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Calcium Influx Through the Mitochondrial Calcium Uniporter Holocomplex, MCU_{cx}

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Abstract

Ca^{2+} flux into the mitochondrial matrix through the MCU holocomplex (MCU_{cx}) has recently been measured quantitatively and with milliseconds resolution for the first time under physiological conditions in both heart and skeletal muscle. Additionally, the dynamic levels of Ca^{2+} in the mitochondrial matrix ($[Ca^{2+}]_m$) of cardiomyocytes were measured as it was controlled by the balance between influx of Ca^{2+} into the mitochondrial matrix through MCU_{cx} and efflux through the mitochondrial Na^+ / Ca^{2+} exchanger (NCLX). Under these conditions $[Ca^{2+}]_m$ was shown to regulate ATP production by the mitochondria at only a few critical sites. Additional functions attributed to $[Ca^{2+}]_m$ continue to be reported in the literature. Here we review the new findings attributed to MCU_{cx} function and provide a framework for understanding and investigating mitochondrial Ca^{2+} influx features, many of which remain controversial. The properties and functions of the MCU_{cx} subunits that constitute the holocomplex are challenging to tease apart. Such distinct subunits include EMRE, MCUR1, MICUx (i.e. MICU1, MICU2, MICU3), and the pore-forming subunits (MCU_{pore}). Currently, the specific set of functions of each subunit remains non-quantitative and controversial. The more contentious issues are discussed in the context of the newly measured native MCU_{cx} Ca^{2+} flux from heart and skeletal muscle. These MCU_{cx} Ca^{2+} flux measurements have been shown to be a highly-regulated, tissue-specific with femto-Siemens Ca^{2+} conductances and with distinct extramitochondrial Ca^{2+} ($[Ca^{2+}]_i$) dependencies. These data from cardiac and skeletal muscle mitochondria have been examined quantitatively for their threshold $[Ca^{2+}]_i$ levels and for hypothesized gatekeeping function and are discussed in the context of model cell (e.g. HeLa, MEF, HEK293, COS7 cells) measurements. Our new findings on MCU_{cx} dependent matrix $[Ca^{2+}]_m$ signaling provide a quantitative basis for on-going and new investigations of the roles of MCU_{cx} in cardiac function ranging from metabolic

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Disclosures

None.

fuel selection, capillary blood-flow control and the pathological activation of the mitochondrial permeability transition pore (mPTP). Additionally, this review presents the use of advanced new methods that can be readily adapted by any investigator to enable them to carry out quantitative Ca^{2+} measurements in mitochondria while controlling the inner mitochondrial membrane potential, $\Delta\Psi_m$.

Introduction

Cytosolic Ca^{2+} signaling is used by diverse cells to regulate a broad range of cellular, subcellular and supra-cellular activities[1, 2]. The diversity and subtlety of these signaling modalities is grand, ranging from contraction in cardiac, skeletal and smooth muscle, to neurotransmission in CNS and peripheral nerve cells, and to multiple other functions in different organs of many eukaryotes. In addition to these fairly direct actions, Ca^{2+} signaling entrains parallel processing that provide support and feedback for the direct actions. Under physiological conditions, it has been hypothesized for many years that Ca^{2+} entry into mitochondria also regulates ATP production in parallel and this ATP production replenishes the energy consumed by the molecular motors and transducers that execute the functions of the cells. Only recently, however, have the details of the regulation of ATP production by mitochondrial matrix calcium ($[Ca^{2+}]_m$) been identified [3]. Importantly, specific putative calcium-sensitive proteins within the matrix, hypothesized to be critical to the Ca^{2+} sensitivity of ATP production, have been ruled out. This was done in mitochondria from cardiac ventricular myocytes under physiological conditions while Ca^{2+} sensitive ATP production was measured simultaneously. There is no question that additional quantitative investigations under alternative physiological conditions, during stressful circumstances and in disease-states still need to be determined. When done this would help us characterize the full spectrum of the Ca^{2+} sensitivity of ATP production by mitochondria. Importantly, however, the first quantitative fluxes of Ca^{2+} through the mitochondrial Ca^{2+} uniporter complex (MCU_{cx}) associated with this physiological activity were also measured in conjunction with the measurements of ATP production. Moreover, the regulation and function of ATP synthase itself by the voltage across the mitochondrial inner membrane, $\Delta\Psi_m$, was also demonstrated and characterized for the first time in a mammalian system.

Here we review the recent literature that examines the mitochondrial calcium uniporter holocomplex, MCU_{cx}. It is formed around four pore-forming subunits (MCU_{pore}) and these are complemented by addition components including four EMRE subunits, and several MICU subunits (from the MICUx family, possibly a MICU1 and MICU2 dimer) [4] and possibly other subunits such as MICUR1[5]. MCU_{cx} works as the holocomplex and is the key mitochondrial Ca^{2+} channel that enables each mitochondrion to continuously monitor cellular activity by reporting the cytosolic $[Ca^{2+}]_i$ level to the matrix. By this means, the mitochondria are able to respond to increased activity with enhanced ATP production (see Figs. 1 and 2 and ref. [3]). In addition, a very important new set of features of the MCU_{cx} have been suggested amidst some controversy. One such features is the hypothesized conductance threshold for Ca^{2+} in the MCU_{cx} which may be under control of one or more of the “non-pore” subunits charged with a “gatekeeping” function, MICU1 and MICU2. In this review, we discuss these matters and place them in the context of our new investigations of

MCU_{cx} [3] along with recent work by others. There are three primary “take-home” conclusions from our treatment of Ca^{2+} influx through MCU_{cx}. *First*, it is risky to generalize by assuming that all MCU holocomplexes (MCU_{cx}) from different tissues are the same. We [3] and others [6] show dramatic differences in the MCU_{cx} from heart and skeletal muscle. *Second*, the gold-standard of function should be the intact MCU_{cx} channels taken from native tissue. Certainly, data on MCU_{cx} obtained from other systems such as model cell lines has informed our understanding of MCUcx function and enabled us to do an impressive array of novel experiments, but these systems have been highly engineered. It remains an important job for those of us who wish to explain physiological function and disease-dependent changes to integrate the model system data into the native tissue function. *Third*, to facilitate reproducibility and discussion, direct conclusion about the function of MCU_{cx} should include *quantitative* measurements of the MCU_{cx} Ca^{2+} flux and the electrochemical gradients driving the MCU_{cx} Ca^{2+} flux.

The biophysical features of MCU_{cx}.

Electrophysiological studies showed that the MCU_{cx} is a highly selective, low conductance Ca^{2+} channel [6, 7]. The single-channel conductance of the MCU_{cx} (g_{mcu}) was found to follow a Michaelis-Menten relationship, with a K_m of 19 mM (much higher than the physiological diastolic levels found in heart of ~100 - ~300 nM) and with a maximal g_{mcu} of ~ 6-7 pS (at 105 mM $[Ca^{2+}]_i$, see [7-9]), ~10,000 fold greater that that observed under physiological conditions. This relationship between the Ca^{2+} conductance of the MCU_{cx} and the Ca^{2+} concentration is a typical biophysical feature of highly selective ion channels [10]. Typically, the conductance of an open channel increases as the availability of the conducting ion(s) increases[10]. Due to these biophysical features of the MCU_{cx}, electrophysiological examination of MCU_{cx} at physiological $[Ca^{2+}]_i$ is particularly challenging. Under such conditions, the single-channel conductance of the MCU_{cx} is low (~0.1 fS at 500 nM $[Ca^{2+}]_i$ and $\Delta\Psi_m = -160$ mV, see [9]). An additional limitation is the low copy number of MCU_{cx} channels in the inner-mitochondrial membrane of several tissues such as the heart [6]. As a result of these difficulties, to date, no electrophysiological examinations of MCU_{cx} current have been reported over the range of $[Ca^{2+}]_i$ of 300 nM to 10 μM, a range of particular interest, in which $[Ca^{2+}]_i$-dependent allosteric gating of the MCU_{cx} is proposed to take place [11-19].

To measure Ca^{2+} flux (J_{Ca}) we carried out calibrated fluorescent measurements of Ca^{2+} to quantify Ca^{2+} influx through MCU_{cx} channels even under physiological conditions [3]. These experiments replaced electrophysiological examinations of mitoplasts which are limited by low signal-to-noise characteristics at physiological $[Ca^{2+}]_i$. To do the quantitative measurements of MCU_{cx} Ca^{2+} influx, fluorescence signals from a large number of mitochondria at physiological $[Ca^{2+}]_i$ were carried out. By contrast, the electrophysiological recordings used patch-clamp methods in a single mitoplast (i.e. the inner-membrane product of a single mitochondrion) but at the "cost" of needing to use about 10,000 times higher $[Ca^{2+}]_i$. The primary cost of the physiological methods we used was the technical challenge of extracting quantitative calibrated signals (also see Table 1). However, this effort enables us to measure and account for all of the Ca^{2+} in all of the relevant compartments. Specifically, instead of the traditional fluorescent estimations of MCU_{cx} activity that are

derived primarily from measuring the free extramitochondrial [Ca] ($[Ca^{2+}]_{extra}$), we measured quantitatively (i.e. with full calibration) all of the Ca^{2+} signals in all of the relevant compartments. This includes the extra-mitochondrial Ca^{2+} (free-Ca^{2+} and Ca^{2+} bound to buffer) and the free Ca^{2+} in the mitochondrial matrix, $[Ca^{2+}]_m$. We also measured $\Delta\Psi_m$. In addition, the rates of change of $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ were also measured. To implement the above method, we measured $[Ca^{2+}]_{extra,\ total}$ (i.e. the total concentration of Ca^{2+} outside the mitochondria) over the range of 0.3 - 5 μM. This required us to eliminate all of the "*invisible" Ca^{2+} buffers* such as EGTA. Thus, the only Ca^{2+} buffer that we used was the Ca^{2+}-sensitive fluorescent indicator Fluo-4. This approach not only enabled us to measure the $[Ca^{2+}]_{extra,\ total}$, but it also avoided the comingling of the effects of "other" Ca^{2+} buffers and the Ca^{2+} flux through MCU_{cx} Ca^{2+} channels. In our approach, the RU360-sensitive time-dependent decline of the $[Ca^{2+}]_{extra,\ total}$ is taken as the total MCU_{cx} Ca^{2+} influx (J_{mcu}). In addition to measuring J_{mcu}, quantitative measurements of $\Delta\Psi_m$ and the free $[Ca^{2+}]$ inside ($[Ca^{2+}]_m$) and outside ($[Ca^{2+}]_{extra}$) the mitochondria were carried out. Together, J_{mcu}, $\Delta\Psi_m$, $[Ca^{2+}]_m$ and $[Ca^{2+}]_{extra}$ provide the means of estimating the physiological conductance of MCU_{cx} (G_{mcu}) with the typical Hodgkin–Huxley model [i.e., $I_{mcu} = G_{mcu}(\Delta\Psi_m - E_{Ca^{2+}})$]. From G_{mcu} that reflects the conductance of all the channels that are simultaneously open and the unitary conductance of a single open MCU (g_{mcu}), the number of open MCU_{cx} channels was estimated.

This approach was undertaken to separate electrochemical effects (thermodynamic) from other inherent properties of the MCU_{cx} channel (e.g. the number of open MCU_{cx}). While electrochemical gradients drive ion movement through the open MCU_{cx} channel, we centered on examining how the opening of the MCU_{cx} channel is gated allosterically by physiological levels of $[Ca^{2+}]_i$. These experiments were carried out with mitochondria isolated from skeletal muscle and heart muscle. In terms of extent, our measurements in mitochondria from heart and skeletal muscle were consistent with published genetic and electrophysiological evidence. Skeletal muscle mitochondria were reported to have the highest MCU_{cx} current density [6] and express the highest copy numbers of MCU_{cx} when compared to other mammalian tissues [20]. Heart has been reported to have one of the lowest. Furthermore, our estimation of the number of open MCU_{cx} channels in a heart mitochondrion (i.e., 5-15) is around the range reported by electrophysiological studies (15-65 per cardiac mitochondrion [6]). Surprisingly, we found that the regulation of the number of open MCU_{cx} channels by $[Ca^{2+}]_i$ is tissue-dependent (See Fig. 2.). We found that the number of open MCU_{cx} channels is regulated by $[Ca^{2+}]_i$ in skeletal muscle but not in heart.

Ca^{2+} signaling in mitochondria under physiological conditions.

In heart muscle cells, the abundant intermyofibrillar mitochondria (IFM) are frequently exposed to large local elevations of cytosolic Ca^{2+} ($[Ca^{2+}]_i$). Each end of the IFM is only about 100 nm away from the release sites containing clusters of ryanodine receptors on the junctional sarcoplasmic reticulum, the intracellular sites of $[Ca^{2+}]_i$ release. About 5,000 - 10,000 of these SR Ca^{2+} release sites open nearly simultaneously during each heartbeat, leading to a cell-wide transient elevation of $[Ca^{2+}]_i$ (i.e., the $[Ca^{2+}]_i$ transient). In the diastolic period between heartbeats, rare releases of Ca^{2+} from individual sites occur

stochastically throughout the cell, giving rise to clearly visualizable Ca^{2+} sparks [21]. During a Ca^{2+} spark, one end of the IFM is exposed to local elevation of $[Ca^{2+}]_i$ from about a 100 nM to as high as about 10 μM. The exposure to high levels of $[Ca^{2+}]_i$ (i.e. μM levels) is brief, lasting only a few milliseconds. During a cell-wide $[Ca^{2+}]_i$ transient, both ends of the IFM mitochondria are frequently exposed to similar spatiotemporal elevations of $[Ca^{2+}]_i$. This locally elevated $[Ca^{2+}]_i$ can enter the mitochondrial matrix via MCU_{cx}, an entry that is further driven by the large negative potential ($\Delta\Psi_m$) across the mitochondrial inner membrane of about −150 mV [3, 7, 22-25].

Recently, Ca^{2+} flux into the mitochondrial matrix through MCU_{cx} has been measured quantitatively and with milliseconds resolution for the first time under physiological conditions [3] (also see Table 1). These measurements were carried out in a manner that enabled estimating the number of open MCU_{cx} and how such number depends on $[Ca^{2+}]_i$. A primary goal of this approach was to test the hypothesis that the opening of the MCU_{cx} is gated allosterically by $[Ca^{2+}]_i$. The tests were carried out over the physiological range of $[Ca^{2+}]_i$, from 0.3 to over 10 μM, a range where $[Ca^{2+}]_i$-dependent gating was suspected to occur. Over this range, these data from cardiac mitochondria suggest that the number of open MCU_{cx} is relatively constant in heart and does not appear to be regulated by $[Ca^{2+}]_i$. These findings contradict earlier generalizations that such $[Ca^{2+}]_i$-dependent gating function is a broad feature of all types of MCU_{cx}'s. Furthermore, in the physiological context of the heart, these findings suggest that $[Ca^{2+}]_m$ is kept tightly regulated without a $[Ca^{2+}]_i$-dependent gating and without a threshold level for conducting Ca^{2+}, features that have been suggested to be imparted by molecular constituents (i.e. subunits) of the MCU_{cx} that together function as "gatekeepers" of the channel [11-19]. Importantly, the putative gatekeeping function has yet to be defined quantitatively or mechanistically in any tissue-identified and testable manner (also see discussion below) and so this should be a near-term goal in reevaluating the gatekeeping hypothesis. As suggested by prior studies, the putative purpose of gatekeeping is to limit Ca^{2+} entry through MCU_{cx}. Thus, "gatekeeping" may be preventing excessively high influx and the development of 'overload' of $[Ca^{2+}]_m$. In heart, however, the constant and low number of open MCU_{cx} in each cardiac mitochondrion (about 5-15 [3]) measured over $[Ca^{2+}]_i$ from ~ 100 nM to ~ 10 μM appears to be sufficient to keep $[Ca^{2+}]_m$ physiologically regulated.

How the Ca^{2+} influx through MCU_{cx} together with the efflux of matrix Ca^{2+} is managed to prevent Ca^{2+} overload in the mitochondrial matrix is a topic of active research. We still know little about how the mitochondrial Na^+/ Ca^{2+} exchanger (NCLX) dynamically regulates the levels of Ca^{2+} in the mitochondrial matrix ($[Ca^{2+}]_m$). To evaluate the dynamics of $[Ca^{2+}]_m$ we carried out measurements in patch-clamped cardiomyocytes under physiologically relevant conditions when both MCU_{cx} and NCLX are active to understand how $[Ca^{2+}]_m$ is controlled by physiological $[Ca^{2+}]_i$ signals. We found that under pronged quiescent conditions when global "diastolic" $[Ca^{2+}]_i$ is stable around 100 nM, $[Ca^{2+}]_m$ was static and slightly higher than $[Ca^{2+}]_i$. However, as soon as $[Ca^{2+}]_i$ transients were electrically evoked, $[Ca^{2+}]_m$ started to rise gradually and slowly, reaching relatively stable steady-state levels over the course of minutes. With additional β-adrenergic stimulation to increase the amplitude of the $[Ca^{2+}]_i$ transients, a higher steady-state levels of $[Ca^{2+}]_m$ were reached [3]. Thus, while cytosolic Ca^{2+} rises abruptly and declines sharply with each

contraction-relaxation cycle, $[Ca^{2+}]_m$ does not. Instead, the $[Ca^{2+}]_m$ dynamics are a low-pass filter version of the $[Ca^{2+}]_i$ signals. In a physiological context, these findings suggest that a single heartbeat-produced $[Ca^{2+}]_i$ transient does not materially change $[Ca^{2+}]_m$. Instead, $[Ca^{2+}]_m$ is controlled by the frequency and amplitudes of multiple $[Ca^{2+}]_i$ transients, therefore tracking changes in the heart rate over time and favoring the influence of the diastolic $[Ca^{2+}]_i$. These measurements also show that under physiological conditions Ca^{2+} influx into the matrix appears to occur through MCU_{cx} even when the diastolic $[Ca^{2+}]_i$ is quite low. This Ca^{2+} influx occurs over the diastolic $[Ca^{2+}]_i$ range of 100 nM to 300 nM and results in a measured increase in matrix $[Ca^{2+}]_m$ from ~200 nM to ~400 nM as shown recently (Fig. 3 in Ref. [3]). These measurements were obtained at low stimulation rates (0.5 Hz) in rat ventricular myocytes and suggest a need to re-examine the hypothesis that there is a "threshold" for Ca^{2+} conductance through the MCU_{cx} below which there is no Ca^{2+} flux. These new data are consistent with the notion that there is not a physiological threshold of the MCUcx between 100 nM and 300 nM and that this channel conducts Ca^{2+} at low diastolic $[Ca^{2+}]_i$ concentrations. Data testing this hypothesis is also presented in Fig. 1 (above).

In other cell types, cytosolic Ca^{2+} signals are profoundly different than those in heart cells [1, 2]. The conduction of such $[Ca^{2+}]_i$ signals through the MCU_{cx} channels is also tissue-specific. The heart mitochondria were found to have the lowest number of MCU_{cx}, the skeletal was found to have the highest of all tested mammalian tissues [6]. Furthermore, we recently found that regulation of the MCU_{cx} by $[Ca^{2+}]_i$ in heart and skeletal muscle is vastly different [3]. The number of open MCU_{cx} channels is regulated by $[Ca^{2+}]_i$ in skeletal muscle, in heart it is not (see Fig 2C). Furthermore, other studies suggested that the regulation of the liver MCU_{cx} by $[Ca^{2+}]_i$ is different than the regulation of the MCU_{cx} in heart and skeletal muscles [19, 26]. These, and other differences that exist between the MCU_{cx} of different tissues are important topics for future research. Better understanding of the tissue-specific MCU_{cx} can potentially further our understanding of the MCU_{cx} complex subunits and broadly advance our understanding of the physiological roles of mitochondrial Ca^{2+} signaling in different tissues.

Regulation of mitochondrial ATP production by matrix Ca^{2+}

Over 40 years ago, Denton, McCormack and co-workers reported that increasing levels of free calcium could augment the enzymatic activity of pyruvate dehydrogenase (PDH) as well as other single enzymatic steps within the Krebs cycle. In later review articles published in the 1990s [27, 28], Denton, McCormack and co-workers surveyed the advances in our understanding of the roles of mitochondrial calcium. Their work highlighted a fundamental need for actual measurements that tested their hypothesis. Such work had yet to be done – it was clear that the net effect of physiologic $[Ca^{2+}]_m$ on the rate of ATP production had not then been determined.

We directly address this gap in our understanding in [3]. Here we presented findings that measured how physiological calcium in the mitochondrial matrix ($[Ca^{2+}]_m$) regulated the rate of mitochondrial ATP production [3]. We used isolated cardiac mitochondria and measured ATP production quantitatively by calibrating the luminescence signal from

luciferin/luciferase. Quantitative measurements of $[Ca^{2+}]_m$ were done using Ca^{2+}-sensitive fluorescent indicators that had been loaded into the mitochondrial matrix. These experiments were carried out over a wide range of $[Ca^{2+}]_m$ levels from quiescent (resting) levels of about 100 nM to a level as high as 2.5 μM $[Ca^{2+}]_m$. We showed that increasing levels of $[Ca^{2+}]_m$ elevated the rate of ATP production in a Michaelis-Menten type of relationship, with a $[Ca^{2+}]_m$ $K_{0.5}$ of around 600 nM. Saturation was reached at about 2 μM. This regulation of ATP production occurred through a $[Ca^{2+}]_m$-dependent modulation of pyruvate dehydrogenase and glutamate dehydrogenase activity and not through any effect of Ca^{2+} on ATP synthase (ETC complex V) or on electron transport chain complexes II, III or IV or on the dehydrogenases within the Krebs cycle itself. Our new findings provide direct evidence contrasting some earlier predictions on where elevated $[Ca^{2+}]_m$ acted to stimulate ATP production [27-38].

Our findings that $[Ca^{2+}]_m$ partly controls ATP production by the mitochondria through only a few pivotal regulatory sites is new and is contrary to the large number of earlier publications that hypothesized many $[Ca^{2+}]_m$-sensitive regulatory sites [27-38]. A key difference between the work by Wescott *et al* (2019) and previous publications is that no prior study had actually carried out time-dependent parallel measurements of $[Ca^{2+}]_m$ and ATP production. Instead, prior studies had measured the rate of oxygen consumption as a surrogate for ATP measurements. $[Ca^{2+}]_m$ was also not measured. Instead, the free Ca^{2+} present in the experimental buffer solutions was calculated using Ca^{2+}-chelation software or other calculation modules or other means.

There is no question that additional quantitative investigations under alternative physiological conditions still need to be carried out to characterize better the full spectrum of Ca^{2+} sensitivity in mitochondria. Furthermore, mitochondrial calcium sensitivity likely occurs with tissue specific features that remain unclear. Such diversity would not be surprising because the features of energy consumption and the daily metabolic rates vary greatly among the major organs and tissues [39, 40]. In addition, mitochondria from different tissues metabolize different proportions of ‘food-stocks’ to fuel ATP production. Furthermore, not all tissues undergo large and acute changes in ATP consumption as do the heart and skeletal muscles. For example, the measured variations in oxygen consumption in the liver and the kidneys is rather modest, rising from the basal O_2-consumption levels of the tissue to levels that are about 25 percent higher [41-48]. In contrast, in the heart, with increased adrenergic tone and increased circulatory demands, the rate of oxygen consumption can rise by 2.5-fold to upward of 4.5-fold [49-58]. Even larger increases in oxygen consumption occurs in the skeletal muscle. In this tissue, depending on the fiber type, a transition from a relaxed muscle to a physiologic tetanic contraction can lead to 6-fold or even 17-fold rise in oxygen consumption [59-62].

The character of the sensitivity of ATP production to mitochondrial calcium in different tissues and cell types remains a critical topic for future work. Equally important is the need to understand how changes in the sensitivity of $[Ca^{2+}]_m$-dependent ATP production may contribute to the pathogenesis of multiple diseases. Indeed, dysfunction of mitochondria related to $[Ca^{2+}]_m$ management has been linked to the etiology of multiple degenerative

diseases and diseases of aging (further reviewed in [40, 63]). Yet, how the dysfunctions of mitochondria contribute to such pathologies remain ill-defined.

The function of the subunits of MCU_{cx}

"MCU_{cx} Threshold".

A large number of publications, many in high impact journals (e.g. [11-19]), have suggested that there is an extramitochondrial [Ca^{2+}] level below which there is no MCU_{cx} Ca^{2+} flux into the mitochondrial matrix. This [Ca^{2+}] level has been given the name "threshold" or "MCU_{cx} threshold". The existence of such MCU_{cx} threshold was first suggested by Mallilankaraman *et al.*, 2012 who also suggested that it serves to prevents excessive mitochondrial Ca^{2+} loading. The threshold was suggested to occur because the MCU_{cx} includes molecular components that act as steric plugs or "gatekeepers" of the channel [16]. Since then the concept of such a threshold has been used as a tool in determining how each of the MCU_{cx}-subunits interacted with the MCU_{pore} itself to better understand the entire MCU_{cx} function. This "threshold" level has been used to "unpack" the details of the MCU_{cx} function based on diverse indirect experiments. Despite the pivotal importance of the putative "threshold" in sorting out the molecular functioning of the MCU_{cx}, the MCU_{cx} Ca^{2+} flux and the critical $[Ca^{2+}]_i$ level of this threshold, to date there appears to be little agreement regarding the numbers. Importantly too, the putative MCU_{cx} threshold has not been quantitatively measured. If indeed such threshold is an important feature of MCU_{cx}, it would need to be determined by direct measurements of the levels of $[Ca^{2+}]_i$ and $[Ca^{2+}]_M$ as well as $\Delta\Psi_M$, and by quantitative measurement of the actual influx of Ca^{2+} into the mitochondria through MCU_{cx} (i.e. $J_{MCU\text{-}cx}$). With this approach, thermodynamic effects and allosteric gating effects can be distinguished and measured. The first such set of such measurements were done in [3] and are shown in Fig. 1a [3], (additional details are in Table 1). As clearly shown in the inset of Fig. 1a (at high resolution), the quantitative measurement of the MCU_{cx} threshold suggests that it does not exist. Put otherwise, the threshold in cardiac myocyte MCU_{cx} is found at 0 nM $[Ca^{2+}]_i$. In skeletal muscle the MCU_{cx} threshold is also found at 0 nM $[Ca^{2+}]_i$ as shown in Fig. 2. In further support of these conclusions, direct quantitative electrophysiological measurements of MCU_{cx} by Garg et al. [64] showed that there is no conductance occlusion in the absence of $[Ca^{2+}]_i$. Under these conditions of nominally zero $[Ca^{2+}]_i$, with Na^+ as the permeant ion, MCU_{cx} readily conducts Na^+ in patch clamped mitoplasts. Put another way, the MCU_{pore} subunits are not blocked in the absence of extramitochondrial Ca^{2+} [64]. This work further demonstrates that a conduction “threshold” is not a feature of the MCU_{cx} in Ca^{2+} - free conditions. In addition, the single-channel recordings in Garg et al., show that MICU1, an auxiliary subunit of the MCU_{cx}, does not function as a plug -- as suggested by earlier estimates [11-19]. Instead, these single-channel recordings demonstrate that MICU1 enable the channel to remain open for longer periods of time. Thus, several independent careful quantitative studies [3, 64] demonstrate that a conduction threshold is not a necessary feature of the MCU_{cx}, that the MCU_{cx} channel conducts Ca^{2+} whenever it is available, and that in some tissues allosteric regulation of MCU_{cx} by cytosolic Ca^{2+} occurs. Furthermore, these findings also indicate a different action of MICU1 on MCUcx than has been reported heretofore. Importantly, a conduction block, or

conduction occlusion, has not been shown to occur by any study that quantitatively examined the conductance of the MCU_{cx} at low Ca^{2+} [6, 7, 65].

Figs. 1-2 provide clear evidence that the MCU_{cx} conduction threshold is indistinguishable from 0 nM $[Ca^{2+}]_i$ in mitochondria from cardiac ventricular myocytes and from mitochondria from fast twitch skeletal muscle. At $[Ca^{2+}]_i$ levels between 300 nM to 3.0 μM the MCU_{cx} flux appear to increase linearly as the concentration of the conducting Ca^{2+} increases. The extrapolated value of the zoomed-in linear fit at values less than 3.0 μM is 0 μM $[Ca^{2+}]_i$. To understand better the spread of the suggested values along with the papers in which the data were presented, Fig. 2b shows the summary of these results graphically. A dashed line with the same slope as the fit shown in the inset in Fig. 1a is used to represent each cluster of findings with "thresholds" at 0.5 μM (red) [11-15], 1.0 μM (purple) [16-18] and 1.5 μM (green) [19]. It is clear that none of the suggested "threshold" lines fit the data better than the working conclusion from experiments on cardiac and skeletal muscle mitochondria. The same value of 0 μM $[Ca^{2+}]_i$ is estimated when there is only a modest influence of the MICUs (in mitochondria from cardiac myocytes) or an overwhelming influence of the MICUs (in mitochondria from skeletal muscle fast twitch myocytes) [66]. Importantly, all of the technologies used in [3] are standard analytic methods and simply involve measuring all Ca^{2+} levels and careful calibration of Ca^{2+}, $\Delta\Psi_M$ and pH. Using this approach, the actual influx of Ca^{2+} into the mitochondria through MCU_{cx} (i.e. the ($J_{MCU\text{-}cx}$) was measured. In sum, these measurements were conducted above and below 0.5 μM $[Ca^{2+}]_i$, which is the lowest "threshold" level proposed in the literature [11-15]. In all cases, the findings contradict the hypothesis that in mitochondria of all tissues there is a "threshold" or minimum non-zero $[Ca^{2+}]_i$ needed to enable a measurable $(J^{Ca})_{MCU\text{-}cx}$.

The MICU's: MICU1, MICU2 and MICU3: "the MICU's".

The function of the MICU's in the MCU_{cx} remain enigmatic. MICU1 appears to be expressed in every tissue tested [13] and mice with MICU1 knocked out are partially [19] or completely [67] peri-natal lethal. Mice that are $MICU2^{-/-}$ [68] develop heart disease. In contrast, knocking out the pore-forming subunit, the MCU_{pore} itself, is not necessarily embryonic lethal when carried out on some genetic backgrounds [69, 70]. However, despite the apparent importance of understanding better the function of MICU1, the investigational results and conclusions are not yet clear and consistent. Knocking down MICU1 has been reported to increase maximum $J_{MCU\text{-}cx}$ for Ca^{2+} in one study [71] but did not change $J_{MCU\text{-}cx}$ in other investigations [11, 16] and blocked it in yet another study [72]. Presumably methodological differences may account for these contradictory results since they were all carried out in the same cell line (HeLa cells). It is also not yet clear what is the degree to which MICU1 is affected by Ca^{2+} inside the mitochondrial matrix to regulate MCU_{cx} ([16, 65, 73, 74], further discussed in [75]). There is also the surprising result that comes from an investigation in *drosophila*, where flies with knockout of the MCU_{pore} ($MCU_{pore}^{-/-}$) live but when MICU1 was also knocked out the creatures were not viable [76]. This finding raises the question differently: what is the vital function that MICU1 fulfills in the absence of the MCU_{pore}? For us, the key approach to resolve the questions raised by these findings is to carry our quantitative experiments that measure critical variables in functional mitochondria. Thus, it remains an important job for those of us who wish to explain disease, to integrate

the model system data into the native tissue function. Importantly, there are stark differences in how the MCU_{cx} of different tissues conduct Ca^{2+} [6] and differences in the molecular composition of the MCU_{cx} among tissues[26]. In addition, while MICU1 is found in all tissues tested there appear to be a unique splice variant of MICU1 with expression that is limited to only a few tissues [66]. Additionally, stress or disease or physical activity may also matter. Thus, when animals are bred with altered MCU_{cx}, sedentary animals should be compared with stressed animals. For maximum understanding, testing exercise stress, stress of specific energy sources and stress of specific kinases and diseases are the kind of investigative approaches that are likely to significantly advance our understanding.

Gating of MCU_{cx}

In 1979 Marco Bragadin, Tullio Pozzan, and Giovanni Felice Azzone [77] were able to measure a much larger mitochondrial Ca^{2+} influx than in any previous studies. These measurements were possible because they identified a key shortcoming of prior approaches. They discovered that mitochondrial Ca^{2+} influx rapidly degraded its own driving force, the mitochondrial membrane potential ($\Delta\Psi_m$), because of the large influx of positive charge carried by Ca^{2+}. To solve this problem they used a mitochondrial "voltage clamp" using the K^+ ionophore valinomycin and the K^+ gradient to set $\Delta\Psi_m$ [78]. With this approach, in isolated liver mitochondria [77], Bragadin et al., (1979) found a very large mitochondrial Ca^{2+} influx that continued to increase with the increasing levels of extramitochondrial Ca^{2+} and only begun to saturate at about 200 μM Ca^{2+}. The observed K_m was around 75 μM, more then 10 times higher than in previous studies. The sigmoidal shape of the Ca^{2+} influx curve and other findings led Bragadin et al., to suggest that allosteric binding cooperativity augments mitochondrial Ca^{2+} influx through a Ruthenium Red sensitive “multi-subunit carrier”, that came to be called the MCU_{cx}. Notably, with this experimental approach, Bragadin et al., also discovered the importance of setting $\Delta\Psi_m$ or quantitatively measuring $\Delta\Psi_m$ when investigating the properties of the MCU_{cx}. Nearly 25 years later, even tighter control of $\Delta\Psi_m$ was achieved by Kirichok et al., 2004 using electrophysiological approach to voltage-clamp the vesicular preparation of the inner mitochondrial membrane of COS-7 cells, the mitoplast [7]. The findings of Kirichok et al., revealed that MCU_{cx} is a Ca^{2+}-selective ion channel, with a much higher K_m than shown before (i.e. 19 mM). This work also enabled discovery of the other central role of $\Delta\Psi_m$ on the MCUcx; it became clear that $\Delta\Psi_m$ not only powers Ca^{2+} movement via the MCU_{cx}, but that the voltage itself also tightly controls the open probability of the MCU_{cx} channel. At $\Delta\Psi_m$ of −80 mV the open probability was only 0.11 but it steeply rises to 0.93 at $\Delta\Psi_m$ of −160 mV. Learning these critical lessons from Bragadin et al., and from Kirichok et al, we measured $\Delta\Psi_m$ quantitatively along with each measurement of MCU_{cx} flux (J_{mcu}). We also used valinomycin to be able to carry out J_{mcu} measurements at high extramitochondrial Ca^{2+} ($[Ca^{2+}]_i$). Like Bragadin et al, we did not observe saturation of J_{mcu} over this range of $[Ca^{2+}]_i$ as shown in Figure 2a. We then used these measurements to assess how $[Ca^{2+}]_i$ allosterically regulates the number of open MCUs. We found that while $[Ca^{2+}]_i$ did not affect the number of open MCUs in heart mitochondria, in skeletal muscle $[Ca^{2+}]_i$ allosterically increased number of open MCUs by about 8 fold, with a $K_{0.5}$ of 7.9 μM. This tissue-dependent $[Ca^{2+}]_i$-sensitivity of the MCU_{cx} is likely due to different molecular

composition of the MCU_{cx} in different tissues [19, 26, 66], but particularly important is the potential involvement of MICU1 as the component of the channel that interacts with $[Ca^{2+}]_i$ [79]. Indeed, recently, Garg et al., 2020, who used a mitoplast preparation from mouse embryonic fibroblasts (MEF) to examine the gating of I_{MCUcx} found that voltage-dependent stochastic opening and closure of the MCU_{cx} channel still occur in the absence of MICU1 [64]. However, Garg et al. also found that in the absence of MICU1, the open probability of the MCU_{cx} was two- to threefold lower than in the WT MCU_{cx} that contain MICU1. This finding further supports the role of MICU1 as a critical component of the activation gate in the MCU_{pore} itself.

The physiological role of MCU_{cx}

An increasing number of recent studies use mice with genetically altered MCU_{cx} channels expressed in their native tissues. These mice may have a subunit knocked out, over-expressed or mutated [18, 69, 70, 82, 83, 90, 91]. Experiments with these mice use native tissues, or cells or mitochondria, that, despite the genetic manipulations and functional consequences, still sustain the life of the mouse. Perhaps, as expected, this approach is not free of controversy [92, 93]. Nevertheless, investigating the MCU_{cx} channels in their native environments has several fundamental advantages that make the effort worthwhile. A key advantage is that one can interpret and discuss the results in the metabolic and physiological contexts of the investigated tissue. Additionally, one can draw from the existing rich literature and have new understanding guided by past metabolic and functional investigations. Such experiments also permit one to compare the results of MCU_{cx} experiments with those done in animals or preparations using fully functional wild-type MCU_{cx} channels.

A broad and consistent finding of all studies is that knocking out the "pore forming subunit" of the MCU_{cx} (i.e. the MCU_{pore}) leads to mitochondria that are incapable of *rapidly* taking up Ca^{2+}. Somewhat similar results are observed when the highly-selective blocker of MCU_{cx} RU360 is used in experiments with the wild-type MCU_{cx}. Unexpectedly, conditional MCU_{pore} knockout in the adult heart and complete embryonic MCU_{pore} knockout mice produced on certain genetic backgrounds display no apparent phenotype when these animals are sedentary [69, 70, 82, 83, 90]. Thus, in all of these mouse models, mitochondrial ATP was produced at sufficiently high rates so that the basic energetic needs of the contracting heart were met. However, when fight-or-flight responses were demanded by acute β-adrenergic stimulation, some of the models performed poorly [82, 83, 90, 91]. Further examination revealed broad transcriptional adaptation that occurs in response to life without a functional cardiac MCU_{cx} [90]. Importantly, such adaptations that accompany genetic deletion of MCU_{pore} include changes of mitochondrial fuel selection, with shift towards mitochondrial fatty-acid metabolism in skeletal muscle [94], as well as augmentation of cardiac fatty-acid oxidation as compensatory source of high-energy to sustain high ATP production [95]. These findings further support the suggested role of MCU_{cx} Ca^{2+} flux as the signal that stimulates pyruvate-fueled ATP production by mitochondria [3]. Thus, in the absence of functional MCU_{cx} channels, as an adaptation, other carbon ‘food-stocks’ are consumed at higher rates.

How does mPTP activation depend on MCU_{cx}?

A huge number of reports in the literature show that prolonged or repeated exposures of mitochondria to high levels of extramitochondrial [Ca^{2+}] underlie the opening of mitochondrial permeability transition pores (mPTP's)[96-99] and the depolarization of mitochondria. This methodological approach to studying mPTP has linked MCU_{cx} to mPTP activation. Activation of mPTP is generally thought to be a catastrophic event but recently it has also been viewed as a reversible physiological "venting" of excessive mitochondrial Ca^{2+} [100, 101]. This venting hypothesis has not been tested rigorously yet and so remains open. The most striking gap in the body of mPTP work, however, it the absence of a viable hypothesis of the molecular identity of the mPTP [99, 102, 103]. The current work on this topic has led to an embroiled high-profile debate that includes the suggestion that one of the most exquisite proteins in biology, the ATP synthase, Complex V, can be transformed to take on the role of mPTP [104-107]. The ATP synthase hypothesis has been strongly disputed by John Walker, well-known for his work on the crystal structure of the ATP synthase[108-111].

A recent provocative experimental result showed that less dramatic mPTP events could be generated by means other than elevated extramitochondrial [Ca^{2+}] and could be gently graded[112]. Furthermore, these more modest mPTP events were shown to be reversible and consistent with the activation of a single channel-like entity. The experimental tool used in these experiments was photon stress [112, 113]. Photon stress is a method that was shown to generate reactive oxygen species (ROS) that activated mPTP. That mPTP was activated in single mitochondria was supported by the rapid and reversible depolarization of a single mitochondrion by mild photon stress. That the photon stress dependent mPTP depolarization was due to elevated ROS was supported by the effective block or slowing of mPTP development by pretreatment with antioxidants. Like the earlier hypothesized source of mPTP, all causes of the mPTP event have been hypothesized to be placed in the inner mitochondrial membrane (IMM). When mPTP's are activated, the increase in the IMM permeability, also called "permeability transition", causes a loss of osmotic balance of the mitochondrion, a degradation of $\Delta\Psi_m$, and diminished capacity to produce ATP by the ATP synthase. MCU_{cx} plays a major role in the Ca^{2+} dependent activation of mPTP because it is the primary pathway by which Ca^{2+} enters the mitochondrial matrix. Another experimental characteristic of the mPTP is its huge conductance, often identified as a "mega-channel" [114, 115]. For example, when activated, substances as large as the fluorescent dye calcein (623 MD) can permeate the mPTP and were shown to enter or leave the mitochondria [116]. Recent work suggests that multiple genetically-distinct complexes can each function as mPTP's. If so, then knocking one complex out may still leave other complexes capable of functioning as mPTP's and making the unique identification exquisitely difficult. Included in the list of such candidates are macromolecular complexes which are otherwise known as selective carriers such as the adenine nucleotide translocase (ANT) and also ATP synthase. These carriers are proposed to mediate or be inherently transformed into non-selective pores that function as mPTP [104-107, 117]. The molecular details remain unclear and controversial both with respect to the mechanism of mPTP activation and the molecular identity of the mPTP. Thus, out of the mix of hypotheses, perhaps the simplest is the ROS

hypothesis because it can account both for photon stress and Ca^{2+} overload. If mitochondrial Ca^{2+} overload works to activate mPTP by profoundly abolishing the reducing power within the matrix, then the effect of MCU_{cx} Ca^{2+} influx is to activate mPTP by increasing matrix ROS. Unfortunately, we are still not sure what the activation mechanism(s) of mPTP is/are and what the identity of the mPTP protein(s) is/are.

OVERVIEW - MCU_{cx}: A flexible regulator of mitochondrial metabolism

MCU_{cx}: lynchpin of metabolic feed-back regulation.

The key to the MCU_{cx} success as a metabolic regulator of mitochondria is customizing the details of the MCU_{cx} components to the tissue itself. It is clear, for example, that the MCU_{cx} Ca^{2+} flux, $J_{MCU\text{-}cx}$, in heart is very different than it is in skeletal muscle MCU_{cx} at $[Ca^{2+}]_i$ above 3 μM. While this difference makes sense from the known biological properties of skeletal muscle, and is implied in Fig. 2, we are only now in a position to explore how the subunit composition of MCU_{cx} in the two tissues enable the functional differences [13, 18, 26]. There are also likely important differences in regulatory kinases, utilization of different carbon sources of energy (e.g. carbohydrate, fat, protein), and circulatory contributions to metabolism.

Novel regulation of blood flow in heart: electro-metabolic regulation -- possible role of MCU_{cx}.

The mitochondria in heart and the structure of the tissue in heart enable the mitochondria to play a pivotal role in controlling local blood flow as well as in making ATP. Clearly one of the vexing issues in heart is matching the supply of blood along with its oxygen and sources of energy to the work-load of the ventricular myocardium. This must occur continuously and flexibly and must match the needs of the animal and the tissue. Recently, Zhao *et al.*, (*PNAS* 2020) [118] described how the cytosolic [ATP] and cytosolic [ADP] in heart can influence local blood flow. How the MCU_{cx} Ca^{2+} channels contribute to this process has not been systematically studied. It seems reasonable, however, to speculate that the MCU_{cx} channels may contribute importantly to this process. If so, when a cell like a ventricular myocyte is driven by action potentials and elevated $[Ca^{2+}]_i$ signals to produce more force (that consumes more ATP), this same Ca^{2+} signal increases ATP production by the mitochondria as presented quantitatively by [3], and briefly reviewed here. If the blood flow should be inadequate, to maintain ATP production, then cytosolic [ATP] will start to decrease (while [ADP] is increasing). These changes in ATP and ADP concentrations activate the K_{ATP} channels in the sarcolemmal membrane of cardiac myocytes by increasing the open probability of the K_{ATP} channels in the myocytes. The larger number of open K_{ATP} channels produce a time-averaged *hyperpolarization* of the cardiac myocytes. This hyperpolarization would then increase the injection of hyperpolarizing current into the cardiac capillary endothelial cells through gap junctions. The resulting relative hyperpolarization of the endothelial cells hyperpolarizes (and relaxes) contractile capillary pericytes and vascular smooth muscle cells. By this means the capillary pericytes and the small arteriole vascular smooth muscle cells relax and increase the blood flow through the arterioles and capillaries supplying the nearby ATP-depleted ventricular myocytes. This electrical network thus works instantaneously upstream to provide increased blood flow to the needy ventricular myocytes.

This vascular electrical network and blood flow control mechanism, recently characterized in heart [118], also enables the removal of metabolic waste and CO_2 and contributes to the relaxation of the local venous system. By this chain of events, the MCU_{cx} Ca^{2+} flux into the mitochondrial matrix not only regulates ATP production by the mitochondria but also regulates blood flow locally to the ventricular myocytes.

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Highlights

- Ca^{2+} flux through the MCU_{cx} (holocomplex) is measured in heart and in skeletal muscle.
- There is no threshold for the dependence of MCU_{cx} Ca^{2+} flux on $[Ca^{2+}]_i$.
- We discuss how matrix $[Ca^{2+}]_m$ regulates ATP production.
- We discuss how MCU_{cx} may influence mPTP and blood flow in heart.
- We discuss the utility of using valinomycin "voltage clamp" in mitochondrial studies.

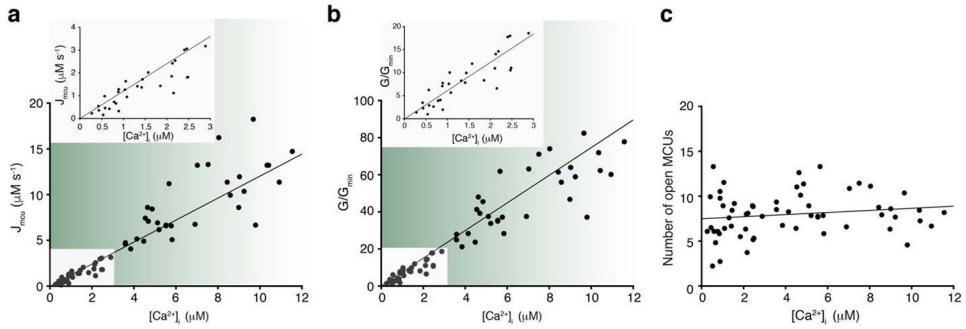

Figure 1. MCU_{cx} flux and its regulation by $[Ca^{2+}]_i$ in heart.

a. Measurements of the MCU_{cx} Ca^{2+} influx (J_{mcu}) in heart mitochondria (units are scaled to liter of cytosol). J_{mcu} (μM s^{-1}) is plotted as a function of measured extramitochondrial, $[Ca^{2+}]_i$. Each of these measurements of J_{mcu} were carried out along with measurements of $[Ca^{2+}]_i$, $[Ca^{2+}]_m$, and $\Delta\Psi_m$. The inset (top) is a zoomed-in view of the region of the plot between 0 and 3 μM $[Ca^{2+}]_i$. Linear least-squares fit to the filled circles is shown (slope = 1.2, going through the origin at 0, 0). **b.** MCU_{cx} conductance (G) for each of 63 experiments shown in **a,** normalized to the minimal conductance (G_{min}) of each dataset (G/G_{min}). G/G_{min} is plotted as a function of $[Ca^{2+}]_i$. Inset (top) is a zoomed-in view of the region between 0 and 3 μM $[Ca^{2+}]_i$. Linear least- squares fit line to the filled circles is shown (slope = 6.1, going through the origin at 0,0). **c**. Number of open MCU_{cx} channels per mitochondrion plotted as a function of $[Ca^{2+}]_i$. Taken from **b** after division by the number of mitochondria per liter cytosol and dividing by the $[Ca^{2+}]_i$-dependent unitary conductance of MCU_{cx} (For additional technical details see [3, 7, 9]). Linear least-squares fit to the filled circles is shown (slope = 0.116, intercept = 7.48). Panels a-c are taken with publisher permission from Wescott *et. al., Nature Metabolism*, 2019 [3].

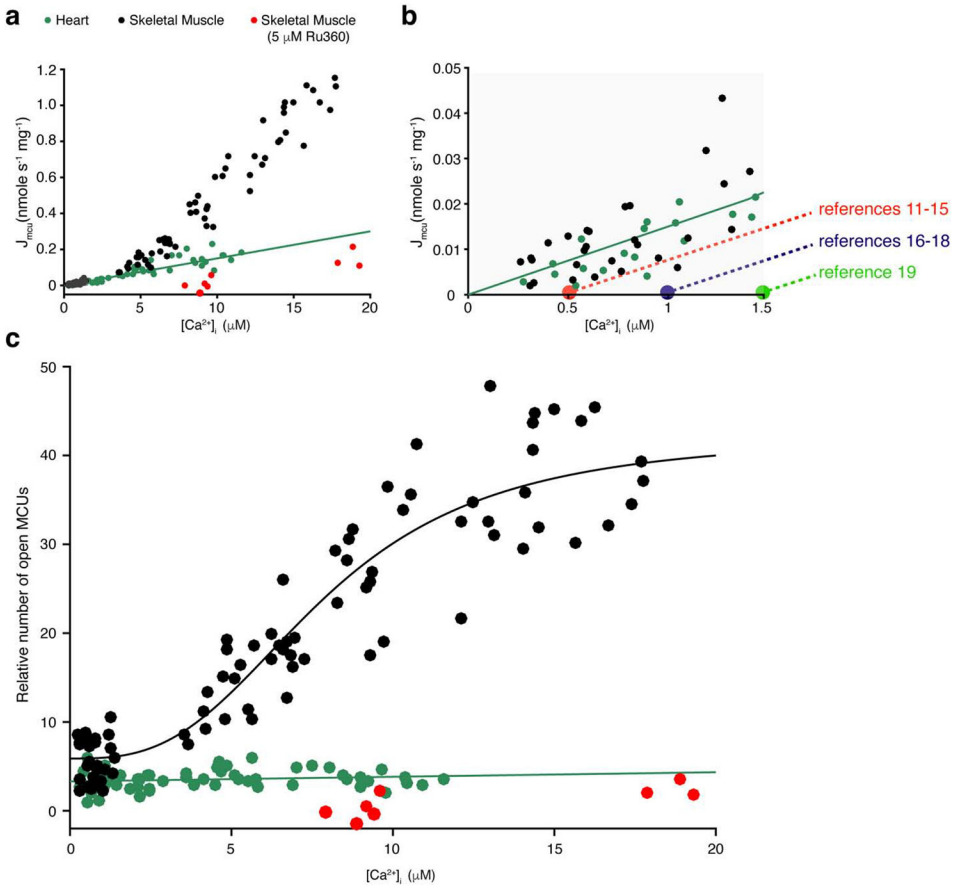

Figure 2. MCU_{cx} and its regulation by $[Ca^{2+}]_i$ in heart and skeletal muscle.

a. Measurement of the MCU_{cx} Ca^{2+} influx ($J_{MCU\text{-}cx}$) in cardiac mitochondria (green circles), skeletal muscle (black circles), and skeletal muscle with Ru360 (5 μM, red circles). For convenience on the graphs, J_{mcu} is used to represent $J_{MCU\text{-}cx}$. Thus J_{mcu} is plotted as a function of measured extramitochondrial $[Ca^{2+}]_i$. Each of these measurements of J_{mcu} were carried out along with measurements of $[Ca^{2+}]_i$, $[Ca^{2+}]_m$, and $\Delta\Psi_m$. Linear least-squares fit to the heart mitochondria data is shown (slope = 0.015). **b**. Zoomed-in view of region between 0 and 3 μM of $[Ca^{2+}]_i$. from a. Black and green filled circles are from [3]. Overlaid dashed lines intercept the vertical axis at the $[Ca^{2+}]_i$ levels where conduction thresholds of the MCU_{cx} were proposed to occur. The levels of $[Ca^{2+}]_i$ at which each threshold was proposed to be are taken from the indicated studies. The red filled circle is based on the threshold value from references [11-15], the purple from[16-18], the green from reference [19] **c**. Relative number of open MCU_{cx} channels per mitochondrion plotted as a function of $[Ca^{2+}]_i$ (For additional technical details see [3, 7, 9]). Linear least-squares fit to the heart mitochondria data is shown (slope = 0.051, intercept = 3.3). Skeletal muscle MCU_{cx} data was fitted to a modified Hill equation yielding a $K_{0.5}$ of 7.9 μM and a Hill coefficient of 2.95. Panels **a-c** are taken with publisher permission from Wescott *et. al., Nature Metabolism*, 2019 [3].

Table 1:

diverse methodologies to measure Ca^{2+} influx via MCU_{CX}.

	General approach	Central tools	Measures	Remarks	Recent	Related
1	**Radioactive mitochondrial Ca^{2+} uptake measurements**	Radiolabeled $^{45}Ca^{2+}$	Cell/mitochondria $^{45}Ca^{2+}$ retention	Insensitive to pH. Can be conducted over a wide range of $[Ca^{2+}]$ (nM to mM), low temporal resolution, cannot be combined with simultaneous measurements of other signals. Uses either isolated mitochondria or permeabilized cells. Does not quantitatively measure total J_{MCUcx} due to dynamic binding to extramitochondrial buffers.	[4, 11, 17, 80]	[22, 81]
2	**Fluorescent measurements of $[Ca^{2+}]_i$**	Ca^{2+}-sensitive fluorescent indicators	Change in free extra-mitochondrial $[Ca^{2+}]$	High temporal resolution can be combined with simultaneous measurements of other signals. Uses either isolated mitochondria or permeabilized cells. Does not quantitatively measure total J_{MCUcx} due to dynamic binding to extram itochondrial buffers.	[5, 11, 14, 16, 18, 26, 67, 74, 82, 83]	[84]
3	**Electrophysiological measurements of I_{MCUcx}**	Patch-clamping the IMM	Whole-mitoplast or single-channel MCU_{cx} currents	High temporal resolution. Quantitative direct measurements of MCU_{cx} currents. Can be combined with simultaneous measurements of other fluorescent signals. Does not have the sensitivity below $[Ca^{2+}]$ of ~10 μM. Requires mitoplast preparation.	[6, 7, 64]	[85, 86]
4	**Fluorescent measurements of total mitochondrial Ca^{2+} influx via MCU_{cx}**	Ca^{2+}-sensitive and voltage-sensitive fluorescent indicators	Total MCU_{cx} Ca^{2+} flux (J_{MCUcx}), MCU_{cx} conductance (G_{MCUcx}).	High temporal resolution. Quantitatively measure total J_{MCUcx} together with simultaneous quantitative measurements of $[Ca^{2+}]_i$, $[Ca^{2+}]_m$, and $\Delta\Psi_m$. Highly sensitivity below $[Ca^{2+}]_i$ of ~10 μM. Carried out in isolated mitochondria but not in permeabilized cells.	[3]	[87], [88, 89]