutral mode of NCLX exchange would be in accordance with this hypothesis. Quantitative studies into the dynamics of $[Ca^{2+}]_m$ and $[Na^+]_i$ may provide insight into this topic and are an area of active research by us and others.

NCLX function during cardiac ischemia and reperfusion—A large body of evidence suggests that the deleterious outcome of ischemia and reperfusion is initiated by $[Ca^{2+}]_m$ overload. The high $[Ca^{2+}]_m$ underlies the opening of the mitochondrial permeability transition pore (mPTP) and loss of $\Delta\Psi_m$ [22,82–87]. During the ischemic phase, it has been shown that mPTP is less likely to open despite the sustained and increased $[Ca^{2+}]_i$ [88,89]. This may be due to the elevated $[H^+]$ present in the cytosol that inhibits mPTP opening [90]. In addition, our analysis here suggests that during the ischemic phase, extrusion of Ca^{2+} from the matrix by NCLX may substantially increase due to high $[Na^+]_i$ [91,92]. Under these conditions NCLX would only extrude Ca^{2+} from the matrix and not contribute to Ca^{2+} influx (even if electroneutral). If NCLX is electrogenic; its function should be more sensitive to $\Delta\Psi_m$ changes (than if it were electroneutral) and this could make NCLX ineffective in preventing mitochondrial Ca^{2+} overload in disease conditions where mitochondria are partially depolarized [14,83,84]).

In conclusion, an electroneutral NCLX may be sufficient under some conditions to regulate $[Ca^{2+}]_m$ and would have the added benefit of being more energetically efficient by

contributing less to depolarization of the mitochondrial inner membrane. Yet, the electrogenic NCLX appears to be more stable and capable of preventing large swings in $[Ca^{2+}]_m$ which could be detrimental for the cell function. Studies into the dynamics of NCLX represent an exciting area for future experimental and computational modeling studies and should include investigations of the stoichiometry.

2.6. NCLX and MCU balance at steady state

Physiologic regulation of mitochondrial function involves changes in $[Ca^{2+}]_m$ that reflect cellular activity. As activity and stress influence cellular behavior so too may $[Ca^{2+}]_m$ influence mitochondrial behavior. Since the MCU Ca^{2+} influx and NCLX Ca^{2+} efflux must be in balance under steady-state conditions, their fluxes of Ca^{2+} must change together. The net effect of changing cellular activity is then the increase or decrease of $[Ca^{2+}]_m$ as activity increases or decreases respectively. As noted in Figs. 3 and 4 and in the related discussion there are important implications for $[Ca^{2+}]_m$ and $[Na^+]_m$ that depend on the stoichiometry of NCLX and the magnitude of the MCU Ca^{2+} flux. If "low" levels of MCU Ca^{2+} influx are the physiological rule, then they will be associated with $[Ca^{2+}]_m$ of 100 – 200 nM under quiescent conditions and 300 – 900 nM during maximum physiological activity. If "high" levels of MCU Ca^{2+} influx (30 to 100 times greater) are found to be physiologic, then $[Ca^{2+}]_m$ will be significantly larger. What is clear, however, is that quantitative experiments and analysis and mathematical models are critical elements in our current and future approaches to resolve questions related to how the mitochondria function under physiological and pathological conditions.

Summary

The net movement of Ca^{2+} across the mitochondrial inner membrane is small at physiological resting $[Ca^{2+}]_i$ (about 100 nM) and also quite modest during physiological $[Ca^{2+}]_i$ transients in all cell types, based on our review of the literature and our own investigations. Published data to date in heart (which has a significant regular $[Ca^{2+}]_i$ transients of about 0.5 to 1.0 μM at 1–5 Hz), $[Ca^{2+}]_m$ in the beating heart cells is probably in the range of 300 – 900 nM with small, virtually imperceptible fluctuations (around 5 – 10 nM per beat). Under physiological conditions in the steady state, NCLX and MCU Ca^{2+} transport must be in balance and transport only modest amounts of Ca^{2+}. While this makes sense from the point of view of energy consumption and metabolic regulation, quantitative experiments still need to be carried out to determine how much Ca^{2+} is moved through the mitochondria via the MCUs in a mitochondrion and how much is pumped out via the NCLX. It is however very important to recognize that differing conclusions have been reached by others groups regarding the influx and efflux of Ca^{2+} into the mitochondria as noted in the review above. Resolution of these differences and the functional implications of this matter require quantitative investigations in living cells with high temporal and spatial resolution of $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$. Such biological investigations will be significantly illuminated by mathematical models of mitochondria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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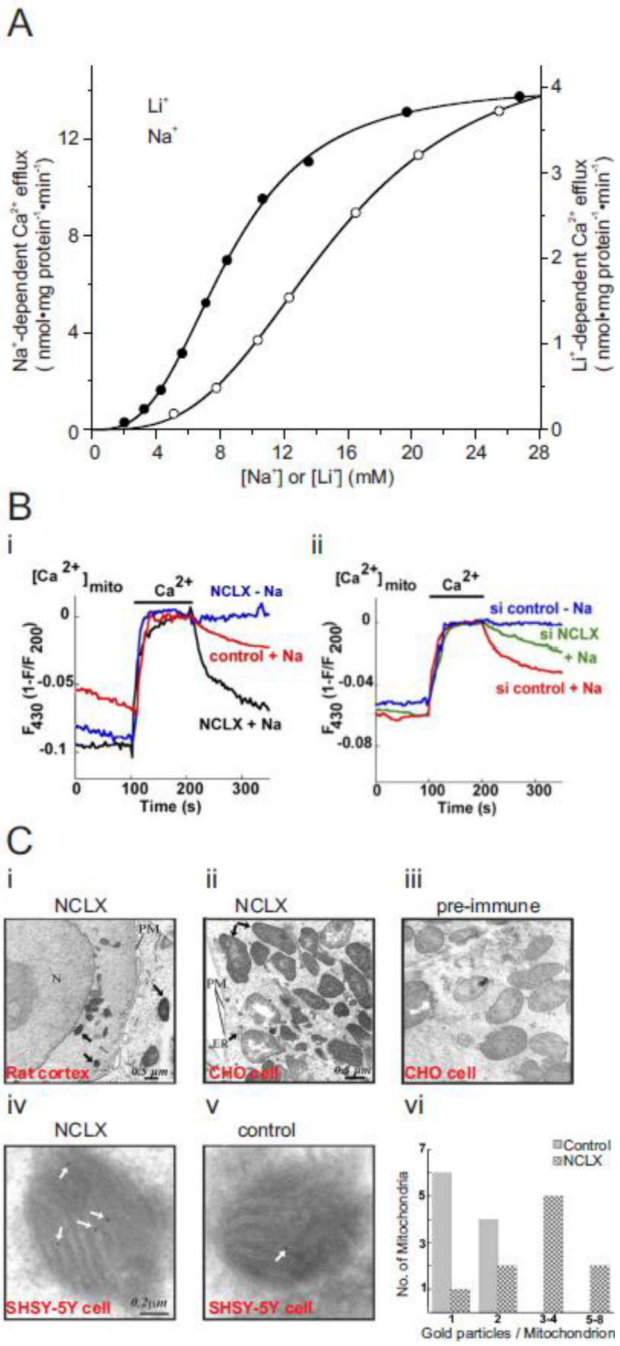

Fig. 1. Function and molecular identity of the mitochondrial Na^+/Ca^{2+} exchanger: NCLX

(A) The early evidence of a mitochondrial Na^+ or Li^+-dependent Ca^{2+} exchange (digitized and re-plotted from[46]). Measurements of Ca^{2+} uptake by isolated heart mitochondria in suspension show a concentration dependent mitochondrial Ca^{2+} efflux following the addition of either Na^+ (filled circles) or Li^+ (open circles). **(B)** The identification of NCLX as the mitochondrial Na^+/Ca^{2+} exchanger (used with permission from [28]). Measurements of $[Ca^{2+}]_m$ in HEK 293 cells using a Ca^{2+} sensitive fluorescent probe that was targeted to the mitochondria (i.e., mito-pericam). The sarcolemmal membrane was permeabilized (0.007% Digitonin) and subsequent experiments had 3 phases: first, for 100 seconds a baseline recording in the absence of Ca^{2+} or Na^+, second, for 100 seconds the mitochondria

was loaded with Ca^{2+} by superfusion with a solution containing 60 μM Ca^{2+} and no Na^{+}, third, Na^{+} dependent Ca^{2+} efflux from mitochondria was induced by superfusion with a solution containing 20 mM Na^{+} (indicated by "+ Na"). Note that in phase 3 when the superfusion solution contains 20 mM of $NMDG^{+}$ (blue traces) instead of 20 mM Na^{+} no change of $[Ca^{2+}]_m$ is observed (indicated by "− Na"). (i) Records of $[Ca^{2+}]_m$ show that overexpression of NCLX enhances the Na^{+} dependent Ca^{2+} efflux. (ii) The Ca^{2+} efflux is diminished by knocking down the expression of NCLX using siNCLX. **(C)** NCLX localization in electron micrographs (reused with permission from [28]). (i) Rat cortical slices stained with anti-NCLX antibodies. (ii) CHO cells stained with anti-NCLX antibodies. (iii) CHO cells stained with NCLX premium serum. Note that in panels i-iii dense DAB (3,3'-Diaminobenzidine) precipitates indicating positive immunolabeling are primarily in the mitochondria. (iv-vi) Immunogold labeling of SHSY-5Y cells overexpressing (iv) or endogenously expressing (i.e., "control") NCLX (v). Note that NCLX labeling is primarily in the mitochondrial cristae. (vi) The number of gold particles per mitochondrion. The number of gold particles in NCLX-overexpressing cells was 35 versus 14 in the control cells. *Abbreviations used; nucleus (N), endoplasmic reticulum (ER) and plasma membrane (PM).*

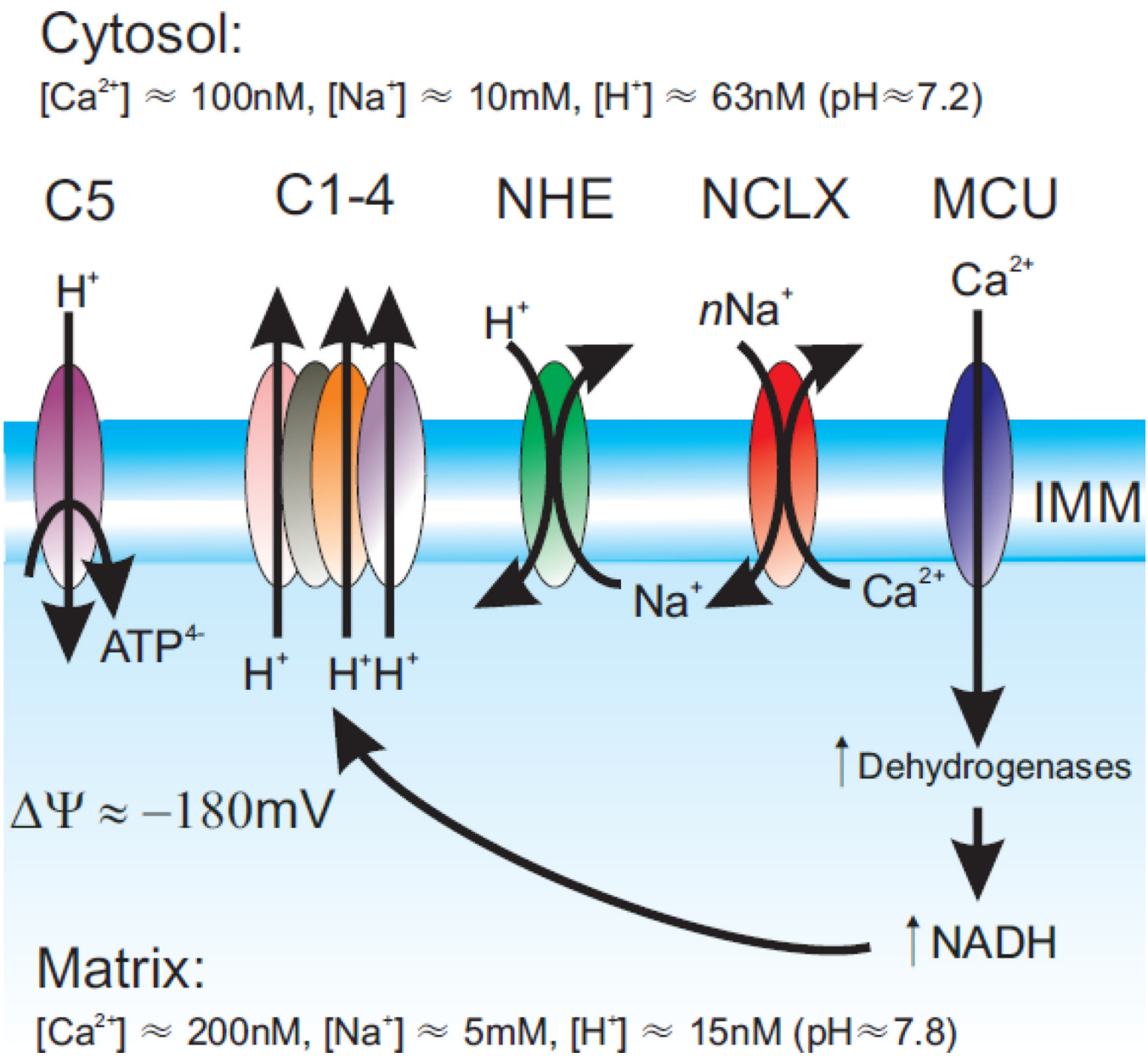

Fig. 2. NCLX and the simplified schematic diagram of the "Ca^{2+} cycle" across the mitochondrial inner membrane

Ca^{2+} enters the mitochondrial matrix through the Ca^{2+} uniporter (MCU), down an electrochemical gradient. The large negative potential across the inner membrane of about −180 mV ($\Delta\Psi_m$) and the overall proton-motive potential across the mitochondrial inner membrane is due to proton extrusion from the matrix by the electron transport chain (C 1–4). Ca^{2+} is removed from the matrix by the mitochondrial Na^+/Ca^{2+} exchanger (NCLX) and Na^+ that enters by NCLX is extruded from the mitochondrial matrix by the mitochondrial Na^+/H^+ exchanger (NHE). Diverse mitochondrial proteins are regulated by $[Ca^{2+}]_m$.

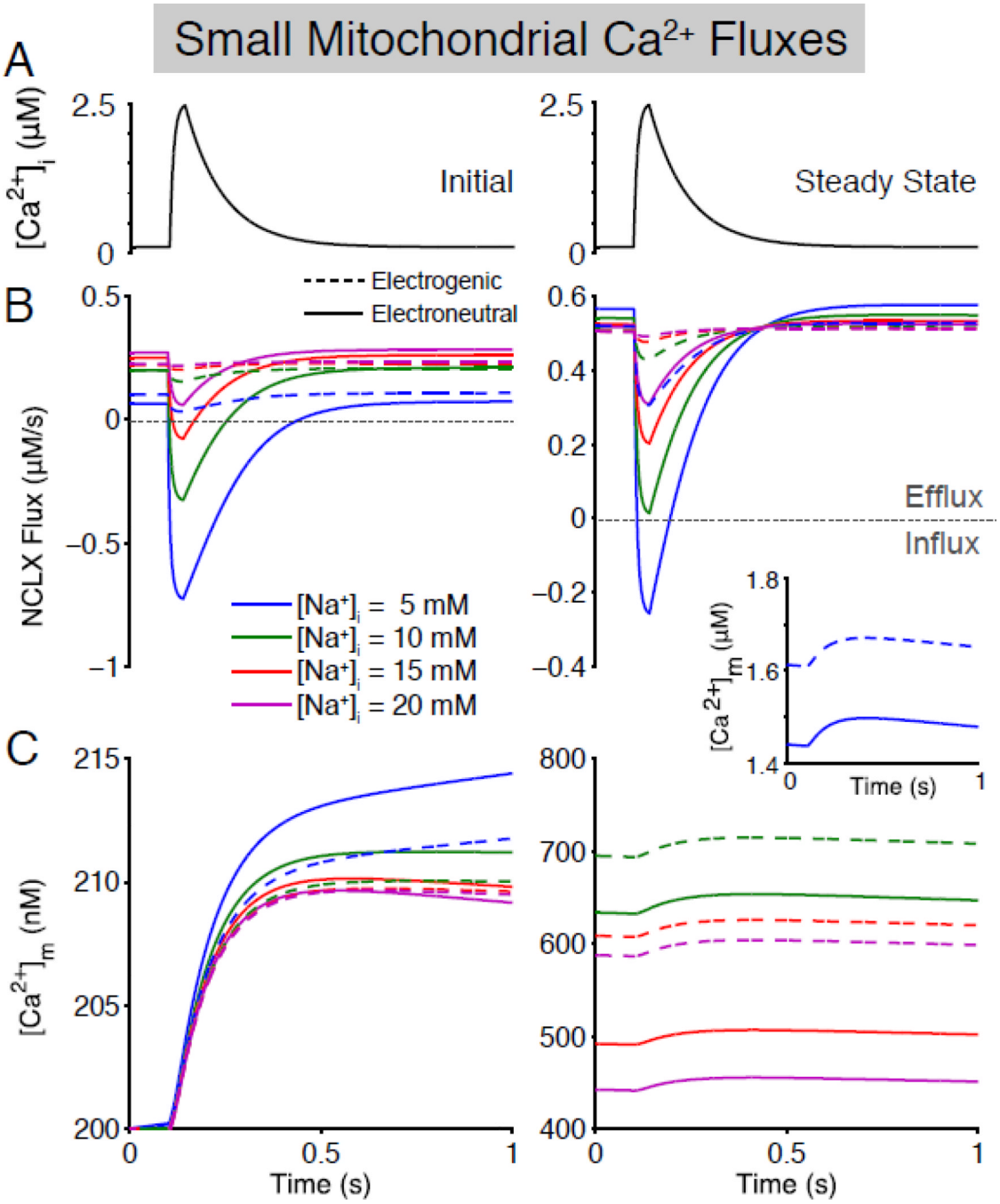

Fig. 3. Effects of NCLX stoichiometry and $[Na^+]_i$ on time-dependent Ca^{2+} signals in cardiac mitochondria - low Ca^{2+} flux

To estimate how the time-dependent mitochondrial $[Ca^{2+}]_m$ depends on NCLX stoichiometry and cytosolic $[Na^+]_i$, a simple computational model of Ca^{2+} movement across the inner membrane was used. Small mitochondrial Ca^{2+} fluxes were assumed, consistent with the results from the Bers group [34]. During normal cardiac activity in the steady state, the MCU-dependent Ca^{2+} influx into the mitochondrial matrix was in balance with the NCLX-dependent Ca^{2+} efflux. Left-hand panels: after a long quiescent period (prolonged diastole), the first $[Ca^{2+}]_i$ transient is shown (top, left) and the first mitochondrial $[Ca^{2+}]_m$ transient is shown (bottom, left). The left-hand middle panel shows the NCLX Ca^{2+} flux

(positive =efflux; negative = influx). Data is shown when NCLX is electrogenic with a 3:1 stoichiometry (dashed lines) and electroneutral with a 2:1 stoichiometry (solid lines). The $[Na^+]_i$ dependencies for four different levels of $[Na^+]_i$ are indicated by the line color (blue = 5, green = 10, red = 15, and magenta = 20 mM). Right-hand panels show the same measures after steady-state heart cell activity at 0.5 Hz. Under all test conditions, $[Na^+]_m$ is assumed to remain constant at 5 mM. (**A**) $[Ca^{2+}]_i$ transients (left and right panel) (**B**) Ca^{2+} flux via NCLX, during the first $[Ca^{2+}]_i$ transient (left panel) and following steady-state $[Ca^{2+}]_i$ transients (right panel) (C) $[Ca^{2+}]_m$ levels during the first $[Ca^{2+}]_i$ transient (left panel) and following steady-state conditions (right panel). Inset shows zoomed in view of steady-state $[Ca^{2+}]_m$ dynamics when cytosolic $[Na^+]_i$ = 5 mM. Model Formulation: This simple compartmental model consists of two compartments; a cytosolic and mitochondrial volume each having a free $[Ca^{2+}]$ (indicated by $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$, respectively). The $[Ca^{2+}]_i$ is "clamped" to follow the idealized $[Ca^{2+}]_i$ transient shown in Fig 3A & 4A). The change in $[Ca^{2+}]_m$ is given by the ordinary differential equation, $\Delta[Ca^{2+}]_m = \beta/\lambda(J_{mcu} - J_{nclx})$ where β is the dynamic buffering fraction (for simplicity, assumed to be similar to the cytosolic buffering levels from [93]) and λ is the mitochondrial to cytosolic volume fraction (λ = 0.5, see [36]). J_{mcu} represents the Ca^{2+} influx into the mitochondria from the cytosol via MCU and J_{nclx} represents the efflux of Ca^{2+} from the mitochondria via NCLX. J_{mcu} was limited to a simple linear function of $[Ca^{2+}]_i$ (i.e., $J_{mcu} = v_{mcu}[Ca^{2+}]_i$ where $v_{mcu} = 2\ s^{-1}$) as these simulations focus on NCLX behavior. Model formulations for NCLX are from Dash et al., 2008. Note, the electrogenic and electroneutral NCLX fluxes are scaled to balance J_{mcu} under "diastolic" conditions when $[Ca^{2+}]_i$ = 100 nM, $[Ca^{2+}]_m$ = 200 nM, $[Na^+]_i$ = 10 mM, and $[Na^+]_m$ = 5 mM.

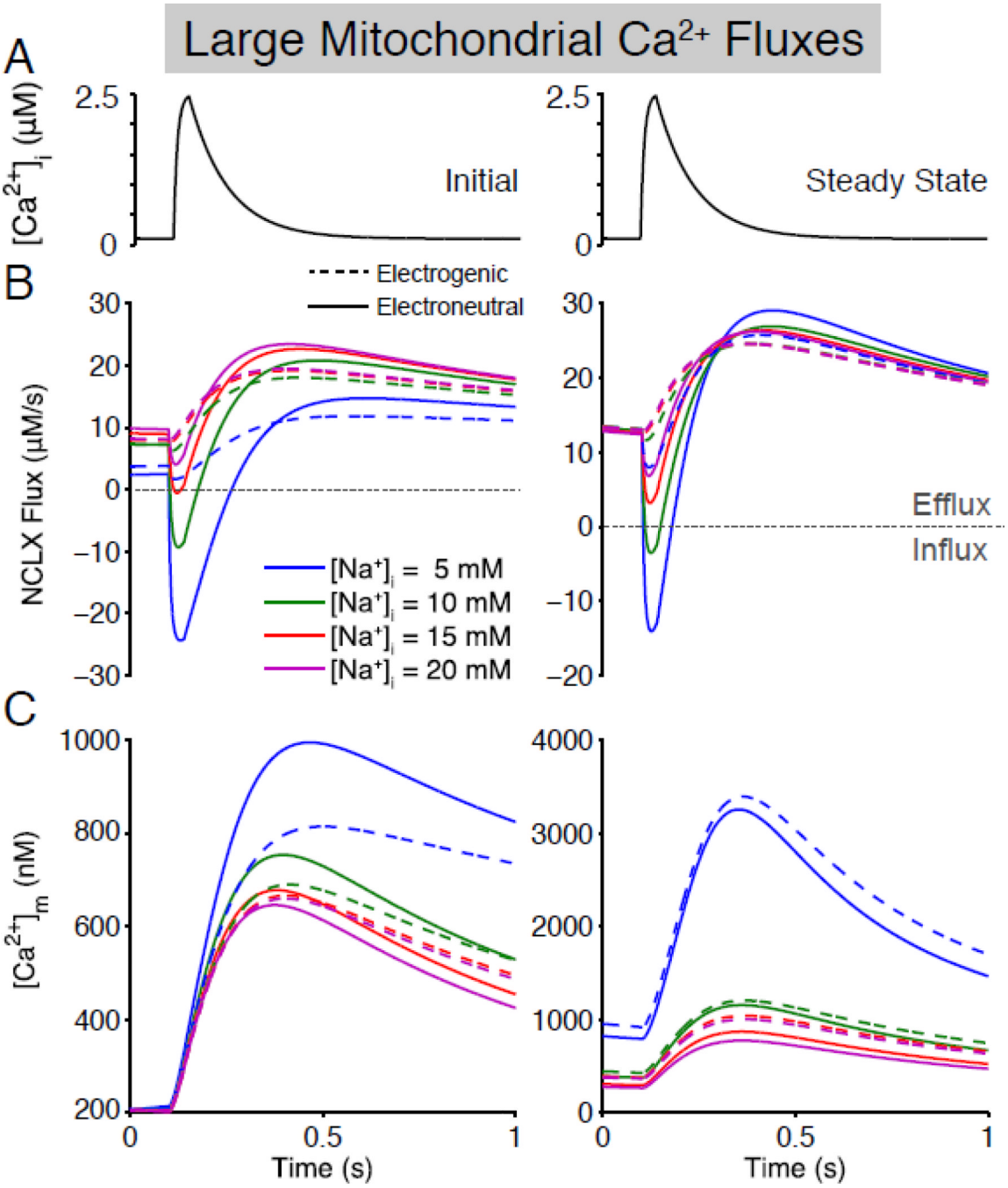

Fig. 4. Effects of NCLX stoichiometry and $[Na^+]_i$ on time-dependent Ca^{2+} signals in mitochondria - high Ca^{2+} flux

The same simple computational model was used as in Fig. 3 except that the mitochondrial Ca^{2+} fluxes (J_{mcu} and J_{nclx}) are assumed to be 36-fold larger. The mitochondrial fluxes used in Fig. 4 are consistent with the published results of [5].