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Mitochondrial Ca^{2+} concentrations in live cells: quantifications and discrepancies

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Abstract

Intracellular Ca^{2+} signaling is a fundamental process that controls many cellular functions. During intracellular Ca^{2+} signaling mitochondria are capable of responding to cytosolic Ca^{2+} changes to regulate their own activities and, in some cell types, shape the spatiotemporal properties of the cytosolic Ca^{2+} signal. Numerous methods have been developed to specifically and quantitatively measure the mitochondrial free Ca^{2+} concentrations ($[Ca^{2+}]_m$), but there are still significant discrepancies in the absolute values of $[Ca^{2+}]_m$ in live cells upon physiological stimulus. These discrepancies may derive from the diverse properties of the method used to measure $[Ca^{2+}]_m$, the calcium-free/bound ratio, and the intrinsic Ca^{2+} dynamics of each cell type in responding to various stimuli. Critical processes happening in the mitochondria, such as the ATP generation, ROS homeostasis, and mitochondrial permeability transition opening, depend directly on certain levels of the $[Ca^{2+}]_m$. Knowing those absolute $[Ca^{2+}]_m$ values precisely is imperative for understanding Ca^{2+} signaling in cell. This review summarizes the reported calibrated $[Ca^{2+}]_m$ values in many cell types and explains the discrepancies between these values. The gap areas for future research are also proposed.

Keywords

Mitochondrial Ca^{2+} Concentrations; Live Cells; Fluorescent Ca^{2+} Indicators; Genetically Encoded Ca^{2+} Indicators

INTRODUCTION

A general cellular response to extracellular stimulus is the rise in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) leading to intracellular Ca^{2+} signaling which plays a key role in multiple cellular processes, including gene expression, differentiation, bioenergetics regulation, contraction, exocytosis, neurotransmission, and cell death [1]. Multiple pathways can lead to this $[Ca^{2+}]_c$ increase, including the Ca^{2+} entry from the extracellular medium through the plasma membrane calcium channels and Ca^{2+} release from the intracellular Ca^{2+}

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stores like the endoplasmic/sarcoplasmic reticulum (ER/SR). The release of Ca^{2+} from the ER/SR can be mediated by depolarization-initiated mechanical coupling of L-type Ca channels with the type 1 ryanodine receptor (RyR1) or by Ca^{2+}- or IP3-induced Ca^{2+} release, depending on the cell type and tissue. These pathways elevate the resting $[Ca^{2+}]_c$ concentration from ~100 nM to a level that varies vastly from report to report (Table 1). Unlike other cellular second messengers, the Ca^{2+} can neither be created nor destroyed; Ca^{2+} can only be transported, released, or bound. Due to the importance of the $[Ca^{2+}]_c$ increases in controlling cell functions, levels of $[Ca^{2+}]_c$ need to be tightly regulated. During a Ca^{2+} transient, mitochondria can uptake Ca^{2+} from the cytosol to modulate the spatiotemporal profiles of $[Ca^{2+}]_c$ mainly via the mitochondrial calcium uniporter complex (MCUC) or through other alternative pathways[2,3]. However, the increase in the mitochondrial Ca^{2+} free concentration ($[Ca^{2+}]_m$) is involved not only in controlling the cytosolic Ca^{2+} signal but also in regulating mitochondrial functions, such as energy metabolism[4]. Cellular activities are expected to change upon stimulation, a process that demands energy. Mitochondria utilize the increase in $[Ca^{2+}]_m$ to enhance ATP production and subsequently the energy generation[5].

Because of its physiological and pathological relevance, mitochondrial Ca^{2+} dynamics have been studied intensively in the last decades. Methods to measure changes in $[Ca^{2+}]_m$ have substantially evolved in recent years. These methods are essentially classified in two major groups: fluorescent dyes and genetically encoded Ca^{2+} indicators (GECIs). GECIs can be subdivided into fluorescent or bioluminescent probes. Ideally, all of these methods are suitable to accurately report $[Ca^{2+}]_m$ values, independent of the cell type or the stimulus, however each has its own advantages and disadvantages, and different methods are preferred to measure $[Ca^{2+}]_m$ depending on the cell type, stimulus, or even on the equipment used to perform the measurements. So, despite the advances in the number of tools available for quantifying $[Ca^{2+}]_m$, discrepancies persist in the absolute values that can be reached inside the mitochondrial matrix upon stimulation in live cells. It is possible that mitochondria from different cell types may reach significantly different $[Ca^{2+}]_m$, due to their own biochemical and physical properties (including localization and/or relation with other cellular organelles). However, under a given identical circumstance, different methods applied should report and confirm the same $[Ca^{2+}]_m$ in the same cell type. In this review, we summarize the importance of knowing absolute values of $[Ca^{2+}]_m$ and most used methods to achieve the measurements; and we compile most of the calibrated $[Ca^{2+}]_m$ values reported in the literature. We also point out the discrepancies between these values and what may underlie these differences. Areas for future research are also proposed.

MITOCHONDRIAL Ca^{2+} AND BIOENERGETICS

Mitochondria are the main ATP producers through oxidative phosphorylation in eukaryotic cells. The reactions of the Krebs cycle and the utilization of the resulting reducing power by oxidative phosphorylation generates up to 32–36 ATP molecules, whereas glycolytic metabolism generates only 2. These reactions are regulated by $[Ca^{2+}]_m$ in a mechanism designed to ensure that the ATP synthesis is coupled with the energy demand of the cell[6].

Due to the great capacity of mitochondria to uptake Ca^{2+}, it was thought that these organelles were essential reservoirs of mobilizable intracellular Ca^{2+} ions[7]. Nevertheless, several independent studies from the 1980s[8–10] showed that total $[Ca^{2+}]_m$ was low under basal conditions, but increases in $[Ca^{2+}]_c$ in response to extrinsic agents were mirrored by increases of $[Ca^{2+}]_m$ [11,12]. The higher $[Ca^{2+}]_m$ in response to increases of $[Ca^{2+}]_c$ would lead to the activation of oxidative metabolism and result in an increased supply of reducing equivalents to drive respiratory chain activity and ATP synthesis. Export of mitochondrial ATP in exchange for ADP will be anticipated to meet the higher ATP demand to fuel energy-requiring processes in the cytosol, such as ion pumping and contraction[13].

Mitochondrial enzymes modulated by $[Ca^{2+}]_m$

- Pyruvate dehydrogenase phosphatase (PDHP).—The pyruvate dehydrogenase (PDH) is a 50 MDa multi-enzyme complex that catalyzes the irreversible reaction of pyruvate, CoA, and NAD to obtain acetyl-CoA, $NADH_2$, and CO_2. The product, acetyl-CoA then enters to the citrate cycle or fatty acid synthesis. The regulation of the PDH complex activity is achieved via end-product inhibition and reversible inhibitory phosphorylation by a PDH kinase[14,15]. A Mg^{2+}/Ca^{2+}-dependent PDH phosphatase, in turn, dephosphorylates and reactivates PDH. It has been described that Ca^{2+} activates the PDH phosphatase in heart mitochondrial extracts with $K_{0.5}$ of 0.7 μM[16,17]. Therefore, $[Ca^{2+}]_m$ regulates the activity of the PDH complex, a rate-limiting step for pyruvate oxidation to acetyl CoA that in turn is reflected in the rate of ATP synthesis in mammalian tissues[6,18].

- 2-Oxoglutarate dehydrogenase (OGDH).—The OGDH is a sizeable multi-subunit complex, which similarly to the PDH complex, consists of multiple copies of three different subunits (E1/E2/E3). The complex catalyzes the multiple step reaction that transforms the 2-oxoglutarate to succinyl-CoA, Co2 and NADH[19]. From the many cofactors involved in the regulation of the OGDH complex, it has been found that Ca^{2+} and adenine nucleotides are important modulators that directly bind to the complex, most likely to the E1 subunit[20] with a described $K_{0.5}$ of ~1 μM in mitochondrial extracts and of ~0.2 μM in permeabilized mitochondria. Additionally, it has been shown that high ADP/ATP ratios decrease not only the K_m of the OGDH complex for the substrate but also the $K_{0.5}$ for the Ca^{2+} [21]. It seems reasonable to think that under sudden high workload conditions, the high ADP/ATP mitochondrial ratios and $[Ca^{2+}]_m$ increases over the same time scale[22,23] stimulate a reduced power supply for oxidative phosphorylation by increasing the activity of the *OGDH*.

- NAD+-isocitrate dehydrogenase (NAD-ICDH).—NAD-ICDH is a hetero-octamer of 2 × (2α,β,γ) subunits which catalyzed the oxidation of isocitrate to 2-ketoglutarate and CO_2[24,25]. This enzyme, located exclusively in the mitochondria in mammalian cells, is a key regulatory enzyme in the TCA cycle. Calcium is one of the main cofactors of the NAD-ICDH. Importantly, the presence of ADP, isocitrate, and Mg^{2+} are required for Ca^{2+} to bind and regulate NAD-ICDH activity. The stoichiometry of Ca^{2+} binding to the enzyme is ~1Ca^{2+} per tetramer (2α,β,γ)[11]. After binding to Ca^{2+} in the presence of ADP, the NAD-ICDH K_m decreased ~8-fold in mitochondrial extracts with a $K_{0.5}$ for Ca^{2+} of 5.4 μM[21,26,27]. In the same direction, it has been observed that the $K_{0.5}$ for Ca^{2+} decreased from 43 μM to 5 μM by increasing ADP/ATP ratios, in toluene-permeabilized

mitochondria[21]. The higher $K_{0.5}$ of the NAD-ICDH compared to the other intra-mitochondrial dehydrogenases (PDHP and OGDH) together with the thigh regulation of its activity depending on the ADP/ATP ratio might reflect here again how $[Ca^{2+}]_m$ mediates the fine-tuning of the oxidative metabolism on these organelles in response to the different energy demands[12].

- F_1–F_O ATP synthase.—This molecular motor couples the ATP turnover with H^+ translocation through the inner mitochondrial membrane. Therefore, the F_1–F_O ATP synthase utilizes the electrochemical proton gradient generated by the mitochondrial electron transport chain to synthesize ATP from ADP+Pi. In tissues with a high energy requirements like the myocardium, ATP synthesis has to match ATP consumption according to energy demands. It has been demonstrated by others that the regulation of the ATP production rate by the F_1–F_O ATP synthase might be independent from changes in mitochondrial membrane potential ($\Delta\psi_m$) or rate of O_2 consumption[28,29], being the enzyme the direct potential target of this regulation. The F_1- F_0 ATP synthase activity regulation by Ca^{2+} fluxes might explain the stabilizing of ATP production during workload that Ca^{2+}-dehydrogenases regulation alone cannot explain. In this concern, Ca^{2+} has emerged as a key regulator of the ATP production. It has been reported that Ca^{2+} directly binds to F_1–F_O ATP synthase, specifically to the F_1–F_O ATP synthase β subunit[30–32] with $K_{0.5}$ for Ca^{2+} in the nM range[29]. Studies on isolated cardiac myocytes from rats have investigated [ATP] and $[Ca^{2+}]$ from both mitochondria and cytosol under different workload conditions. These measurements were performed by adenoviral infection on isolate cardiomyocytes with luciferase ([ATP]) and aequorin ($[Ca^{2+}]$) specifically targeted to each cellular compartment[22]. Upon different workload conditions, no changes in the free [ATP] were observed in neither of the studied compartments. Interestingly, when cardiomyocytes were subjected to a sudden workload after a resting period, an initial drop in $[ATP]_m$ was observed before reaching equilibrium again. This drop in $[ATP]_m$ was accompanied with a simultaneous increase in $[Ca^{2+}]_m$ indicating an ATP synthesis regulation mediated by $[Ca^{2+}]_m$ variations in response to changes in myocardial workload[22].

- The mitochondrial permeability transition pore (mPTP).—Mitochondrial PTP is a non-specific channel that is known to allow the diffusion of molecules up to 1500 Da, but its molecular nature remains elusive [33–45]. The mPTP has important physiological[46,47] and pathological roles[48,49]. Transient openings of the mPTP could participate in ROS generation[46,50,51] and Ca^{2+}[52] handling. It has been postulated that the mPTP itself might also influence $[Ca^{2+}]_{m.}$ Mathematical models suggest that under high intracellular calcium cycling during periods of stress, the mitochondrial calcium efflux pathway mediated by the mNCX may be unable to prevent calcium overload, suggesting possible different mechanisms of $[Ca^{2+}]_m$ extrusion[52,53]. The observation of the mitochondrial calcium efflux inhibition in rat and mouse cardiomyocytes treated with mPTP blockers (cyclosporine A) or lacking cyclophilin D (CyP-D)[54,55] supported the possible role of mPTP on the mitochondrial calcium extrusion process. Furthermore, an elevated $[Ca^{2+}]_m$ has been found to be related with a shift in substrate utilization from fatty acid oxidation to glycolysis in the working heart, suggesting that the mPTP might constitute a control point that links mitochondrial metabolism with myocardial workload through changes in the $[Ca^{2+}]_m$[53].

According to these observations, mPTP can modulate and sense changes in $[Ca^{2+}]_m$, acting as a mediator of cell energy demand adaptation or triggering cell death.

$[Ca^{2+}]_m$ MEASUREMENT METHODS

As reported above, $[Ca^{2+}]_m$ handling is essential in cell life. $[Ca^{2+}]_m$ is not only essential for mitochondrial biogenetics and energy production but also crucial for buffering and shaping $[Ca^{2+}]_c$ transients. Additionally, $[Ca^{2+}]_m$ is directly involved in preventing or triggering cell death both by necrosis or apoptosis[56]. Therefore, it is important to accurately know the $[Ca^{2+}]_m$ dynamics to fully understand all these physiological and pathological processes. Several methods are currently available to measure $[Ca^{2+}]$, from fluorescent dyes and proteins to bioluminescent sensors and radioisotopes of Ca^{2+}. Those methods that are most frequently used to monitor mitochondrial Ca^{2+} dynamics in live cells and their pros and cons will be explained further in detail.

Fluorescent Dyes

A number of fluorescent dyes to measure intracellular $[Ca^{2+}]$ are currently available. All of them are based on the first fluorescent Ca^{2+} probe, Quin-2, developed back in the 1980s by Roger Tsien as a modification of the Ca^{2+} chelator EGTA[57]. A diverse group of Ca^{2+} fluorescent indicators has been developed since then, covering multiple combinations of spectral (single/double-wavelength or UV/visible excitation) and chemical (Ca^{2+} affinity, Kd) properties[58]. Some of these dyes are ratiometric indicators, like the Fura-2 and Indo-1 families. The Oregon Green family provides a resting signal while others like the Fluo family have no resting signal. Finally, the Rhod family are suitable to be used in the long wavelength range[59]. These dyes work with the principle of changing the fluorescence intensity according to changes in $[Ca^{2+}]$. Most of them can be easily loaded into the cytosol. The non-invasive acetoxymethyl (AM) ester loading technique is the most popular to achieve this aim[60]. The acetoxymethyl ester group lends hydrophobic properties to the fluorescent dyes, allowing them to cross the cytosolic and mitochondrial membranes. To further promote mitochondrial accumulation, a positively charged molecule is then attached to the Ca^{2+} indicators (e.g., Rhod-2). The cationic nature of these dyes results in $\Delta\psi_m$-driven uptake into the mitochondrial matrix. Once inside the matrix, mitochondrial esterases hydrolyze the AM groups, releasing the acids groups, retaining the probe in the mitochondrial matrix and conferring it the capability to respond to changes in the $[Ca^{2+}]_m$. The use of fluorescent dyes as Ca^{2+} sensors have many advantages. All of them are commercially available, and they are easy and fast to load in any type of cells, from established cell lines to primary cultures. The loading conditions must be optimized for each fluorescent dye, enabling high spatiotemporal resolution for imaging experiments. Most of them show a high Ca^{2+} affinity that goes from 0.1 μM to 1μM and a few show Ca^{2+} affinity at a low micromolar range (< 30 μM) and exceptionally at a micromolar range of > 30 μM. Despite of their versatility and easy handling, they also have strong disadvantages. Even with the AM technique, they are difficult to target and confine in the mitochondrial matrix. Incomplete AM ester hydrolysis, extracellular AM ester hydrolysis, and leakage are common problems related with these fluorescence dyes. The most concerning issue is that the dye, still localized in the cytosol, causes a strong signal contamination. Some strategies

can be used to optimize the mitochondrial compartmentalization. The cold loading-warm incubation protocol[61] can help to reduce the artifactual cytosolic signal, and so can manganese treatments[62], cobalt treatments[63], and membrane permeabilization[64]. Despite the number of fluorescent dyes, Fura2-FF AM and Rhod-2 AM are the default selections to measure $[Ca^{2+}]_m$. The Fura2-FF is a ratiometric dye excited at 340/380 nm, with an emission peak at 510 nm. Thanks to its ratiometric condition, artifacts created during the acquisition can be avoided so that more reliable Ca^{2+} measurements can be obtained. According to the manufacturer, the Kd of Fura2-FF is 5μM, however, in vivo studies determined the Kd is approximately 4μM[65]. The Rhod-2 has a very high Ca^{2+} affinity (Kd = 570 nM), but some of his family members have lower affinity like Rhod-FF Kd = 19 μM or the extreme low Ca^{2+} affinity Rhod-5N with Kd = 350 μM. Previous studies have reported that Rhod family members are toxic for the cells when loaded at higher concentrations than 2 μM and suggest that Rhod-2 is unable to respond to two or more $[Ca^{2+}]_m$ repetitive increases[66].

Genetically Encoded Calcium Indicators (GECIs)

Unlike the fluorescent dyes, GECIs are protein-like probes that can be easily targeted to different organelles by including a target sequence before the probe sequence. Another general advantage of the GECIs is that the sequence of their Ca^{2+}-bound domain can be modified to reduce or adjust the probe affinity for Ca^{2+}. This feature has allowed the generation of GECIs that cover a wide range of $[Ca^{2+}]$, from the low nM to high mM, making them suitable to perform $[Ca^{2+}]$ measurements in every cell organelle, including in the largest intracellular Ca^{2+} store, the ER/SR. The most significant disadvantage of GECIs is that they need to be expressed by the cell's own machinery, meaning that the plasmid encoding for the probe needs to be delivered within the cell. Many cells lines do not present difficulties with transfection protocols, however others require different techniques, such as infection or electroporation. After the probe internalization, at least 24 – 48 h of probe expression is required prior to the experimental procedure. The waiting time for the expression of the probe is not a problem in cell lines, nevertheless it becomes critical when working with primary cells. Some of the primary cultures, like cardiomyocytes, are difficult to maintain without major structural changes for the time frame required for the probe's expression. Alternative approaches exist to achieve expression of GECIs in primary cultures. Especially in rodent models, adenovirus or adeno-associated virus can be injected in the live animal to make the primary cell culture express the probe by the time the cell is isolated. Another disadvantage of GECIs is that, unlike with fluorescent dyes, the probe may be not present in every cell, a factor that can limit single cell imaging experiments. Two major types of GECIs have been developed, one type based on bioluminescent proteins and another type based on fluorescent proteins. Further individual characteristics and functional details will be provided in the following sections.

Bioluminescent based GECIs

The aequorin was the first organelle-targeted engineered Ca^{2+} indicator[67]. It was used for the first time during the 1960s and 1970s[68], and it is the most widely used bioluminescent Ca^{2+} probe. Aequorin emits light in a Ca^{2+}-dependent reaction where the cofactor coelenterazine is oxidized, releasing CO_2 and a single photon (470 nm) per aequorin

molecule. Using a calibration curve, the amount of light emitted can be easily transferred to [Ca^{2+}]. The native aequorin possesses 3 Ca^{2+} binding sites and a high Ca^{2+} affinity. This native version can only measure with reliability [Ca^{2+}] between 0.1 μM and 5μM. Single point mutations were introduced in the first and second Ca^{2+} binding sites, reducing its Ca^{2+} affinity by ten times (disabling only the second binding site) or by 100 times (disabling both the first and second Ca^{2+} binding sites)[69]. The three aequorin variants (wtAEQ, mutAEQ, and 2mutAEQ) in combination with different types of coelenterazine allow the measurement of [Ca^{2+}] from 0.1 μM to > 1 mM. Because of the bioluminescent nature of the aequorin, excitation illumination is not required; therefore, there are no autofluorescence or phototoxicity artefacts. However, the bioluminescent enzymatic reaction is irreversible in the experimental period, so the aequorin is "consumed". This "consumption" was a problem when the aequorin was exposed to high [Ca^{2+}], because it could lead to miscalculation of the real [Ca^{2+}] values, however this problem was solved with the low Ca^{2+} affinity version of aequorin. The main disadvantage to aequorin is that the amount of light emitted is extremely low, and the experiments should be performed most of the times in a cell population. On the other hand, working with cell populations eliminates the cell-to-cell variability. Measurements of $[Ca^{2+}]_m$ can be performed in individual cells expressing aequorin, and although the spatial resolution is very poor, the results are reliable due to its precise intracellular location[70]. The three aequorin variants mentioned above have been targeted to mitochondria, although the most frequently used is the mutAEQ in combination with the coelenterazine n. All the mitochondrial-targeted aequorins have been calibrated and report absolute $[Ca^{2+}]_m$ values. Despite the fact that aequorin is the most commonly used bioluminescent Ca^{2+} probe, other chemiluminescent Ca^{2+} sensors are worth mentioning, including obelin[71], mitrocomin, photina[72], and clytin[73]. The recent advances in genetic engineering allowed the generation of other bioluminescent Ca^{2+} indicators, such as the RLuc8 based indicator BRAC[73], and the Split RLuc8-based Nano-Lantern[74]. Further details about these bioluminescent Ca^{2+} indicators were described in a review[75].

Fluorescent-based GECIs

Fluorescent-based GECIs emerged from three critical achievements: the discovery of the green fluorescent protein (GFP) [76]; the development of color variants suitable for fostering resonance energy transfer (FRET) between two fluorescent proteins[77]; and the finding that Ca^{2+} binding to the calmodulin (CaM) fused with the M13 peptide derived from the myosin light chain kinase[78]. The first developed FRET-based GECIs were FIP-CB_{SM} and Cameleon. FIP-CB_{SM} is characterized by the localization of the M13 peptide between BGFP and FGFP. Cameleon possesses yellow and cyan fluorescent proteins (FP) fused directly to the CaM and M13 peptide[79,80]. In this type of ratiometric GECI, Ca^{2+} binding causes a conformational change in the probe that alters the fluorescence intensity of both the donor and the acceptor and therefore the FRET efficiency.

Since their initial development, GECIs have been evolved in multiple directions to improve their detection efficacy. These improvements include the development of single-fluorescence Ca^{2+} sensors (Pericams, Camgaroos, GCaMPs, Cepias, and GECOs), the implementation of circulated permuted FP to enhance FRET-based indicators, and the remodeling the CaM-M13 interface (Dcpv family) or the replacing of the CaM as a Ca^{2+} sensor for troponin C

(TN-indicators family) [81–87]. Most of the GECIs use the CaM-M13 as Ca^{2+} binding site, but two fluorescent GECIs, the GFP-aequorin (GA) and the GFP and apo-aequorin (GAP), bind the Ca^{2+} to the aequorin [88,89]. The probes here work as a BRET system, where the photons emitted by aequorin are transferred to the GFP, as happens in jellyfish. Due to the massive list of fluorescence GECIs, detailed features of most of them have already been compiled in recent reviews [75,90–92].

Fluorescent GECIs have some significant advantages over bioluminescent. The Ca^{2+} dynamics can be visualized with high resolution at the subcellular level due to their high fluorescence emission compared to the extremely low emission of photons by the bioluminescent probes. Another benefit over the bioluminescent probes is that the fluorescent reaction is reversible and does not require any cofactors. Despite recent improvements in fluorescent GECIs, the Ca^{2+} affinity in most of them is relatively high. Only a few display a K_d in the 1 μM to 10 μM range and even fewer > 10 μM. Many fluorescent GECIs from diverse families have been targeted to mitochondria and found to report reliable $[Ca^{2+}]_m$ dynamics. Nevertheless, most of them have not been calibrated to absolute $[Ca^{2+}]_m$ values.

REPORTED $[Ca^{2+}]_m$ VALUES WITH DIFFERENT MEASUREMENT METHODS

Despite the abundance of tools to measure the $[Ca^{2+}]_m$, few researchers have calibrated the probes, so records reveal only qualitative changes in the $[Ca^{2+}]_m$. So while absolute $[Ca^{2+}]_m$ values are critical for our understanding of Ca^{2+}-mediated regulatory mechanisms of mitochondrial activities, ironically, they can barely be found in the published literature. The following paragraphs present the reported $[Ca^{2+}]_m$ values found, the method used to measure $[Ca^{2+}]_m$, the cell type in which the experiments were performed, and the physiological stimulus applied to achieve the $[Ca^{2+}]_m$ rise. In most of these experiments, intact cells were used. However, some studies in permeabilized cells are also mentioned. Although there are numerous reports on absolute values of $[Ca^{2+}]_m$ in isolated mitochondria, these results are not discussed in the present review.

In 2001, Collings et al.[93] performed experiments in intact HeLa cells using 1μ M Rhod-2 as a probe. Their work reported a maximum $[Ca^{2+}]_m$ of 3.5 μM upon histamine (100 μM) stimulation. The maximum $[Ca^{2+}]_m$ reached was also measured upon ER Ca^{2+} leak (using the addition of thapsigargin to inhibit the SERCA pump) and during the capacitative Ca^{2+} entry. The values obtained were 0.4 μM and 1μM, respectively. These authors showed that even with prolonged stimulation (20 min) with histamine, $[Ca^{2+}]_m$ barely reached 3μM. It is worth mentioning that the mitochondrial Rhod-2 fluorescent did not decrease significantly after 2 min following the maximum mitochondrial Ca^{2+} peak. The Rhod-2 signal was reduced to resting levels 25 min after the mitochondrial peak, pointing to the possibility that Rhod-2 cannot follow the mitochondrial Ca^{2+} dynamics properly. A mitochondrial calibration curve is also reported in the manuscript, showing a Kd of 1.3 μM for the Rhod-2. To obtain the minimum Rhod-2 fluorescence, 4 mM of EGTA was applied and a 10 μM Ca^{2+} pulse was used to reach the maximum Rhod-2 fluorescence.

In 2002, Pitter et al.[94] also used Rhod-2 (2 μM) to measure $[Ca^{2+}]_m$ in permeabilized glomerulosa cells and in an INS-1/EK-3 cell line. The authors increased the $[Ca^{2+}]_c$ progressively from 60 nM to 740 nM to determine mitochondrial Ca^{2+} uptake. The calibrated $[Ca^{2+}]_m$ values were always similar to or even smaller than the $[Ca^{2+}]_c$ added, despite a large electrochemical gradient favoring mitochondrial Ca^{2+} accumulation. This work used the calibration equation $[Ca^{2+}]$ = Kd (F-Fmin)/(Fmax-F)[58]. The basal fluorescence emission of the buffer with no Ca^{2+} added (no Ca^{2+} chelator present either) was consider as the minimum fluorescence. To obtain the maximum fluorescence, 500 μM Ca^{2+} was added to the buffer (in the presence of ionomycin and the uncoupler FCCP). A Kd value of 490 nM was reported for Rhod-2 and then used in the equation.

In 2012, De la Fuente et al.[95] used the extremely low Ca^{2+} affinity dye Rhod-5N to measure $[Ca^{2+}]_m$ in intact HeLa cells. The stimulus used was histamine (100 μM) and histamine plus the MCUC activator Kaempferol. The $[Ca^{2+}]_m$ values registered in individual cells (with histamine only) ranged from 10 to 40 μM, and the average of multiple cells was 30 μM. When the histamine was applied together with kaempferol, the average values were higher, reaching levels around 80 μM. Since the spatial resolution of the fluorescent dyes is high enough, $[Ca^{2+}]_m$ values were also quantified in subcellular mitochondrial regions. The calibrated Rhod-5N signal showed values close to 120 μM, ~34 times higher than the value obtained by Collings et al., in those mitochondria surrounding the nucleus. Instead of using the Grynkiewicz calibration equation, De la Fuente et al. created an intracellular calibration curve, relating known $[Ca^{2+}]$ with normalized Rhod-5N fluorescence intensity. The calibration was done in the presence of FCCP, and the minimum fluorescent value was taken with 0.5 mM EGTA present and the maximum fluorescence with $[Ca^{2+}]$ 10 mM. The Kd reported in vivo for Rhod-5N in the described conditions was 470 μM.

In 2009, Andrienko et al.[96] used the high Ca^{2+} affinity dye Fura-2 (10 μM) and Rhod-2 (concentration not specified) to measure $[Ca^{2+}]_m$ in permeabilized adult rat cardiomyocytes. Spontaneous SR Ca^{2+} release events occurring at regular frequency were used as mitochondrial Ca^{2+} uptake stimulus. According to the calibrated Fura-2 signals, $[Ca^{2+}]_m$ increased to 110 nM after 8 waves and to 216 nM after 20 waves. The calibrated Rhod-2 signals were inferred from the obtained Fura-2 data. Therefore, the authors concluded that the largest $[Ca^{2+}]_m$ rise during SR release was 10 nM, with the average rise in the 2 to 4 nM range. The Fura-2 calibration was done using Ca^{2+} free solution and Ca^{2+} 50 μM to 100 μM for minimum and maximum fluorescence, respectively; then the Grynkiewicz calibration equation was used (no Kd was reported).

In 2001, Csordas & Hajnoczky[65] utilized RBL-2H3 mucosal mast cells loaded with Fura-FF (5 μM) to measure mitochondrial Ca^{2+} uptake evoked by IP3-mediated ER Ca^{2+} release. The Kd obtained by a Ca^{2+} calibration kit (Calcium calibration kit2&3, molecular probes) was 4 μM, a similar value reported by others[97]. The imaging studies performed showed that in these conditions, $[Ca^{2+}]_m$ reached 10–20 μM.

In the same year, Arnaudeau *et al.*[98] used three different variants of the fluorescent GECI Camaleon to measure $[Ca^{2+}]_m$ in intact HeLa cells. The following Kd variants were reported: YC2mit 1.26 μM, YC3.1mit 3.98 μM, and YC4.1 104 μM. These Kd values were

determined by applying increasing [Ca^{2+}] from 1 nM to 10 mM (1 μM thapsigargin, 1 μM ionomycin, and 1 μM CCCP were present in the calibration buffer). The Hela cells were stimulated with histamine 50 μM, and the $[Ca^{2+}]_m$ peak as wells as the % of saturated pixels were recorded. The study showed that $[Ca_{2+}]^m$ increased as the Ca^{2+} affinity of the probe decreased. The Ca^{2+} values were 3.19 μM for YC2mit, 49.4 μM for YC3.1mit, and 106 μM for YC4.1mit. The saturated pixels were 24.8%, 17.8%, and 3.19% respectively, pointing to the fact that a low Kd affinity of the probe is critical to correctly and accurately measure $[Ca^{2+}]_m$ values.

In 2012, Lu et al.[99] performed imaging experiments in intact adult rabbit cardiomyocytes using the fluorescent GECI Mitycam (inverse Pericam). At the end of every experiment, the Mitycam was calibrated in situ with low and high Ca^{2+} (concentrations not specified) to obtain the $[Ca^{2+}]_m$ values. The results showed a gradient of Ca^{2+} within an individual mitochondrion with higher $[Ca^{2+}]_m$ in the Z-lines (next to the SR Ca^{2+} releasing sites) than in M-lines. The reported $[Ca^{2+}]_m$ were 37 nM in the Z-lines versus 26 nM in the M-lines. These values are higher but not significantly different from the ones obtained previously by the same group using Fura-2 and Rhod-2[96].

In 2017, Wust et al.[100] delivered the GECI 4mtD3cpv Camaleon (MitoCam) into adult rat cardiomyocytes by viral infection to estimate $[Ca^{2+}]_m$. Upon electrical stimulation (0.1–4 Hz), the $[Ca^{2+}]_m$ values ranged from 300 nM to 800 nM while in presence of the Na^+/Ca^{2+} exchanger inhibitor CGP-37157, the values vary from 580 nM to 1125 nM. The manuscript shows a MitoCam calibration where the Kd (470 nM) was applied to a modified Grynkiewicz equation[86].

Lastly, multiple publications in several cell types can be found where the bioluminescent GECI aequorin was used as a mitochondrial Ca^{2+} probe. The calibration of the different aequorins was performed as described by De la Fuente et al.[69]. Several Ca^{2+} concentrations were added in presence of thapsigargin, ionomycin, oligomycin and FCCP to obtain the calibration curve values. Ca^{2+} 10mM addition achieved the release of the total luminescence. The calibrated $[Ca^{2+}]_m$ values from the aequorin and coelenterazine combinations differ significantly depending on the cell type used for the measurements. As such, the reported values of $[Ca^{2+}]_m$ range from 1μM in cardiomyocytes to 600 μM in bovine chromaffin cells[101]. A list of reported $[Ca^{2+}]_m$ using aequorins, including further details on the cell type, the stimulus, and the $[Ca^{2+}]_m$ reached, can be found in Table 1.

POSSIBLE EXPLANATIONS FOR THE DISCREPANCIES IN $[Ca^{2+}]_m$ REPORTED.

Despite many new tools and efforts to measure the $[Ca^{2+}]_m$ accurately, there is still no consensus about the absolute values of $[Ca^{2+}]$ reached in the mitochondrial matrix during physiological stimulation, even in cases where the same cell types with the same stimulus were used. The discrepancies may result from a variety of factors. One of the most critical is the Kd of the probe for Ca^{2+}. If the Kd is too low for the range of $[Ca^{2+}]_m$ changes, then the dye will be saturated or close to the saturation; conversely, if the Kd is too high, then the changes may not be detected. This is because most of the probes follow a sigmoidal

saturation curve, meaning that, when the Ca^{2+} levels start to deviate from Kd significantly, changes in the fluorescence intensity would be extremely low even when changes in $[Ca^{2+}]$ are still high. This phenomenon could lead to misinterpretation of the data since a non-saturated fluorescence signal does not necessarily translate to a correctly calibrated $[Ca^{2+}]$. According to Arnaudeau et al.[98], higher $[Ca^{2+}]_m$ values occurred when a lower Ca^{2+}-affinity probe was used. Note that in this work none of the YC Camaleon signals were saturated at the $[Ca^{2+}]_m$ peak. However, the variant with the lowest Kd resulted in measurement of the highest $[Ca^{2+}]_m$. The kinetics of the probes can also lead to different detecting signals, a factor that is especially critical for a rapid Ca^{2+} change, such as in beating cardiac muscle cells. If the probes are slower in picking up the real-time Ca^{2+} changes, then the signals will be distorted. Another source of discrepancy is the probe calibration performed by the different groups. Because calibration protocols are not standardized, every group carries out their own calibrations, leading to slight variations among them. One of the primary cause of discrepancies is that the Fmax and Fmin used for the Grynkiewicz equation are not always obtained under the same conditions. For the Fmin, some groups only omit the Ca^{2+} from the medium while others add the Ca^{2+} chelator EGTA in different concentrations to avoid any Ca^{2+} contamination. For the Fmax. a similar situation is faced. While some groups apply a $[Ca^{2+}]$ believed to saturate the probe, others go further and apply $[Ca^{2+}]$ in the 10 mM range to ensure the full saturation. Another possible explanation resides in the nature of the cells by themselves. The published data suggest a quite stable rise in the $[Ca^{2+}]_c$ values (~ 1 μM) independently on the cell type, so the cytosolic Ca^{2+} is not a probable source of inconsistency. The high $[Ca^{2+}]_c$ microdomains have not been as extensively studied as the global cytosolic Ca^{2+} signal[102]. Nevertheless, it does not appear to be the source of the variability either[103].In the other hand, mitochondria from diverse cell types may uptake Ca^{2+} in a different manner. The total amount of MCUC expressed in that particular cell type, or the proximity between mitochondria and the Ca^{2+} source may affect the mitochondrial Ca^{2+} uptake capabilities substantially[104]. Here is where the expression and the specific localization of the MCUC within the cell becomes critical, as does the concept of mitochondrial ‘contactology’ with other organelles, especially with the main intracellular Ca^{2+} reservoir, the ER/SR[105].

Another controversial point that may affect the reported free $[Ca^{2+}]_m$ is the role of the mitochondrial Pi and its capacity to buffer and form Ca^{2+} phosphate precipitates[106]. It has been shown that the presence of phosphate facilitates the mitochondrial Ca^{2+} uptake leading to 10–30 times more Ca^{2+} accumulation[107]. However, this large Ca^{2+} mitochondrial load is simultaneously buffered by Pi having as a consequence mitochondrial morphological changes and Ca^{2+} precipitation[69]. While specific studies suggest that free $[Ca^{2+}]_m$ higher than 2 μM sufficiently generates calcium phosphate precipitates[107], others claim that at least 1.5 mM Ca^{2+} is required to form the same precipitates[69]. These values align with the reported data on the solubility of the product of $[HPO_4^{2-}]\bullet[Ca^{2+}]$[108]. Additionally, the same study showed that the presence of 1 mM or 3 mM phosphate precludes $[Ca^{2+}]_m$ to reach higher concentrations because of the above mentioned Ca^{2+} buffering capacity of the phosphate. A third study also showed that the presence of Pi reduced by almost 50% the $[Ca^{2+}]_m$ reached (from an original value of 50 μM in the absence of Pi)[109]. The role of the Pi as a mitochondrial Ca^{2+} buffer as well as the still under debate calcium-free/bound

ratio[107,110,111] creates an extremely complex scenario where accurate and precise measurement of $[Ca^{2+}]_m$ is critical. Lastly, we can not forget that all Ca^{2+} sensors are Ca^{2+} buffers due to their Ca^{2+} binding capability. Therefore, for the sensors with a lower quantum yield, the higher loading concentrations could alter the original Ca^{2+} signals at some degree. However, it is hard to find studies reporting, discussing or making a comparison between the buffering capacities among the Ca^{2+} sensors[112].

CONCLUSIONS AND PERSPECTIVES

Mitochondrial Ca^{2+} uptake was first described almost 40 years ago. Alternations in $[Ca^{2+}]_m$ regulate crucial processes such as enzyme activities, bioenergetics, and cell death. Despite the importance of the dynamic range of $[Ca^{2+}]_m$, the absolute values that can be reached upon stimulation are still not well known. The probes available for precisely measuring $[Ca^{2+}]$ inside the mitochondria have individual pros and cons. The ideal probe to measure $[Ca^{2+}]_m$ requires some essential characteristics. It needs to be specifically and efficiently targeted to the mitochondrial matrix to avoid cytosolic signal contaminations. It should have fast enough kinetics, to be able to track both fast Ca^{2+} uptakes as well as fast Ca^{2+} release. It should have high quantum yields to avoid buffering. It should provide enough spatial resolution at both the cellular and subcellular levels. It needs to have the appropriate Ca^{2+} affinity to not to suffer saturation upon high $[Ca^{2+}]$. Finally, it should be easy to deliver to the mitochondrial matrix, independently on the cell type. Further investigations are required to obtain this “perfect” mitochondrial Ca^{2+} sensor and use it to elucidate the $[Ca^{2+}]_m$ reached upon physiological stimulation as well as the mitochondrial Ca^{2+} dynamics in the different cell types. As mentioned, mitochondria from different cell types may uptake Ca^{2+} in a quite variable range of concentrations. It is not possible to cover the full range of $[Ca^{2+}]$ changes (from <10 nM to >1mM) with only one “ideal” sensor. The decision of which one is better depends on all factors described above and the cell type where the $[Ca^{2+}]_m$ will be measured. When its expression is feasible, we would recommend the use of fluorescent GECIs due to its specific organelle targeting. Among these, the ratiometric ones are preferred over the single-wavelength to ensure the interference of experimental artifacts. Accurate knowledge in these Ca^{2+} values will resolve current discrepancies in the amount of $[Ca^{2+}]_m$ required to control essential cellular processes, including energy production and mPTP opening, and even to prevent the formation of calcium phosphate precipitates.

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Table 1.

Reported mitochondrial calcium concentrations

PROBE	Kd (μM)	CELL TYPE	STIMULUS	$[Ca^{2+}]_m$ reported	References
Rhod-2	0.57	HeLa	Histamine	3.5 μM	[93]
			Thapsigargin	900 nM	
			CCE	1.6 μM	
		Glomerulosa cells	Extracellular Ca^{2+} 750nM	Equilibrated with Cyto	[94]
Fura-FF	5.50	RBL	Ip3	10–20 μM	[65,113]
		Rat myocytes	Electrical Stimulation	10 nM	[96]
Rhod-5N	320	HeLa	Histamine	20–80 μM	[95]
Yc2	1.26	HeLa	Histamine	3.2–10 μM	[98]
Yc3	3.98	HeLa	Histamine	49.5 μM	
Yc4	104	HeLa	Histamine	106 μM	
Mitycam	0.2	Rabbit cardiomyocytes	Electrical Stimulation	40 nM	[99]
4mtD3cpv	0.47	Rat cardiomyocytes	Electrical Stimulation	0.3–0.9 μM simulated	[100]
			Extracellular Ca^{2+}	0.3–2.4 μM simulated	
AEQ variants	260–1000	Hela	Histamine	20–150 μM	[66,69,114–116]
		Adipocytes	Bradykinin	2 μM	[117]
		TNBC Cells	ATP	80 μM	[118]
		Human Fibroblast	Histamine	45 μM	[119]
		Cromafin	K^+	200–600 μM	[101]
		Cardiomyocytes	Angiotensin	1 μM	[120]
		Pleura mesothelioma	Bradykinin	50 μM	[121]
		SH-SY5Y neuroblastoma	Bradykinin	50 μM	[116]
		N2a neuroblastoma	Bradykinin	85 μM	[122]
		Smooth muscle cells	0 Na^+	3.5 μM	[123]
			ATP	2 μM	
		STH Huntington estriatial (virus)	Extracellular Ca^{2+}	60–450 μM	[124]
			SOCE	25 μM	