Published in final edited form as:

J Mol Cell Cardiol. 2009 June ; 46(6): 767–774. doi:10.1016/j.yjmcc.2008.12.011.

Mitochondrial Ca^{2+} uptake: tortoise or hare?

Brian O'Rourke, PhD and

Division of Cardiology, Department of Medicine, The Johns Hopkins University, Baltimore, MD 21205

Lothar A. Blatter, M.D., Dr. med.

Dept. Molecular Biophysics & Physiology, Rush University medical Center, Chicago, IL 60612

Abstract

Mitochondria are equipped with an efficient machinery for Ca^{2+} uptake and extrusion and are capable of storing large amounts of Ca^{2+} ions. Furthermore, key steps of mitochondrial metabolism (ATP production) are Ca^{2+} -dependent. In the field of cardiac physiology and pathophysiology, two main questions have dominated the thinking about mitochondrial function in the heart: 1) how does mitochondrial Ca^{2+} buffering shape cytosolic Ca^{2+} levels and affect excitation-contraction coupling, particularly the Ca^{2+} transient, on a beat-to-beat basis, and 2) how does mitochondrial Ca^{2+} homeostasis influence cardiac energy metabolism. To answer these questions a thorough understanding of the kinetics of mitochondrial Ca^{2+} transport and buffer capacity is required. Here, we summarize the role of mitochondrial Ca^{2+} signaling in the heart, discuss the evidence either supporting or arguing against the idea that Ca^{2+} can be taken up rapidly by mitochondria during excitation-contraction coupling and highlight some interesting new areas for further investigation.

Keywords

calcium uniporter; sodium-calcium exchange; mitochondrial inner membrane; Ca^{2+} transients

Intro

Like historic vintages, certain great questions of physiology have managed to span more than a century and continue to generate interest and debate. An example is the mechanism behind the Frank-Starling "law of the heart"[1], which in an early form described the relationship between diastolic cardiac volume and oxygen consumption (an indirect measurement of mechanical energy expenditure)[2], evolving more recently into current

© 2008 Elsevier Ltd. All rights reserved.

Correspondence to: Lothar A. Blatter, M.D., Dr.med., Dept. Molecular Biophysics & Physiology, Rush University Medical Center, 1750 W. Harrison St., Chicago, IL 60612, Tel.: 312-563-3238, Fax.: 312-942-8711, Lothar_Blatter@rush.edu. Brian O'Rourke, PhD, Johns Hopkins University, Institute of Molecular Cardiobiology, Division of Cardiology, 720 Rutland Ave, 1060 Ross Bldg., Baltimore, MD 21205-2195; USA, Tel.: 410-614-0034, Fax.: 410-502-5055, bor@jhmi.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

discussions about how the Ca^{2+} sensitivity of myofilament force generation is determined by changes in sarcomere length. The second component of this question, which is also incompletely understood at the present time, is the mechanism by which “the metabolism of the heart increases or diminishes in proportion to the mechanical demands made upon this organ, so that the chemical sources of energy are drawn upon in proportion to its requirements”, as stated by Starling and coworkers in 1914[1].

Based on studies of suspensions of isolated mitochondria, several potential mechanisms have been elucidated by which mitochondrial ATP production can be regulated to match energetic demand. Respiratory (or acceptor) control by ADP and P_i availability was described in the 1950s[3, 4] and is indisputably a robust determinant of respiration in isolated mitochondria. On the other hand, the inability to detect changes in the bulk adenine nucleotide phosphorylation potential (i.e., $[\text{ATP}]/([\text{ADP}] + [\text{P}_i])$) in intact hearts at different workloads using phosphorous NMR[5] has led investigators to examine other potential factors regulating oxidative phosphorylation. For example, control of mitochondrial function through the allosteric effects of P_i on the rates of both NADH production by the tricarboxylic acid (TCA) cycle and electron transport has been put forward as a potentially important metabolic signal[6].

Since mitochondria have a high permeability to Ca^{2+} [7] and key enzymes in the tricarboxylic acid (TCA) cycle are known to be Ca^{2+} sensitive, metabolic signaling by Ca^{2+} has been a leading hypothesis to explain the control of mitochondrial oxidative phosphorylation *in vivo*. Moreover, evidence suggests that Ca^{2+} might also regulate various sites of the electron transport chain and the mitochondrial ATP synthase[8, 9]. Changes in cardiac output often (but not always) involve changes in the time averaged cytoplasmic Ca^{2+} concentration, so it is important to determine the kinetics and magnitude of mitochondrial Ca^{2+} transport in intact cardiomyocytes in order to answer two main questions: 1) how does mitochondrial Ca^{2+} buffering shape the Ca^{2+} transient and 2) how does mitochondrial Ca^{2+} uptake influence metabolism. This subject has been a subject of intense investigation and numerous reviews[10, 11]. Here, we will briefly summarize some of the evidence either supporting or arguing against the idea that Ca^{2+} can be taken up rapidly by mitochondria during excitation-contraction coupling and highlight some interesting new areas for further investigation.

O'RourkeBrian

POINT: Evidence in favor of fast mitochondrial Ca^{2+} uptake in the heart

The development of new tools and techniques of measuring mitochondrial Ca^{2+} over the years has led to a gradual accumulation of support for the idea that mitochondria can respond rapidly to changes in Ca^{2+} during the heartbeat. This evidence spans from the level of isolated mitochondria to the intact heart, as described below.

Isolated mitochondria

Ca^{2+} is taken up by the mitochondria via the Ca^{2+} uniporter (mCU), a still undefined protein in the inner membrane, driven by the large electrochemical gradient for Ca^{2+} across the inner mitochondrial membrane ($\Psi_m \approx -180 \text{ mV}$). The mCU studied in isolated

mitochondria has second order kinetics, indicating an allosteric effect of Ca^{2+} on the transport rate. The turnover for Ca^{2+} uptake was previously estimated to be approximately $2 \times 10^4 \text{ Ca}^{2+} \text{ s}^{-1}$ per single mCU molecule, in the range of a fast gated pore [7, 10]. In support of the notion that the mCU is an ion channel, a recent patch clamp study of intact mitoplasts has identified a mitochondrial Ca^{2+} channel (MiCa) possessing a high affinity binding site for Ca^{2+} (2 nM) that confers high Ca^{2+} selectivity at resting cytoplasmic Ca^{2+} but with an enormous capacity for Ca^{2+} uptake (half-saturation of I_{MiCa} was $\sim 20 \text{ mM}$) [12]. The channel activated almost instantaneously at negative membrane potentials with some inward rectification and showed partial fast inactivation and block by known mCU inhibitors like ruthenium red. Notably the V_{max} of MiCa was significantly higher than earlier estimates of mCU ($V_{\text{max}} = 5 \times 10^6 \text{ Ca}^{2+} \text{ s}^{-1}$ per single mCU molecule), as was the channel density (~ 10 – 40 channels per μm^2). The authors suggested that the true mCU V_{max} is usually underestimated in intact mitochondria due to the fact that Ψ_{m} will be rapidly dissipated at high Ca^{2+} concentrations, a problem which is circumvented by voltage clamping the membrane. Similarly, the true K_{m} of Ca^{2+} uptake through the mCU, which is on the order of ~ 10 – $20 \mu\text{M}$ for intact isolated mitochondria, might be much higher.

Indirect support for the idea that Ca^{2+} uptake is very fast in isolated mitochondria comes from experiments examining the effects of Ca^{2+} on respiration using rapid solution switching or stopped-flow methods. As long ago as 1965, Chance [12] reported that the oxidation of cytochrome b occurs with a halftime of $\sim 70 \text{ msec}$ (an upper limit estimate) in isolated liver mitochondria exposed to a $200 \mu\text{M}$ pulse of Ca^{2+} . In more recent experiments, Territo et al [13] showed that changes in mitochondrial oxygen consumption, NADH, and light scattering (a measure of mitochondrial matrix volume) occurred with an initial response time of $< 100 \text{ msec}$ after the rapid application of an optimal level of 535 nM Ca^{2+} in porcine heart mitochondria.

A rapid mode of mitochondrial Ca^{2+} uptake, termed RaM, has been reported [14], with kinetics approximately 100-fold faster than those measured for mCU (which are in the range of seconds). Rapid mitochondrial Ca^{2+} uptake via RaM was observed on the first of a series of Ca^{2+} pulses delivered by rapid mixing in liver or heart mitochondria [14]. RaM is less sensitive to ruthenium red than the mCU, but it is rapidly inactivated if the Ca^{2+} concentration in the medium exceeds $\sim 100 \text{ nM}$ [15]. Recovery from inactivation of this mode of Ca^{2+} transport occurs over 10s of seconds, so it is unlikely to account for beat-to-beat mitochondrial Ca^{2+} transients in paced cells.

Other cytosolic factors can dramatically influence the apparent affinity for mitochondrial Ca^{2+} uptake in isolated mitochondria, including the concentrations of Na^+ and Mg^{2+} in the medium. For example, the addition of 10 mM Na^+ plus 1 – 2 mM Mg^{2+} , levels not very different from physiological conditions, can shift the midpoint of Ca^{2+} uptake from $\sim 0.1 \mu\text{M}$ to $> 1.5 \mu\text{M}$ [16]. Moreover, the midpoint of activation of TCA cycle enzymes by extramitochondrial Ca^{2+} can be shifted to the right by about one log unit when 0.5 mM Mg^{2+} plus 15 mM Na^+ is present [17]. The effects of Mg^{2+} are thought to be mediated by a direct interaction of the divalent ion with the mCU, and Ca^{2+} current through MiCa $^{2+}$ is inhibited by almost 60% in the presence of 0.5 mM Mg^{2+} . The Na^+ effect follows logically from the consensus that the primary mitochondrial Ca^{2+} efflux mechanism (at least in cardiac

mitochondria) is the mitochondrial Na⁺/Ca²⁺ exchanger (mNCE)[7, 17], which is presumably electrogenic[18, 19], exchanging 1 Ca²⁺ for 3 Na⁺, with a K_m for [Na⁺]_i of ~8 mM[20]. Steady-state matrix Ca²⁺ concentration would therefore depend on the balance between fast mitochondrial Ca²⁺ uptake and slow efflux via mNCE.

Electron-probe microanalysis

Electron-probe microanalysis studies were among the first to suggest that mitochondrial Ca²⁺ uptake in intact cardiac myocytes can be several orders of magnitude faster than that in traditional isolated mitochondrial suspensions. Wendt-Gallitelli and Isenberg[21, 22] reported transients in total mitochondrial Ca²⁺ that peaked ~15–20 ms after the cytosolic transient in shock frozen guinea-pig cardiac, with flux estimates on the order of ~100 nmol/s/mg protein for the mCU. Mitochondrial Ca²⁺ decayed with a rate of ~36 nmol/s/mg protein, accompanied by a mitochondrial Na⁺ transient, consistent with matrix Ca²⁺ rapidly exchanging with Na⁺ via the mNCE[22]. This extra increment of matrix [Na⁺] is then removed by exchange of Na⁺ with H⁺ via the mitochondrial Na⁺/H⁺-exchanger (mNHE) at the expense of the pH gradient[7, 16, 23]. Notably, the observation of fast mitochondrial transients was only possible after paired-pulse stimulation, which will enhance the SR Ca²⁺ load.

Loading the mitochondrial compartment with fluorescent Ca²⁺ indicators

The other main strategy for examining the kinetics of mitochondrial Ca²⁺ uptake has been to load the mitochondrial matrix with fluorescent Ca²⁺ indicators. Among the inorganic dyes, Rhod-2 is often used, because its negative charge helps to preferentially retain it in the matrix due to the large negative Ψ_m . The challenge with these methods is to avoid contamination from dye in the cytosolic component when intact myocytes are employed. Several papers from the Lemasters group tackled this issue by using confocal microscopy in rabbit cardiac myocytes loaded with either fluo-3/AM[24, 25], rhod-2/AM-[26, 27] or indo-1/AM[25]. They argued that the high spatial resolution of the confocal permitted them to distinguish cytosolic and mitochondrial signals and they found that Ca²⁺ signals arising from mitochondria (i.e., colocalizing with the Ψ_m probe TMRE) had rapid kinetics almost identical to the cytosolic Ca²⁺ transient (although the time resolution of the recordings was not sufficient to analyze the rising phase of the transients in detail). β -Adrenergic stimulation increased amplitudes of both the cytosolic and mitochondrial Ca²⁺ transients[24, 25]. Although it has been argued that this experimental design inadequately excludes contamination of the fluorescence signal from the cytosolic compartment[11], it was shown that the mCU-inhibitor ruthenium red preferentially inhibited the mitochondrial Ca²⁺ signal without altering the cytoplasmic transient[26].

To more rigorously exclude contamination of mitochondrial Ca²⁺ signals with cytoplasmic dye, others have either quenched the cytosolic fluorescence by incubation with Mn²⁺, at concentrations that don't significantly impair contraction[28–33], allowed the cytoplasmic component to leak out over hours at warm temperatures[32], or equilibrated the cytoplasm with dye-free pipette solution using the whole cell patch clamp method[33]. The majority of these studies came to the conclusion that mitochondrial Ca²⁺ uptake occurred over seconds rather than milliseconds, when myocytes were electrically-stimulated, even at rates up to

4Hz with β -adrenergic activation electrical stimulation of the myocyte[28, 29]. When the experimental protocols of these papers are specifically broken down and critically analyzed, there is still room to argue in favor of fast mitochondrial Ca^{2+} uptake. For instance, Mn^{2+} quench methods do not take into account the inhibitory effect of Mn^{2+} on the mCU and oxidative phosphorylation that could alter the kinetics of mitochondrial Ca^{2+} uptake. Additionally, some of the evidence for a high K_m and slow uptake of Ca^{2+} by mitochondria in several studies was based on changing the cytosolic Ca^{2+} through slow transsarcolemmal process, that is, Ca^{2+} was raised by activating reverse-mode NCX current by removing external Na^+ [28] or by giving long voltage clamp pulses[33]. In the latter instances, the extracellular location of the source of Ca^{2+} , and the diffusion pathway to reach the mitochondria may be very different from Ca^{2+} released as a spark from the SR right next to the mitochondrion.

The importance of the kinetics, amplitude and location of the Ca^{2+} pulse applied to the mitochondria cannot be understated, as emphasized by studies of partially-permeabilized cells. Sharma et al.[34], loaded rat cardiomyocytes with rhod-2/AM and then permeabilized the sarcolemma with saponin. Ca^{2+} in the bath solution was measured using fura-2 salt. SR Ca^{2+} release initiated by the application of caffeine caused bath Ca^{2+} to rise rapidly in a few seconds and then decay within 13 seconds. Mitochondrial Ca^{2+} rose with similar kinetics, had a longer peak and then also decayed rapidly within 15s. The mitochondrial location of the rhod-2 signal was confirmed by the differential sensitivity of the transient to Ruthenium Red and its abolishment by dissipation of the protonmotive force. Most notably, while the caffeine-induced increase in free Ca^{2+} in the medium could be prevented by adding the fast Ca^{2+} buffer BAPTA to the bath, the mitochondria still took up Ca^{2+} , indicating that the sites of mitochondrial Ca^{2+} uptake were in a microdomain that was very close to the release sites. The rate of mitochondrial Ca^{2+} uptake upon caffeine exposure was strictly controlled by the rate of SR Ca^{2+} release, which could be slowed by the addition of thapsigargin, an inhibitor of SERCA. In a more recent study, Belmonte et al[35] also reported that caffeine-induced SR Ca^{2+} release rapidly loads the mitochondrial compartment in adult rat cardiomyocyte, even if the sarcolemma is permeabilized and bulk Ca^{2+} is buffered. The important novel finding of this study was that the mitochondrial Ca^{2+} store could be discharged back into the cytoplasm under the mechanical strain imposed by a change in the perfusion flow (a “pressure puff”).

Similar evidence for an SR-mitochondrial Ca^{2+} microdomain was also obtained by Szalai et al.[36] in cardiac-derived H9c2 cells. In permeabilized cells, SR Ca^{2+} release induced by caffeine or ryanodine produced rapid increases in mitochondrial Ca^{2+} synchronized with the rising phase of the cytosolic transient and these mitochondrial Ca^{2+} transients were not inhibited by adding Ca^{2+} buffer to the medium. Addition of $30\mu\text{M}$ Ca^{2+} to the bath was required to match the rates of rise of the caffeine-induced mitochondrial Ca^{2+} transients, again indicating that the local Ca^{2+} in the microdomain near the mitochondria during SR Ca^{2+} release is much higher than the average cytoplasmic concentration. In another study by the Hajnóczky group, more direct evidence of local fast communication between the SR Ca^{2+} release units and the mitochondria was obtained. In permeabilized H9C2 myotubes, in which the mitochondria were loaded with rhod-2, low concentrations of caffeine elicited repeated Ca^{2+} sparks that generated Ca^{2+} transients in adjacent single mitochondria, referred

to as Ca^{2+} marks (i.e. mitochondrial Ca^{2+} sparks)[37]. Marks had fast rise times of $<50\text{ms}$ and decayed very slowly, with a half width of 215 msec. The duration of the Ca^{2+} marks was prolonged to 374 msec by the mNCE inhibitor CGP-37157, while the decay of cytosolic Ca^{2+} sparks was unaffected. More recently, this group reported that the functional complex containing the SR Ca^{2+} release unit is retained even after isolation of the mitochondria[38]. Acute caffeine treatment could initiate a rise in mitochondrial Ca^{2+} even after partial purification of mitochondria to remove SR membranes, indicative of a close physical coupling between the SR Ca^{2+} release apparatus and the mitochondria.

Although most of the early studies using Mn^{2+} quench to eliminate the cytoplasmic component of the Ca^{2+} indicators did not support fast mitochondrial Ca^{2+} transients, Griffiths[39] made the observation that indo-1 loaded guinea-pig, but not rat, cardiac myocytes showed beat-to-beat mitochondrial Ca^{2+} transients using the heat treatment method. In fact, this species selectivity may provide an important clue to explaining the disparate results reported in the literature. Considering potential explanations, either there is an intrinsic difference in the mechanism of mitochondrial Ca^{2+} uptake in rats versus guinea pigs or there are different regulatory factors present that influence SR-mitochondrial Ca^{2+} transport.

In light of our own recent studies, one such factor could be the resting cytosolic Na^+ concentration ($[\text{Na}^+]_i$). As discussed above, it is well known that $[\text{Na}^+]_i$ shifts the apparent K_m for mitochondrial Ca^{2+} uptake, presumably by affecting the Ca^{2+} efflux rate via the mNCE. It is also known that resting $[\text{Na}^+]_i$ is highly species dependent[40]. To study the effects of changing $[\text{Na}^+]_i$ on mitochondrial Ca^{2+} uptake in intact guinea pig cardiac myocytes, we recently developed a method to monitor both cytosolic and mitochondrial Ca^{2+} in guinea pig myocytes using a combination of differential dye loading and whole cell dialysis into the cytoplasmic and mitochondrial matrix compartments[41]. We found that a rapid mitochondrial Ca^{2+} transient could be observed during every cytosolic Ca^{2+} transient elicited by voltage clamp pulses to initiate SR Ca^{2+} release. Remarkably, the upstroke of the $[\text{Ca}^{2+}]_m$ transient preceded the upstroke of $[\text{Ca}^{2+}]_c$ by several ms, whereas the decay of $[\text{Ca}^{2+}]_m$ was ~ 2.5 -fold slower than of $[\text{Ca}^{2+}]_c$. The fast uptake, slow release kinetics led to a stepwise accumulation $[\text{Ca}^{2+}]_m$ during rapid pacing (up to 4Hz), particularly in the presence of concomitant β -adrenergic stimulation. Directionally opposite effects on the mitochondrial and cytosolic Ca^{2+} transients were affected by inhibitors of mitochondrial Ca^{2+} uptake or efflux, confirming the mitochondrial origin of the signal and indicating that a significant fraction of the Ca^{2+} released by the SR is buffered by the mitochondria on each beat. Most relevant to the present discussion, the slope of the relationship between the amplitude of the cytosolic Ca^{2+} transient and the mitochondrial Ca^{2+} transient was strongly dependent on $[\text{Na}^+]_i$. An increase of $[\text{Na}^+]_i$ from 5 mM to 15 mM, within the range of measured values of Na^+ in intact myocytes or muscles, blunted the amplitude of the mitochondrial Ca^{2+} transient and decreased the extent of mitochondrial Ca^{2+} loading upon electrical stimulation. Importantly, the diminished mitochondrial Ca^{2+} response at high $[\text{Na}^+]_i$ impaired the ability of the mitochondria to balance NADH production to increased workload. We proposed that this mechanism could contribute to an energy supply and demand mismatch in cardiac pathologies associated with elevated resting $[\text{Na}^+]_i$, for example, in hypertrophied or failing hearts. We have recently confirmed that resting $[\text{Na}^+]_i$ is elevated to a similar extent and the

NADH response to pacing is defective in myocytes from failing hearts in a guinea pig aortic banding model[42].

Our recent findings, taken together with reports in the literature that rats have a higher resting $[Na^+]_i$ (~ 10 mM) than guinea-pigs (5mM in our measurements, <7 mM from other laboratories; see refs. within[40]), could explain why some investigators have failed to see fast mitochondrial Ca^{2+} transients, although other effectors (e.g., Mg^{2+} , spermine, etc.) could certainly play a role as well. This speculative explanation will have to be confirmed by additional direct investigation.

Targeted molecular probes

Genetic targeting of fluorescent Ca^{2+} indicators has offered an alternative method to measure the kinetics of Ca^{2+} uptake into mitochondria. These constructs typically incorporate a peptide leader sequence taken from an electron transport protein that is recognized by the protein import machinery of mitochondria, used to direct the trafficking of Ca^{2+} -sensitive reporters like the photoprotein aequorin or FRET-based sensors like the pericams[43]. Although this method has its own limitations, the consensus finding has been that mitochondria take up Ca^{2+} rapidly, roughly in synchrony with cytosolic Ca^{2+} release in cardiac myocytes[44, 45]. Again, even taking into account nonlinearities in the kinetic response of aequorin to Ca^{2+} , mitochondrial Ca^{2+} decay is observed to be noticeably slower than that of the cytoplasm. Interestingly, in a study by Bell et al[45], transient changes in mitochondrial [ATP], reported by targeted luciferase, were observed within the first minute of rapid pacing while mitochondrial Ca^{2+} oscillated on a beat-to-beat basis.

Indirect methods

Direct measurements of fast mitochondrial Ca^{2+} transients using the methods described above are perhaps the most convincing arguments for a special spatiotemporal relationship between the sites of SR Ca^{2+} release and mitochondrial Ca^{2+} uptake; however, other indirect observations also support this notion. Several studies indicate that mitochondria are effective local Ca^{2+} buffers of the cytosolic Ca^{2+} transient [22, 37, 41, 46–49]. When blocking mitochondrial Ca^{2+} uptake, an increase of cytosolic Ca^{2+} transients is often observed[22, 49] and impairs the recovery of inactivation of the L-type Ca^{2+} current during a train of pulses[50].

Spatial considerations

In reviewing the points in favor of rapid mitochondrial Ca^{2+} uptake, it is worth mentioning the common sense argument that the sites of Ca^{2+} release are generally located in the space between two mitochondria (Fig. 1), and spikes of Ca^{2+} of 50 μ molar or so are released into a narrow space roughly 40nm away from the nearest mitochondrial membrane. It is therefore reasonable to assume that at least part of the mitochondrion experiences a Ca^{2+} gradient exceeding that of the bulk space. How rapidly this pulse of Ca^{2+} might be transmitted into and distributed across the convoluted architecture of the matrix cristae is a question that will require further investigation and more detailed modelling of Ca^{2+} diffusion with realistic structural models to answer.

BlatterLothar A.

Rebuttal

The preceding article comprehensively discussed the ongoing controversy surrounding mitochondrial Ca^{2+} uptake kinetics. Several elegant empirical studies ([22, 41, 44]; see also reviews[11, 51]) were presented that have generated convincing evidence that mitochondria indeed can respond to rapid cytosolic Ca^{2+} oscillations with rapid changes of mitochondrial $[\text{Ca}]$ ($[\text{Ca}]_m$), or in other words, that the kinetics of the mitochondrial Ca^{2+} transport machinery are fast enough to allow for (nearly) beat-to-beat changes of $[\text{Ca}]_m$ in the heart. Nonetheless, as also discussed in the preceding article a number of studies that supported rapid mitochondrial Ca^{2+} uptake are plagued by experimental shortcomings and flaws. For example, fluorescence microscopy imaging studies attempting to measure cytosolic $[\text{Ca}]$ and $[\text{Ca}]_m$ simultaneously are particularly prone to misinterpretation of experimental results. Limitations in spatial resolution and thus sufficient signal separation are the crux of such light microscopic approaches because mitochondria are smaller than the resolvable volume. As a consequence cytosolic and mitochondrial Ca^{2+} transients have (suspiciously) similar kinetics due to signal 'contamination'. While experimental limitations may have hampered such studies, from a teleological perspective one may contemplate the question whether a beat-to-beat change in $[\text{Ca}]_m$ is desirable and/or even required for mitochondrial function. Shuttling of Ca^{2+} across the inner mitochondrial membrane (IMM) is energetically expensive and occurs under consumption of energy that is deprived from the cell and not available to cover other cellular energy demands. In the following COUNTERPOINT we will compile and discuss experimental evidence in support of a model of slow integration of cytosolic Ca^{2+} oscillations by mitochondrial Ca^{2+} transport and buffering, and we address the question whether from a point of view of cellular energetics this model is favorable over rapid changes of $[\text{Ca}]_m$.

BlatterLothar A.

Counterpoint: Evidence against fast mitochondrial Ca^{2+} uptake in the heart

Historically, two different theories have evolved on how mitochondria decode rapid cardiac Ca^{2+} transients (for review see for example ref.[51]). In model I, originally induced by Crompton[52], Ca^{2+} uptake into mitochondria is slow and coupled to an even slower release of accumulated Ca^{2+} ions. Fast cytosolic Ca^{2+} oscillations are integrated by the Ca^{2+} transport machinery of the IMM. The response to a train of cytosolic Ca^{2+} transients is a frequency-dependent net accumulation of Ca^{2+} in the matrix compartment until a new steady-state is reached when Ca^{2+} uptake during a single cycle equals Ca^{2+} efflux. Consequently, beat-to-beat changes in $[\text{Ca}]_m$ are small, and energetic requirements of integrated mitochondrial Ca^{2+} transport are minimal. In contrast, model II explains how fast cytosolic Ca^{2+} oscillations are efficiently translated into beat-to-beat changes of $[\text{Ca}]_m$. As a requirement, rapid mitochondrial Ca^{2+} uptake as well as fast Ca^{2+} efflux mechanisms are a mandatory prerequisite, with Ca^{2+} uptake being large enough to overcome the buffering capacity of both the cytosol and mitochondrial matrix. In this scenario, mitochondrial Ca^{2+} uptake would effectively buffer cytosolic Ca^{2+} transients during e-c coupling. This, in turn, would require SR Ca^{2+} release and reuptake to be large enough to compensate for this additional fast buffering and to still provide sufficient Ca^{2+} for contraction. With

approximately one third of cell volume being occupied by mitochondria in cardiac cells, the additional SR Ca^{2+} fluxes (and linked energy consumption) would be substantial[11].

Slow integration of cytosolic Ca^{2+} transients by mitochondrial Ca^{2+} transport and buffering

As has been discussed in the preceding article the controversy surrounding beat-to-beat changes in $[\text{Ca}]_m$ in the heart is certainly (at least in part) related to experimental limitations of available methods for reliable measurements of $[\text{Ca}]_m$. This caveat applies to many such studies (for an overview of methods and results see Table 1 in ref.[51]) irrespective whether the outcome favored model I or II (e.g. contamination of mitochondrial Ca^{2+} signal by fast cytosolic Ca^{2+} signals due to insufficient signal separation). Evidence in support of slow mitochondrial integration of cytosolic Ca^{2+} transients are based on studies utilizing electron probe microanalysis (EPMA) and fluorescent techniques. EPMA uses flash-frozen tissue samples and has the advantages of a resolution close to electron microscopy, however it measures total mitochondrial Ca^{2+} ($[\text{Ca}]_{m,\text{tot}}$) instead of free $[\text{Ca}]_m$. Studies using EPMA performed on hamster[53, 54] and rat[55] papillary muscles were not able to resolve fast changes in $[\text{Ca}]_{m,\text{tot}}$, not even after β -adrenergic stimulation of hamster papillary muscle[53] or whole hearts[56].

In a majority of studies, fluorescent dyes (such as the membrane-permeable ester forms of indo-1, rhod-2 or fluo-3) known to compartmentalize in mitochondria were used to monitor $[\text{Ca}]_m$ directly. To eliminate the cytosolic component of the fluorescent signals, cells were treated with MnCl_2 [28, 29, 33, 57], exposed to high temperature[32, 39] or permeabilized[58, 59]. Using indo-1 loaded rat[28, 39], hamster[29], ferret and cat[33] ventricular myocytes with subsequent Mn-quenching of cytosolic dye, it was shown that an increase in the stimulation frequency from 0.2 to 4 Hz in the presence of β -adrenergic stimulation[28, 32] or cellular loading of Ca^{2+} through sarcolemmal Na/Ca^{2+} exchange[33] led to a slow rise of $[\text{Ca}]_m$ from ~100–200 to ~500–800 nM, however no beat-to-beat changes in $[\text{Ca}]_m$ were observed. In the absence of β -adrenergic stimulation, only modest increases in $[\text{Ca}]_m$ could be achieved in rat myocytes by electrical stimulation at 2 Hz[29], which indicated that only large amplitude Ca^{2+} transients were sensed by mitochondria. Miyata *et al.*[28] demonstrated an exponential relationship between $[\text{Ca}]_m$ and $[\text{Ca}]_i$, with the threshold for activation of mitochondrial Ca^{2+} uptake being at a $[\text{Ca}]_i$ of ~500 nM. Similarly, Zhou *et al.*[33] reported that under conditions of high cellular Ca^{2+} load imposed by membrane depolarization in ferret and cat myocytes, phasic increases of $[\text{Ca}]_m$ could be detected, although they were slow and only observed at diastolic $[\text{Ca}]_i > 400$ nM. The authors concluded that mitochondria of intact cells did not take up detectable amounts of Ca^{2+} during individual contractions. A general criticism of the experimental approach outlined above is the possible interference of Mn with Ca^{2+} transporters of the IMM. First, Mn can be sequestered rapidly by mitochondria since it competes with Ca^{2+} for the mitochondrial Ca^{2+} uniporter (MCU), and Mn can potentially quench the fluorescence of the dye compartmentalized inside of mitochondria. While Miyata *et al.*[28] provided evidence that Mn had no effect on mitochondrial Ca^{2+} uptake as well as cell shortening, other studies demonstrated that Mn significantly inhibits Ca^{2+} efflux[60]. Nonetheless, removal of cytosolic indo-1 by heat treatment gave similar results to the Mn quench technique[32, 39].

Moreover, when fura-2 loaded rat heart mitochondria were exposed to Ca^{2+} oscillations at 100 cycles/min, $[\text{Ca}]_m$ increased proportionally to the average rise in extramitochondrial $[\text{Ca}]$, but independently of oscillation frequency[61]. Furthermore, Griffiths[39] revealed that there might be a species-dependent difference in the kinetics of $[\text{Ca}]_m$. Utilizing the heat treatment protocol, the author demonstrated $[\text{Ca}]_m$ transients in indo-1 loaded quinea-pig myocytes, while no changes in $[\text{Ca}]_m$ were observed in rat cardiomyocytes.

A novel experimental approach was developed in our laboratory by Sedova *et al.*[58] which allowed to simulate fast cytosolic Ca^{2+} transients in membrane-permeabilized cells. $[\text{Ca}]_m$ was measured with fluo-3 entrapped inside mitochondria and after removal of cytosolic indicator by plasma membrane permeabilization with digitonin. Permeabilized cells have the unique advantage that the cytosolic environment can be controlled precisely while the arrangements and interaction between intracellular membranes and organelles (SR, mitochondria) remain structurally and functionally intact[62, 63]. Control experiments indicated that mitochondrial Ca^{2+} uptake was initiated only when extramitochondrial $[\text{Ca}]$ was elevated to more than 0.5 μM , confirming the existence of a threshold for MCU activation[33, 59]. Ca^{2+} entry via the uniporter exhibited a sigmoidal extramitochondrial $[\text{Ca}]$ dependence ($K_{0.5}=4.4 \mu\text{M}$). To simulate cytosolic Ca^{2+} transients, cells were placed in the laminar flow of Ca^{2+} free solution containing 1 mM EGTA and exposed to rapid ejections of 100 μM Ca^{2+} from a micropipette positioned upstream of the cell with regard to the direction of the bulk flow. Calculations revealed that with the applied technique cells were exposed to $\sim 5 \mu\text{M}$ extramitochondrial $[\text{Ca}]$ during each pulse of stimulation, which is in the range of physiological cytosolic Ca^{2+} transients amplitude and close to the estimated $K_{0.5}$ for the MCU. With this technique rapid beat-to-beat changes in $[\text{Ca}]_i$ (with Ca^{2+} transient durations of 100 to 500 ms) could be simulated by rapid switching (0.25 – 1 Hz) extramitochondrial $[\text{Ca}]$ to high levels. Exposure to a train of Ca^{2+} transients evoked a gradual, frequency- and Ca^{2+} transient duration-dependent elevation of $[\text{Ca}]_m$, but did not lead to $[\text{Ca}]_m$ oscillations. These data suggest that in cat ventricular myocytes fast cytosolic $[\text{Ca}]$ transients are integrated by mitochondrial Ca^{2+} transport systems resulting in a frequency-dependent net accumulation of Ca^{2+} in the matrix, thus supporting model I of mitochondrial decoding of fast cytosolic Ca^{2+} signals.

Is Fast mitochondrial Ca^{2+} cycling energetically desirable?

The process of mitochondrial Ca^{2+} cycling involves continuous fluxes of Ca^{2+} and Na^+ ions, as well as protons (H^+) driven by the respiratory chain[64]. Ca^{2+} influx dissipates the mitochondrial membrane potential (Ψ), whereas efflux dissipates the mitochondrial pH (indirectly via mitochondrial Na/Ca^{2+} and Na/H exchange)[52]. Generally, a biphasic time course of mitochondrial Ca^{2+} uptake is expected from its electrogenic nature. Rapid Ca^{2+} entry is slowed to a level at which Ca^{2+} influx is balanced by H^+ extrusion[65]. Moreover, rapid H^+ pumping by the F_1F_0 ATP-synthase may contribute to maintaining Ψ during high rates of Ca^{2+} import at the expense of ATP[66], therefore making fast beat-to-beat changes in $[\text{Ca}]_m$ energetically expensive. However, no beat-to-beat changes in mitochondrial ($[\text{ATP}]_m$) or cytosolic ($[\text{ATP}]_i$) free ATP levels were observed in beating rat ventricular myocytes in the absence or presence of isoproterenol (i.e. upon increased workload)[45]. Only when myocytes were stimulated at high frequencies (2 Hz from rest) in the presence of

isoproterenol, significant (on average to 10% of the initial signal) decreases in $[ATP]_m$ were observed. This $[ATP]_m$ drop, however, lasted only 30–60 s, and was followed by a slower recovery that exceeded the initial level and resulted in a sustained rise in $[ATP]_m$. These data are in agreement with mitochondrial NADH or NAD(P)H measurements[67–69] as indirect indicators of the energetic state of mitochondria. Similar to the results presented above, physiological increases in the workload of whole hearts did not evoke any changes in NAD(P)H[67]. Nonetheless, data obtained on isolated rat ventricular trabeculae indicate that with a sudden increase in stimulation frequency (3 Hz from rest) or extracellular $[Ca]_i$, there was a transient decrease in mitochondrial NADH ($[NADH]_m$), consistent with NADH production not keeping up with the increased ATP and NADH consumption[69]. However, this $[NADH]_m$ decline was followed by a recovery toward previous levels. This slow rise of $[NADH]_m$ was dependent on increased average $[Ca]_i$ and was paralleled by an equivalently slow increase of $[Ca]_m$ [70]. It was concluded that the increased average $[Ca]_i$ caused an increase in $[Ca]_m$ and stimulation of dehydrogenases and NADH production. The kinetics of the measured $[NADH]_m$ changes in response to increased pacing frequency did not require a rapid transmission of cytosolic Ca^{2+} signals into the mitochondrial matrix. In fact, the time course of the delayed $[NADH]_m$ recovery was reminiscent of the slow changes in $[Ca]_m$ reported by Miyata and co-workers[28] under comparable conditions. The same is true for the direct $[ATP]_m$ measurements reported by Bell *et al.*[45], where the time course of $[ATP]_m$ changes closely followed the slower changes in $[Ca]_m$.

In conclusion, experimental data do not support an absolute requirement for a rapid (beat-to-beat) transmission of cytosolic Ca^{2+} signals into the mitochondrial matrix to regulate the mitochondrial response to altered cellular energy demands. While the following conclusion may be more teleological rather than empirical, it appears that a slow integration of cytosolic Ca^{2+} oscillations is cost-efficient and, at the same time, sufficient to fully respond to the cellular energetic requirements of the heart.

O'RourkeBrian

Rebuttal

Experimental limitations of measuring mitochondrial Ca^{2+}

Certainly, it is agreed that the resolution of the central question posed in this article will be resolved eventually by improvements of the experimental methods for assessing intramitochondrial Ca^{2+} . I would argue that great improvements have been made already with respect to the development of genetically-targeted Ca^{2+} sensors[43, 45] and, in our own experiments, careful attention to elimination of cytosolic Ca^{2+} signals and simultaneous measurement of both compartments with dual sensors[41]. Moreover, the directionally opposite effects of an inhibitor of the mitochondrial Ca^{2+} uniporter on the fast mitochondrial transient (inhibition) versus the cytosolic transient (enhancement), along with the inverse effects of a mitochondrial Na^+/Ca^{2+} exchange inhibitor, provides unequivocal (in my mind) evidence that the indicators are localized as expected.

While one can always fall back on the argument that previous evidence in favor of fast mitochondrial Ca^{2+} uptake was artifactually due to signal contamination, it is equally easy to challenge whether inadequate experimental design or lack of control over important factors

could have resulted in a failure to observe fast mitochondrial Ca^{2+} transients. I have already discussed the important role of intracellular Na^+ , and undoubtedly other modulators (e.g., P_i [41, 42]) will also prove to be important. With respect to two of the methods discussed above (the work of Zhou et al[33] and Sedova et al[58]), there is an essential limitation in how the Ca^{2+} gradients driving mitochondrial Ca^{2+} uptake are applied. In both cases, the Ca^{2+} is increased by first driving up bulk Ca^{2+} (either by activation of sarcolemmal $\text{Na}^+/\text{Ca}^{2+}$ exchange in the first study or by increasing bath Ca^{2+} in the second study) and then relying on Ca^{2+} diffusion to the mitochondrial membranes. This ignores the complex spatiotemporal factors associated with native SR Ca^{2+} release from the two juxtaposed compartments (cf. Fig. 1). Indeed, under the right conditions, when Ca^{2+} release is initiated by triggering the ryanodine receptor, mitochondrial Ca^{2+} can rise rapidly without much change in bulk Ca^{2+} [34].

Energetic cost

The latter argument is that it would be energetically costly for the mitochondria to respond rapidly to Ca^{2+} on each beat. This would be true if there were no mechanistic requirement for the Ca^{2+} signal to be transmitted rapidly to the bioenergetic subsystem. However, as we have recently shown, matching energy supply with demand does require a rapid mitochondrial Ca^{2+} response[42]. If fast mitochondrial Ca^{2+} transients are inhibited (e.g., by blocking the mitochondrial Ca^{2+} uniporter or increasing $[\text{Na}^+]_i$), an increase in workload results in net oxidation of the mitochondrial NADH pool, indicating that the stimulation of oxidative phosphorylation is very fast and requires an almost immediate increase in NADH production to remain in balance. Fast mitochondrial Ca^{2+} uptake is thus required to stimulate the tricarboxylic acid cycle dehydrogenases. Furthermore, impaired mitochondrial Ca^{2+} uptake and high $[\text{Na}^+]_i$ contribute to altered NADH responses in cardiomyocytes from failing hearts[42], underscoring the pathophysiological implications of this mechanism. Hence, Ca^{2+} cycling across the mitochondrial membrane does result in an energetic cost, but it pays for a fast bioenergetic response. We would argue that a rapid mitochondrial effect on NADH production (mediated by fast Ca^{2+} uptake) is necessary to respond to frequent and nearly instantaneous changes in the metabolic demand of the heart, which accounts for the lack of an observable change in the NADH redox potential under a variety of conditions.

Fast versus slow: a straw man for both sides

Finally, while I have argued in favor of a fast component of mitochondrial Ca^{2+} uptake, the integrated effect on matrix Ca^{2+} depends on both fast and slow processes. As we have reported previously in a computational model of mitochondrial Ca^{2+} uptake in response to brief ($\sim 20 \mu\text{M}$) pulses of Ca^{2+} to simulate local Ca^{2+} release[41], fast Ca^{2+} influx coupled with slow efflux permits the mitochondrion to respond to changes in either the frequency or amplitude of SR Ca^{2+} release synergistically. Because the Ca^{2+} pulses were brief (50 ms), their effects on bioenergetics were rapidly compensated by proton pumping, so that the drop in Ψ_m during uptake was quickly reversed and did not substantially change the average electrochemical driving force.

In the end the real argument is not whether mitochondria take up Ca^{2+} on each beat, I believe we both agree that some Ca^{2+} must enter during each transient, but is rather about

the kinetics and magnitude of the uptake. The answer to the latter question requires more precise measurements of the compartmentalized Ca^{2+} pools and a better understanding of the influence of the ultrastructural arrangement of the organelles involved.

References

1. Patterson SW, Piper H, Starling EH. The regulation of the heart beat. *J Physiol.* 1914 Oct 23; 48(6): 465–513. [PubMed: 16993269]
2. Evans CL, Matsuoka Y. The effect of various mechanical conditions on the gaseous metabolism and efficiency of the mammalian heart. *J Physiol.* 1915 Jul 5; 49(5):378–405. [PubMed: 16993302]
3. Lardy HA, Wellman H. Oxidative phosphorylations; role of inorganic phosphate and acceptor systems in control of metabolic rates. *J Biol Chem.* 1952 Mar; 195(1):215–24. [PubMed: 14938372]
4. Chance B, Williams GR. A method for the localization of sites for oxidative phosphorylation. *Nature.* 1955 Aug 6; 176(4475):250–4. [PubMed: 13244669]
5. Katz LA, Swain JA, Portman MA, Balaban RS. Relation between phosphate metabolites and oxygen consumption of heart in vivo. *Am J Physiol.* 1989 Jan; 256(1 Pt 2):H265–74. [PubMed: 2912189]
6. Bose S, French S, Evans FJ, Joubert F, Balaban RS. Metabolic network control of oxidative phosphorylation: multiple roles of inorganic phosphate. *J Biol Chem.* 2003 Oct 3; 278(40):39155–65. [PubMed: 12871940]
7. Gunter TE, Pfeiffer DR. Mechanisms by which mitochondria transport calcium. *Am J Physiol.* 1990 May; 258(5 Pt 1):C755–86. [PubMed: 2185657]
8. Balaban RS, Bose S, French SA, Territo PR. Role of calcium in metabolic signaling between cardiac sarcoplasmic reticulum and mitochondria in vitro. *Am J Physiol Cell Physiol.* 2003 Feb; 284(2):C285–93. [PubMed: 12529248]
9. Territo PR, Mootha VK, French SA, Balaban RS. Ca^{2+} activation of heart mitochondrial oxidative phosphorylation: role of the F(0)/F(1)-ATPase. *Am J Physiol Cell Physiol.* 2000 Feb; 278(2):C423–35. [PubMed: 10666039]
10. Gunter TE, Gunter KK, Sheu SS, Gavin CE. Mitochondrial calcium transport: physiological and pathological relevance. *Am J Physiol.* 1994 Aug; 267(2 Pt 1):C313–39. [PubMed: 8074170]
11. Huser J, Blatter LA, Sheu SS. Mitochondrial calcium in heart cells: beat-to-beat oscillations or slow integration of cytosolic transients? *J Bioenerg Biomembr.* 2000 Feb; 32(1):27–33. [PubMed: 11768759]
12. Kirichok Y, Krapivinsky G, Clapham DE. The mitochondrial calcium uniporter is a highly selective ion channel. *Nature.* 2004 Jan 22; 427(6972):360–4. [PubMed: 14737170]
13. Territo PR, French SA, Dunleavy MC, Evans FJ, Balaban RS. Calcium activation of heart mitochondrial oxidative phosphorylation: rapid kinetics of mVO_2 , NADH, AND light scattering. *J Biol Chem.* 2001 Jan 26; 276(4):2586–99. [PubMed: 11029457]
14. Sparagna GC, Gunter KK, Sheu SS, Gunter TE. Mitochondrial calcium uptake from physiological-type pulses of calcium. A description of the rapid uptake mode. *J Biol Chem.* 1995 Nov 17; 270(46):27510–5. [PubMed: 7499209]
15. Buntinas L, Gunter KK, Sparagna GC, Gunter TE. The rapid mode of calcium uptake into heart mitochondria (RaM): comparison to RaM in liver mitochondria. *Biochim Biophys Acta.* 2001 Apr 2; 1504(2–3):248–61. [PubMed: 11245789]
16. McCormack JG, Halestrap AP, Denton RM. Role of calcium ions in regulation of mammalian intramitochondrial metabolism. *Physiol Rev.* 1990 Apr; 70(2):391–425. [PubMed: 2157230]
17. Denton RM, McCormack JG. Ca^{2+} transport by mammalian mitochondria and its role in hormone action. *Am J Physiol.* 1985 Dec; 249(6 Pt 1):E543–54. [PubMed: 2417490]
18. Baysal K, Jung DW, Gunter KK, Gunter TE, Brierley GP. Na^{+} -dependent Ca^{2+} efflux mechanism of heart mitochondria is not a passive $\text{Ca}^{2+}/2\text{Na}^{+}$ exchanger. *Am J Physiol.* 1994 Mar; 266(3 Pt 1):C800–8. [PubMed: 8166244]
19. Jung DW, Baysal K, Brierley GP. The sodium-calcium antiport of heart mitochondria is not electroneutral. *J Biol Chem.* 1995 Jan 13; 270(2):672–8. [PubMed: 7822294]

20. Paucek P, Jaburek M. Kinetics and ion specificity of Na(+)/Ca(2+) exchange mediated by the reconstituted beef heart mitochondrial Na(+)/Ca(2+) antiporter. *Biochim Biophys Acta*. 2004 Nov 4; 1659(1):83–91. [PubMed: 15511530]
21. Wendt-Gallitelli MF, Isenberg G. Total and free myoplasmic calcium during a contraction cycle: x-ray microanalysis in guinea-pig ventricular myocytes. *J Physiol*. 1991 Apr; 435:349–72. [PubMed: 1770441]
22. Isenberg G, Han S, Schiefer A, Wendt-Gallitelli MF. Changes in mitochondrial calcium concentration during the cardiac contraction cycle. *Cardiovasc Res*. 1993 Oct; 27(10):1800–9. [PubMed: 8275527]
23. Hansford RG. Relation between mitochondrial calcium transport and control of energy metabolism. *Rev Physiol Biochem Pharmacol*. 1985; 102:1–72. [PubMed: 2863864]
24. Chacon E, Ohata H, Harper IS, Trollinger DR, Herman B, Lemasters JJ. Mitochondrial free calcium transients during excitation-contraction coupling in rabbit cardiac myocytes. *FEBS Lett*. 1996 Mar 11; 382(1–2):31–6. [PubMed: 8612759]
25. Ohata H, Chacon E, Tesfai SA, Harper IS, Herman B, Lemasters JJ. Mitochondrial Ca²⁺ transients in cardiac myocytes during the excitation-contraction cycle: effects of pacing and hormonal stimulation. *J Bioenerg Biomembr*. 1998 Jun; 30(3):207–22. [PubMed: 9733088]
26. Trollinger DR, Cascio WE, Lemasters JJ. Mitochondrial calcium transients in adult rabbit cardiac myocytes: inhibition by ruthenium red and artifacts caused by lysosomal loading of Ca(2+)-indicating fluorophores. *Biophys J*. 2000 Jul; 79(1):39–50. [PubMed: 10866936]
27. Trollinger DR, Cascio WE, Lemasters JJ. Selective loading of Rhod 2 into mitochondria shows mitochondrial Ca²⁺ transients during the contractile cycle in adult rabbit cardiac myocytes. *Biochem Biophys Res Commun*. 1997 Jul 30; 236(3):738–42. [PubMed: 9245725]
28. Miyata H, Silverman HS, Sollott SJ, Lakatta EG, Stern MD, Hansford RG. Measurement of mitochondrial free Ca²⁺ concentration in living single rat cardiac myocytes. *Am J Physiol*. 1991 Oct; 261(4 Pt 2):H1123–34. [PubMed: 1928394]
29. Di Lisa F, Gambassi G, Spurgeon H, Hansford RG. Intramitochondrial free calcium in cardiac myocytes in relation to dehydrogenase activation. *Cardiovasc Res*. 1993 Oct; 27(10):1840–4. [PubMed: 8275533]
30. Di Lisa F, Fan CZ, Gambassi G, Hogue BA, Kudryashova I, Hansford RG. Altered pyruvate dehydrogenase control and mitochondrial free Ca²⁺ in hearts of cardiomyopathic hamsters. *Am J Physiol*. 1993 Jun; 264(6 Pt 2):H2188–97. [PubMed: 8322950]
31. Miyata H, Lakatta EG, Stern MD, Silverman HS. Relation of mitochondrial and cytosolic free calcium to cardiac myocyte recovery after exposure to anoxia. *Circ Res*. 1992 Sep; 71(3):605–13. [PubMed: 1499108]
32. Griffiths EJ, Stern MD, Silverman HS. Measurement of mitochondrial calcium in single living cardiomyocytes by selective removal of cytosolic indo 1. *Am J Physiol*. 1997 Jul; 273(1 Pt 1):C37–44. [PubMed: 9252440]
33. Zhou Z, Matlib MA, Bers DM. Cytosolic and mitochondrial Ca²⁺ signals in patch clamped mammalian ventricular myocytes. *J Physiol*. 1998 Mar 1; 507(Pt 2):379–403. [PubMed: 9518700]
34. Sharma VK, Ramesh V, Franzini-Armstrong C, Sheu SS. Transport of Ca²⁺ from sarcoplasmic reticulum to mitochondria in rat ventricular myocytes. *J Bioenerg Biomembr*. 2000 Feb; 32(1):97–104. [PubMed: 11768767]
35. Belmonte S, Morad M. “Pressure-flow”-triggered Cai-transients in rat cardiac myocytes: possible mechanisms and role of mitochondria. *J Physiol*. 2008 Jan 10.
36. Szalai G, Csordas G, Hantash BM, Thomas AP, Hajnoczky G. Calcium signal transmission between ryanodine receptors and mitochondria. *J Biol Chem*. 2000 May 19; 275(20):15305–13. [PubMed: 10809765]
37. Pacher P, Thomas AP, Hajnoczky G. Ca²⁺ marks: miniature calcium signals in single mitochondria driven by ryanodine receptors. *Proc Natl Acad Sci U S A*. 2002 Feb 19; 99(4):2380–5. [PubMed: 11854531]
38. Garcia-Perez C, Hajnoczky G, Csordas G. Physical coupling supports the local Ca²⁺ transfer between SR subdomains and the mitochondria in heart muscle. *J Biol Chem*. 2008 Sep 12.

39. Griffiths EJ. Species dependence of mitochondrial calcium transients during excitation-contraction coupling in isolated cardiomyocytes. *Biochem Biophys Res Commun.* 1999 Sep 24; 263(2):554–9. [PubMed: 10491330]
40. Pieske B, Houser SR. $[Na^+]_i$ handling in the failing human heart. *Cardiovasc Res.* 2003 Mar 15; 57(4):874–86. [PubMed: 12650866]
41. Maack C, Cortassa S, Aon MA, Ganesan AN, Liu T, O'Rourke B. Elevated cytosolic Na^+ decreases mitochondrial Ca^{2+} uptake during excitation-contraction coupling and impairs energetic adaptation in cardiac myocytes. *Circ Res.* 2006 Jul 21; 99(2):172–82. [PubMed: 16778127]
42. Liu T, O'Rourke B. Enhancing mitochondrial Ca^{2+} uptake in myocytes from failing hearts restores energy supply and demand matching. *Circ Res.* 2008 Aug 1; 103(3):279–88. [PubMed: 18599868]
43. Rizzuto R, Duchen MR, Pozzan T. Flirting in little space: the ER/mitochondria Ca^{2+} liaison. *Sci STKE.* 2004 Jan.13(215):re1. 2004. [PubMed: 14722345]
44. Robert V, Gurlini P, Tosello V, Nagai T, Miyawaki A, Di Lisa F, Pozzan T. Beat-to-beat oscillations of mitochondrial $[Ca^{2+}]$ in cardiac cells. *Embo J.* 2001 Sep 3; 20(17):4998–5007. [PubMed: 11532963]
45. Bell CJ, Bright NA, Rutter GA, Griffiths EJ. ATP regulation in adult rat cardiomyocytes: time-resolved decoding of rapid mitochondrial calcium spiking imaged with targeted photoproteins. *J Biol Chem.* 2006 Sep 22; 281(38):28058–67. [PubMed: 16882672]
46. Pacher P, Csordas P, Schneider T, Hajnoczky G. Quantification of calcium signal transmission from sarco-endoplasmic reticulum to the mitochondria. *J Physiol.* 2000 Dec 15; 529(Pt 3):553–64. [PubMed: 11118489]
47. Duchen MR, Leyssens A, Crompton M. Transient mitochondrial depolarizations reflect focal sarcoplasmic reticular calcium release in single rat cardiomyocytes. *J Cell Biol.* 1998 Aug 24; 142(4):975–88. [PubMed: 9722610]
48. Mackenzie L, Roderick HL, Berridge MJ, Conway SJ, Bootman MD. The spatial pattern of atrial cardiomyocyte calcium signalling modulates contraction. *J Cell Sci.* 2004 Dec 15; 117(Pt 26): 6327–37. [PubMed: 15561771]
49. Seguchi H, Ritter M, Shizukuishi M, Ishida H, Chokoh G, Nakazawa H, Spitzer KW, Barry WH. Propagation of Ca^{2+} release in cardiac myocytes: role of mitochondria. *Cell Calcium.* 2005 Jul; 38(1):1–9. [PubMed: 15993240]
50. Sanchez JA, Garcia MC, Sharma VK, Young KC, Matlib MA, Sheu SS. Mitochondria regulate inactivation of L-type Ca^{2+} channels in rat heart. *J Physiol.* 2001 Oct 15; 536(Pt 2):387–96. [PubMed: 11600674]
51. Dedkova EN, Blatter LA. Mitochondrial $Ca(2+)$ and the heart. *Cell Calcium.* 2008 Jul; 44(1):77–91. [PubMed: 18178248]
52. Crompton, FM. The role of Ca^{2+} in the function and dysfunction of heart mitochondria. In: Langer, GA., editor. *Calcium and the Heart.* New York: Raven Press; 1990. p. 167-98.
53. Moravec CS, Bond M. Effect of inotropic stimulation on mitochondrial calcium in cardiac muscle. *J Biol Chem.* 1992 Mar 15; 267(8):5310–6. [PubMed: 1544913]
54. Moravec CS, Bond M. Calcium is released from the junctional sarcoplasmic reticulum during cardiac muscle contraction. *Am J Physiol.* 1991 Mar; 260(3 Pt 2):H989–97. [PubMed: 2000992]
55. Horikawa Y, Goel A, Somlyo AP, Somlyo AV. Mitochondrial calcium in relaxed and tetanized myocardium. *Biophys J.* 1998 Mar; 74(3):1579–90. [PubMed: 9512053]
56. Moravec CS, Desnoyer RW, Milovanovic M, Schluchter MD, Bond M. Mitochondrial calcium content in isolated perfused heart: effects of inotropic stimulation. *Am J Physiol.* 1997 Sep; 273(3 Pt 2):H1432–9. [PubMed: 9321835]
57. Schreier JH, Figueredo VM, Miyamae M, Shames DM, Baker AJ, Camacho SA. Cytosolic and mitochondrial $[Ca^{2+}]$ in whole hearts using indo-1 acetoxymethyl ester: effects of high extracellular Ca^{2+} *Biophys J.* 1996 Jun; 70(6):2571–80. [PubMed: 8744296]
58. Sedova M, Dedkova EN, Blatter LA. Integration of rapid cytosolic Ca^{2+} signals by mitochondria in cat ventricular myocytes. *Am J Physiol Cell Physiol.* 2006 Nov; 291(5):C840–50. [PubMed: 16723510]
59. Fry CH, Powell T, Twist VW, Ward JP. Net calcium exchange in adult rat ventricular myocytes: an assessment of mitochondrial calcium accumulating capacity. *Proceedings of the Royal Society*

- of London Series B, Containing papers of a Biological character. 1984 Dec 22; 223(1231):223–38.
60. Gavin CE, Gunter KK, Gunter TE. Manganese and calcium efflux kinetics in brain mitochondria. Relevance to manganese toxicity. *Biochem J*. 1990 Mar 1; 266(2):329–34. [PubMed: 2317189]
 61. Leisey JR, Grottyhann LW, Scott DA, Scaduto RC Jr. Regulation of cardiac mitochondrial calcium by average extramitochondrial calcium. *Am J Physiol*. 1993 Oct; 265(4 Pt 2):H1203–8. [PubMed: 8238406]
 62. Saks VA, Veksler VI, Kuznetsov AV, Kay L, Sikk P, Tiivel T, Tranqui L, Olivares J, Winkler K, Wiedemann F, Kunz WS. Permeabilized cell and skinned fiber techniques in studies of mitochondrial function in vivo. *Molecular and cellular biochemistry*. 1998 Jul; 184(1–2):81–100. [PubMed: 9746314]
 63. Fiskum G, Kowaltowski AJ, Andreyev AY, Kushnareva YE, Starkov AA. Apoptosis-related activities measured with isolated mitochondria and digitonin-permeabilized cells. *Methods in enzymology*. 2000; 322:222–34. [PubMed: 10914020]
 64. Crompton M, Heid I. The cycling of calcium, sodium, and protons across the inner membrane of cardiac mitochondria. *Eur J Biochem*. 1978 Nov 15; 91(2):599–608. [PubMed: 32035]
 65. Kauffman RF, Lardy HA. Biphasic uptake of Ca^{2+} by rat liver mitochondria. *J Biol Chem*. 1980 May 10; 255(9):4228–35. [PubMed: 7372676]
 66. Brand MD, Lehninger AL. Superstoichiometric Ca^{2+} uptake supported by hydrolysis of endogenous ATP in rat liver mitochondria. *J Biol Chem*. 1975 Oct 10; 250(19):7958–60. [PubMed: 1176454]
 67. Heineman FW, Balaban RS. Effects of afterload and heart rate on NAD(P)H redox state in the isolated rabbit heart. *Am J Physiol*. 1993 Feb; 264(2 Pt 2):H433–40. [PubMed: 8447459]
 68. Brandes R, Bers DM. Increased work in cardiac trabeculae causes decreased mitochondrial NADH fluorescence followed by slow recovery. *Biophys J*. 1996 Aug; 71(2):1024–35. [PubMed: 8842239]
 69. Brandes R, Bers DM. Intracellular Ca^{2+} increases the mitochondrial NADH concentration during elevated work in intact cardiac muscle. *Circ Res*. 1997 Jan; 80(1):82–7. [PubMed: 8978326]
 70. Brandes R, Bers DM. Simultaneous measurements of mitochondrial NADH and Ca^{2+} during increased work in intact rat heart trabeculae. *Biophys J*. 2002 Aug; 83(2):587–604. [PubMed: 12124250]

Biographies

Dr. Lothar A. Blatter is a Professor of Molecular Biophysics and Physiology at Rush University Medical Center Chicago. He received his M.D. degree and the degree of Doctor of Medicine from the University of Bern, Switzerland. He received postdoctoral training in physiology and pharmacology at the University of Bern, Switzerland, at the Mayo Clinic in Rochester, MN, and at the University of Maryland at Baltimore. He was recognized as an Established Investigator of the American Heart Association. He has authored over 90 peer-reviewed articles and he serves on the Editorial Board of *The Journal of Physiology*. Since 1993 he has maintained an active, independent and extramurally funded (NIH, AHA) research program and is actively involved in graduate and medical teaching. His research focuses on cellular calcium signaling pathways in the cardiovascular system (cardiac cells, vascular endothelial cells, vascular smooth muscle cells), using high-resolution imaging techniques, electrophysiological methods and molecular biology approaches.

Brian O'Rourke, PhD, FAHA

Professor of Medicine

Dr. Brian O'Rourke is a Professor in the Division of Cardiology, Department of Medicine of the Johns Hopkins University. Dr. O'Rourke's laboratory uses an integrated approach to study the biophysics and physiology of cardiac cells in normal and diseased states. A major emphasis of Dr. O'Rourke's work is to characterize the mechanisms of control and modulation of mitochondrial function. In this regard, he has elucidated how some ion channels in the mitochondrial inner membrane play an instrumental role in protecting myocytes from necrotic and apoptotic cell death (e.g., the mitochondrial K_{ATP} and $K_{Ca^{2+}}$ channels), while others contribute to mitochondrial dysfunction (e.g., permeability transition pores and inner membrane anion channels). Over the course of his career, Dr. O'Rourke has been most interested in studying the dynamics of mitochondrial energetics by taking a cross-disciplinary approach, working with collaborators in the areas of computational biology, proteomics, biophysics and molecular biology to gain a comprehensive understanding of the integrative physiology of the cardiomyocyte. A main theme is to understand how failures at the subcellular level scale to produce global electrical and contractile dysfunction in the heart. Rational strategies can then be devised to correct dysfunction during the progression of disease through a comprehensive understanding of basic mechanisms. Dr. O'Rourke currently directs a Program Project on mitochondrial function in cardiac disease (P01 HL081427), holds an NIH MERIT award (R37HL54598), and participates in a Systems Biology grant on sudden cardiac death (R33 HL087338).

Figure 1.

Left panel: Transmission electron micrograph of bat myocardium illustrating close juxtaposition of junctional SR (jsr) Ca^{2+} release sites at the t-tubules (t) and the mitochondria (Mito) (Porter KR. Mitochondria and transverse tubules of bat heart. ASCB Image & Video Library. August 2006: FND-10. Available at: <http://cellimages.ascb.org/u/?p4041coll12,56>. All rights reserved.

Reprinted under license from The American Society for Cell Biology). Right panel: Schematic representation of the key components of excitation-contraction-bioenergetic coupling. Arguing in favor of a rapid component of Ca^{2+} uptake, mitochondria are in close proximity to the SR junction containing L-type Ca^{2+} channels (L) and ryanodine receptors (RyR), which could drive fast uptake due to a large local Ca^{2+} gradient, despite a relatively high K_m for Ca^{2+} uptake via the mitochondrial uniporter (mCU). Slower Ca^{2+} extrusion from the mitochondria via the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCE) results in Ca^{2+} accumulation during rapid pacing. The fast Ca^{2+} signals may be required to provide rapid compensation for increased metabolic demand (and NADH oxidation) during high workloads through the activation of tricarboxylic acid (TCA) cycle dehydrogenases.