Slow activation of fast mitochondrial Ca\textsuperscript{2+} uptake by cytosolic Ca\textsuperscript{2+}

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Mitochondrial Ca\textsuperscript{2+} uptake through the mitochondrial Ca\textsuperscript{2+} uniporter (MCU) is a tightly controlled process that sustains cell functions mainly by fine-tuning oxidative metabolism to cellular needs. The kinetics of Ca\textsuperscript{2+} fluxes across the mitochondrial membranes have been studied both in vitro and in vivo for many years, and the discovery of the molecular components of the MCU has further clarified that this Ca\textsuperscript{2+} uptake mechanism is based on a complex system subject to elaborate layers of controls. Alterations in the speed or capacity of the in-and-out pathways can have detrimental consequences for both the organelle and the cell, impairing cellular metabolism and ultimately causing cell death. Here, we report that pretreatment of deenergized mitochondria with low-micromolar Ca\textsuperscript{2+} concentrations for a few minutes markedly increases the speed of mitochondrial Ca\textsuperscript{2+} uptake upon re-addition of an oxidizable substrate. We found that this phenomenon is sensitive to alterations in the level of the MCU modulator proteins mitochondrial calcium uptake 1 (MICU1) and 2 (MICU2), and is accompanied by changes in the association of MICU1–MICU2 complexes with MCU. This increased Ca\textsuperscript{2+} uptake capacity, occurring under conditions mimicking those during ischemia/reperfusion in vivo, could lead to a massive amount of Ca\textsuperscript{2+} entering the mitochondrial matrix even at relatively low levels of cytosolic Ca\textsuperscript{2+}. We conclude that the phenomenon uncovered here represents a potential threat of mitochondrial Ca\textsuperscript{2+} overload to the cell.

Ca\textsuperscript{2+} is central to the life and death of every eukaryotic cell type, and mitochondria actively participate in sensing and spreading Ca\textsuperscript{2+} signals throughout the cell. Mitochondria have the ability to shape the cellular Ca\textsuperscript{2+} signaling by taking up and releasing Ca\textsuperscript{2+} ions and are conveniently positioned at sites of Ca\textsuperscript{2+} entry on the plasma membrane (1, 2) or at sites of Ca\textsuperscript{2+} release from the (endoplasmic reticulum (3, 4). They can thus take up the cation with different speeds and in different amounts, depending on their location within the cell (5).

Basal, intramitochondrial Ca\textsuperscript{2+} levels are kept low, similar to the concentration present in the cytosol in resting conditions (≤100 nM) (6, 7). Increases in cytosolic Ca\textsuperscript{2+} are mirrored in the mitochondria, where the main entry pathway is the calcium uniporter (MCU), an electrogenic channel that exploits the driving force of the steep electrochemical gradient present across the inner mitochondrial membrane (IMM). Two main efflux routes, namely the Na\textsuperscript{+}/Ca\textsuperscript{2+} and H\textsuperscript{+}/Ca\textsuperscript{2+} exchangers, control the accumulation of free Ca\textsuperscript{2+} in the mitochondrial matrix and guard against Ca\textsuperscript{2+} overload (8, 9). Ca\textsuperscript{2+}, in turn, controls mitochondrial metabolism. The increase of free Ca\textsuperscript{2+} within the matrix activates mitochondrial dehydrogenases (pyruvate dehydrogenase, isocitrate dehydrogenase, oxoglutarate dehydrogenase), and intermembrane Ca\textsuperscript{2+} concentration modulates the activity of carriers such as citrin and aralar (10); the net results are the increase of the respiratory rate, H\textsuperscript{+} extrusion, and ATP synthesis. Tightly controlled Ca\textsuperscript{2+} fluxes enable mitochondria to fine-tune energy production to the necessities of the cell. Unregulated Ca\textsuperscript{2+} handling, on the other hand, can cause alterations in mitochondrial morphology and major organelle dysfunctions that can have dire consequences for cells and the whole organism (11–13). Mitochondrial Ca\textsuperscript{2+} uptake was studied for more than half a century (14, 15) before the molecular nature of this process was unraveled; its properties have been investigated thoroughly, and its characteristic features, such as high capacity, selectivity (16), and cooperativity (17), have been clarified. The major components of the uniporter have been recently identified, including the pore-forming subunit (MCU) (19, 20), a dominant-negative form (MCUb) (21), a regulatory subunit (EMRE) (22, 23), and Ca\textsuperscript{2+}-sensitive regulators (MICU1 (24–27) and MICU2 (28–30)). MICU1 and MICU2 complexes (homo- or heterodimers) have been suggested to regulate Ca\textsuperscript{2+} uptake, determining both the threshold and cooperative activation of MCU (28, 29, 31), although the specific role of each component is still debated; for recent reviews, see also De Stefani et al. (32), Pendin et al. (33), and De Stefani et al. (34).

It is well-established that the kinetics of Ca\textsuperscript{2+} transport are governed principally by the inner membrane potential difer-

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This article contains Fig. S1.

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3 The abbreviations used are: MCU, mitochondrial calcium uniporter; MICU1, mitochondrial calcium uptake 1; MICU2, mitochondrial calcium uptake 2; CAMCU, Ca\textsuperscript{2+}-dependent activation of mitochondrial Ca\textsuperscript{2+} uptake; bis-tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; DDM, n-dodecyl β-D-maltopyranoside; IMM, inner mitochondrial membrane; MAPK, mitogen-activated protein kinase; KO, knockout; TMDP, N,N,N’,N’-tetramethyl-p-phenylenediamine; TPEN, N,N,N’,N’-tetrakis(2-pyridylmethyl)ethane-1,2-diamine.

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ence (limited by the speed of the respiratory chain complexes) (18) and by the extramitochondrial Ca\(^{2+}\) concentration. An unusual phenomenon was described in the late 1980s: a slow (complete in several minutes) activation of the uniporter with the transformation of the classical cooperative kinetics into an almost hyperbolic one, even at low-micromolar [Ca\(^{2+}\)] \((17, 35)\). This potent activation occurs after exposure of isolated, de-energized mitochondria to micromolar Ca\(^{2+}\) levels for a few minutes \((36)\). This process, however, has not been further investigated, and its mechanism and role have not been clarified.

Based on the new knowledge of the Ca\(^{2+}\)-uniporter complex components, we re-examined the accelerated Ca\(^{2+}\) uptake mode of the uniporter to investigate whether and, if so, which of the components might be responsible for this effect. The ultimate aim is to unravel strategies for modulating the uniporter activity that may have potential therapeutic applications.

**Results**

**Ca\(^{2+}\)-dependent activation of mitochondrial Ca\(^{2+}\) uptake (CAMCU)**

We employed a standard Ca\(^{2+}\) uptake protocol to monitor the effect on the rate of matrix Ca\(^{2+}\) accumulation (substrate oxidation-driven) when nonrespiring mitochondria have stood prolonged preincubation time with fixed extramitochondrial [Ca\(^{2+}\)]. Briefly, isolated mouse liver mitochondria were incubated in the absence of substrates and supplemented with rotenone (to block the creation of membrane potential via the oxidation of endogenous substrates) and with oligomycin (to prevent the build up of the mitochondrial membrane potential). The mitochondrial Ca\(^{2+}\) uptake was clearly demonstrated by Calcium Green-5N \((\text{CAMCU})\) \((\text{CAMCU})\) (Fig. 1A). Where indicated, 8 \(\mu M\) CaCl\(_2\) (3 \(\mu M\) free Ca\(^{2+}\)) was added together with succinate to activate the electron flow in the respiratory chain. Alternatively, succinate was added 1, 2, 5, or 10 min after the addition of CaCl\(_2\). The inset in Fig. 1A shows representative traces of mitochondrial Ca\(^{2+}\) uptake following the different incubation times. The rate of Ca\(^{2+}\) decrease in the medium (dependent on the accumulation of the cation by mitochondria) was clearly slower when Ca\(^{2+}\) and succinate were added together (time 0) compared with mitochondria exposed to 3 \(\mu M\) Ca\(^{2+}\) for increasing periods of time before the active uptake took place. Ca\(^{2+}\) uptake speed was measured after the addition of the substrate by calculating the slope of the trace during its initial linear phase. The rate of Ca\(^{2+}\) accumulation appears to reach a maximum at 5 min of preincubation, and it does not significantly increase further after 10 min of incubation. Fig. 1B shows the measurements (plus calculation of mean and S.D.) of Ca\(^{2+}\) uptake rates at different incubation times, whereas Fig. 1C shows the values normalized to the Ca\(^{2+}\) uptake rate measured after 10 min of preincubation. On average, we observed about a 3-fold increase in the rate of mitochondrial Ca\(^{2+}\) uptake between 0 and 5 min of incubation.

**[Ca\(^{2+}\)] dependence and reversibility of CAMCU**

The mitochondrial Ca\(^{2+}\) uptake rate increases with the increase of the external Ca\(^{2+}\) concentration; we analyzed the dependence of the activation of mitochondrial Ca\(^{2+}\) uptake on the concentration of external Ca\(^{2+}\) during a fixed preincubation period. Fig. 2A shows that, as expected, the initial rate of Ca\(^{2+}\) accumulation increased as Ca\(^{2+}\) concentration rose, but the acceleration due to the preincubation period was observed up to 7 \(\mu M\) free Ca\(^{2+}\). Above this value, the rate of mitochondrial Ca\(^{2+}\) uptake was no longer influenced by the preincubation period. As an example, the inset table presents the values of Ca\(^{2+}\) uptake speed obtained with 7 and 10 \(\mu M\) external free Ca\(^{2+}\); at 10 \(\mu M\), the difference between the 0- and 2-min incubation times is no longer significant. These results obtained with isolated mitochondria confirm and extend the observation made by H. Krömer \((17, 35)\) demonstrating that a process requiring several minutes (up to 5 min to reach the maximum) is activated if nonrespiring mitochondria are first exposed to low-micromolar Ca\(^{2+}\), and afterward, active Ca\(^{2+}\) uptake is triggered with the addition of an oxidizable substrate. Henceforth, this phenomenon \(\text{(i.e. the Ca}^{2+}\)-dependent activation of mitochondrial Ca\(^{2+}\) uptake) will be referred to as CAMCU.

CAMCU is not substrate-specific, because it can be observed with succinate (substrate feeding reducing equivalents at the level of complex II of the respiratory chain) or with ascorbate plus TMPD (artificial substrate that feeds electrons at the level of complex IV) as the energy source (results not shown). It is more difficult to observe CAMCU in isolated mitochondria utilizing substrates for complex I, because the presence of endogenous substrates carried over during mitochondria extraction prevents the possibility of controlling Ca\(^{2+}\) uptake, unless mitochondria are kept for several hours on ice until endogenous substrates are spontaneously consumed. Moreover, CAMCU does not depend on the tissue from which mitochondria are isolated, as the phenomenon was observed in mitochondria from mouse liver and heart (not shown).

The question then arises whether CAMCU is reversible or irreversible. To address this issue, nonrespiring mitochondria were first allowed 5 min in the presence of 3 \(\mu M\) free Ca\(^{2+}\), followed by the addition of succinate (Fig. 2B). When the steady state was reached, 4 \(\mu M\) EGTA was added, causing the almost instantaneous drop of free Ca\(^{2+}\) in the medium to \(-0.3 \mu M\). The addition of 7 \(\mu M\) CaCl\(_2\) about 40 s after EGTA (to reach 3 \(\mu M\) free Ca\(^{2+}\)) resulted in a rate of Ca\(^{2+}\) uptake that was similar to that of cells not preincubated with 3 \(\mu M\) free Ca\(^{2+}\) (Fig. 2B, representative trace \((\text{left})\) and quantification \((\text{right})\)).

**CAMCU in permeabilized cells**

CAMCU is a phenomenon observable not only with isolated mitochondria. It can also be detected in permeabilized cells. We used mainly the human immortalized cell line HeLa; as proof of principle, some experiments were also repeated with SHSY5Y cells, yielding the same results (not shown). Fig. 3A shows representative traces from cells in culture. The cells were transfected with aequorin targeted to the mitochondrial matrix. Accordingly, in these experiments, we measured the rate of intramitochondrial Ca\(^{2+}\) increase and not that of the decrease of medium [Ca\(^{2+}\)]. Intact cells were first perfused with a high-[KCl] medium, with no added Ca\(^{2+}\) and in the presence of 50 \(\mu M\) EGTA; 50 \(\mu M\) digitonin was added for 1 min, which caused the permeabilization of the plasma membrane while leaving the mitochondrial membranes intact. After permeabilization, the
Figure 1. Prolonged incubation time of isolated mitochondria with 3 μM free Ca\textsuperscript{2+} increases mitochondrial Ca\textsuperscript{2+} uptake speed. A, representative trace of mitochondrial Ca\textsuperscript{2+} uptake. Where indicated (m), 0.5 mg/ml mouse liver mitochondria were added to the incubation medium supplemented with 1 μM rotenone, 1 μg/ml oligomycin, 5 μM EGTA, 1 μM Calcium Green-5N. After 2 min of incubation, substrate, together with 3 μM free Ca\textsuperscript{2+} (s/Ca\textsuperscript{2+}), was added to start active mitochondrial Ca\textsuperscript{2+} uptake. The inset shows three representative traces (a–c) of active Ca\textsuperscript{2+} uptake following increasing incubation times with 3 μM free Ca\textsuperscript{2+}: 0, 2, and 5 min, respectively. B, values and quantification (mean ± S.D. (error bars)) of mitochondrial Ca\textsuperscript{2+} uptake speed following increasing incubation time with 3 μM free Ca\textsuperscript{2+}. C, mitochondrial Ca\textsuperscript{2+} uptake speed normalized to the values measured after 10 min of incubation with 3 μM free Ca\textsuperscript{2+}. The results shown are the mean ± S.D. of at least three independent preparations of mitochondria and multiple probing for each time point. Analysis of significance was done with Kruskal–Wallis and Dunn’s multiple comparison test. Box size represents 25th and 75th percentiles of data; the small square inside of the box represents mean of data, whereas the horizontal bar represents median data. Whiskers, S.D. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
cells were perfused with the KCl medium without added Ca²⁺ (in the presence of oligomycin and rotenone, again to prevent the creation of membrane potential via the oxidation of endogenous substrates or hydrolysis of ATP), and after 2 min, 3 μM CaCl₂ together with succinate were added (trace a; 0 min). As in the experiments with isolated mitochondria, presented in Fig. 1, the addition of succinate was delayed by 1 min (trace b) or 5 min (trace c) after that of 3 μM free CaCl₂ (Ca²⁺ and substrate additions not represented in the figure). The acceleration in the maximal rate of Ca²⁺ accumulation produced by prolonging the preincubation time with 3 μM free CaCl₂ was even more evident in permeabilized cells than with isolated mitochondria. Indeed, the increase in the rate of mitochondrial [Ca²⁺] in some experiments was, after 5 min of preincubation, as large as 20-fold and on average 15-fold. As in the case of isolated mitochondria, CAMCU was not dependent on the type of substrate oxidized, as it was observed also with glutamate malate (in the absence of rotenone; results not shown). The inset in Fig. 3A shows a quantification of multiple measurements performed.

The use of cell lines offers the opportunity to genetically manipulate the mitochondrial Ca²⁺ uptake machinery and, thus, to determine whether and, if so, which of the known components of the MCU complex is involved in CAMCU. In Fig. 3B, the cells were transfected with MCU itself or with two of the known uniporter’s partners: MICU1 and MICU2. On average, the amount of each overexpressed protein was about 4-fold that of the native one. The rate of Ca²⁺ accumulation when 3 μM CaCl₂ was added to the medium together with succinate was slightly faster in cells overexpressing MCU compared with mock-transfected controls, but the time required to reach the maximal rate of uptake and the percentage increase at 5-min incubation were not significantly different from those observed in controls. On the contrary, in cells overexpressing MICU1, the rate of Ca²⁺ accumulation at time 0 (3 μM CaCl₂ added together with succinate) was notably accelerated compared with controls, and the time necessary to reach the maximal rate of uptake was significantly shorter than in controls; in fact, after 1 min of incubation, the Ca²⁺ uptake rate
Figure 3. Incubation with 3 μM free Ca^{2+} increases calcium uptake speed in permeabilized HeLa cells. 

A (left), representative traces of permeabilized HeLa cells perfused for various times with 3 μM free Ca^{2+}: 0 min (a), 1 min (b), and 5 min (c). Ca^{2+} or substrate addition is not shown. Inset, quantification of the Ca^{2+} uptake rate (box–whiskers representation as described before); n = 39 for each time point. The fast decrease in matrix Ca^{2+} in trace c could reflect the opening of the permeability transition pore as a consequence of matrix Ca^{2+} overload. 

B, quantification of the Ca^{2+} uptake rate in permeabilized HeLa cells overexpressing MCU, MICU1, or MICU2, normalized to their respective controls at 5-min incubation time. Values are presented as mean ± S.D. (error bars) of at least three independent experiments and multiple probing for each time point. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; n = 12 for each time point (Mann–Whitney test). 

C, Western blots of protein extracts of control cells and cells overexpressing the indicated proteins and respective loading controls.
had reached almost its maximal value. The rate of Ca\(^{2+}\) increase in MICU1-overexpressing cells was in fact increased also after 5 min of preincubation, compared with controls, and the accelerated rate was evident also when the values were normalized to the maximal uptake rate. Finally, overexpression of MICU2 reduced the rate of Ca\(^{2+}\) accumulation at all of the preincubation times examined, and the kinetics required to reach the maximal rate, when normalized, were slightly slower than in controls. It is noteworthy that, in these and all of the following experiments, we verified that the maximal Ca\(^{2+}\) influx rate elicited by CAMCU activation at the extracellular \([Ca^{2+}]_o\) of 3 \(\mu\)M was lower than the \(V_{max}\) obtainable at higher \([Ca^{2+}]_o\) (e.g. at 20 \(\mu\)M), meaning that in our experimental conditions, it was not limited by the rate of oxygen consumption.

**Effects of mutated MICU1 on CAMCU**

Given that the overexpression of MICU1 resulted in a significant alteration of the process of Ca\(^{2+}\)-dependent activation, we next tested (Fig. 4) the effect of the expression of two mutants of the protein: MICU1\(^{C465A}\) (where the mutation in cysteine 465 impairs the dimerization of MICU1) and MICU1\(^{D233A,E244K,D423A,E434K}\) (abbreviated MICU1\(^{EFmut}\); where the mutations abolish the Ca\(^{2+}\)-binding properties of the EF-hand domains within the protein). In cells expressing MICU1\(^{C465A}\), the extent of activation was similar to that observed for the cells overexpressing MICU1, whereas the kinetics were notably downsized, because maximum activation could be observed at 5 min of incubation and not at shorter incubation time. In cells expressing MICU1\(^{EFmut}\), on the contrary, the presence of the mutant abolished the stimulatory effect observed with WT MICU1, and the mutated protein seems to behave as a dominant negative isoform that quashes the effect of the native WT protein.

**Contribution of the different uniporter components to CAMCU**

To better understand the effect that each of the uniporter complex components has on CAMCU, in the next series of experiments, each one of them was independently down-regulated by siRNA. As expected, reducing the level of MCU by about 51 ± 5% (average of at least 5 measurements) reduced the rate of Ca\(^{2+}\) accumulation at all preincubation times, but no significant difference in the kinetics and percentage of activation was observed between controls and MCU-down-regulated cells (Fig. 5A). Down-regulation of either MICU1 (Fig. 5B) or
MICU2 (Fig. 5C) (on average 65 ± 8% and 73 ± 10% of total, respectively) had a series of complex and partially unexpected effects. In particular, (i) the maximal rates of Ca^{2+} uptake, observed after a 5-min incubation with 3 μM Ca^{2+}, were very similar to those of controls (treated with the universal negative siRNA sequence); (ii) on the contrary, down-regulation of either MICU1 or MICU2 increased the rates by about 2–3 fold, both at time 0 and at 1 min; and (iii) the time dependence of
Time-dependent Ca\(^{2+}\) modulation of mitochondrial Ca\(^{2+}\) uptake

Figure 6. Effects of kaempferol and SB202190 on mitochondrial Ca\(^{2+}\) uptake rate. A. mitochondrial Ca\(^{2+}\) uptake speed in permeabilized HeLa cells normalized to control at time 0. Left, controls; right, overexpressing MICU1, perfused with 3 \(\mu\)M Ca\(^{2+}\), without (open symbols) or with 10 \(\mu\)M kaempferol (closed symbols) after different preincubation times with 3 \(\mu\)M Ca\(^{2+}\) alone. B. Ca\(^{2+}\) uptake rate in permeabilized HeLa cells normalized to untreated samples (controls) (open symbols) or to overexpressing MICU1 (gray symbols), exposed to 40 \(\mu\)M kaempferol or 10 \(\mu\)M SB202190 and 3 \(\mu\)M Ca\(^{2+}\). Quantification is presented as mean ± S.D. (error bars); *, \(p = 0.05\); **, \(p = 0.01\); ***, \(p < 0.001\) (Mann–Whitney test).

CAMCU activation was much faster in MICU-down-regulated cells compared with controls. This effect was particularly striking in cells where MICU1 was down-regulated; in fact, after 1 min of preincubation, the rate of Ca\(^{2+}\) uptake was about 75% of the rate achieved at 5 min. By comparing the results shown in Figs. 3 and 5, it is clear that the down-regulation of MICU1 and -2 and the overexpression of MICU1 qualitatively have the same effect both on the rate of Ca\(^{2+}\) accumulation at time 0 and on the speed of CAMCU activation. These paradoxical results (see “Discussion”) might derive from the fact that down-regulation of MICU1 drastically reduces also the expression of its partner MICU2 (28, 29). As shown in Fig. S1, when MICU1 is down-regulated, as in HeLa cells via siRNA, or completely absent, as in HEK-293T KO cells (22), not only is the high-molecular weight MICU1–MICU2 complex markedly diminished or absent, but also, the MICU2 monomer is drastically reduced.

Pharmacological activation of Ca\(^{2+}\) uptake

A few years ago, Montero et al. (37) showed that a series of MAPK inhibitors (among which the most potent were kaempferol and SB202190) are able to strongly activate the rate of mitochondrial Ca\(^{2+}\) uptake in both intact and permeabilized cells. They concluded, however, that this effect was not dependent on the inhibition of MAPKs, but it was rather a side effect of the drugs. They also showed that these drugs increased the apparent affinity for Ca\(^{2+}\) of the Ca\(^{2+}\) uptake mechanism of mitochondria. To investigate whether these MAPK inhibitors and the Ca\(^{2+}\) preincubation protocol affected the same target, in the experiment presented in Fig. 6A (and B), permeabilized cells were perfused with 10 or 40 \(\mu\)M kaempferol (a dietary flavonoid and phyto-estrogen) or, alternatively, with 10 \(\mu\)M SB202190, either together with the 3 \(\mu\)M Ca\(^{2+}\) buffer or after different preincubation time periods with Ca\(^{2+}\). The rate of Ca\(^{2+}\) uptake at time 0 was increased by about 4-fold by pre-treatment with the drug without measurable delay, whereas after 1 or 5 min of Ca\(^{2+}\) preincubation, the effect of the drug was almost negligible. The observed acceleration was more pronounced in control cells, as compared with cells overexpressing MICU1, suggesting that kaempferol, MICU1, and the preincubation period affect the same target(s). As shown in Fig. 6B, in control cells, SB202190 (without preincubation with Ca\(^{2+}\)) was slightly more potent than kaempferol, but the difference observed is not statistically significant. To better understand whether kaempferol interacts with either MICU in promoting its effect on the activation of the Ca\(^{2+}\) uptake rate, we took advantage of the availability of MICU1 KO HEK-293T (22) cells. In this case, no MICU1 was expressed, and a possible confounding effect of protein residual expression (as in the case of siRNA treatment) was abolished. It is noteworthy that, in MICU1 KO cells, a substantial reduction in MICU2 was observed, as in the case of HeLa cells treated with MICU1 siRNA. The rate of Ca\(^{2+}\) accumulation in digitonin-permeabilized HEK cells was extremely variable; for this reason, the next experiments were carried out in intact cells. As in the previous experiments, the cells were transfected with mitochondrially targeted aequorin, and mitochondrial Ca\(^{2+}\) uptake was elicited by treatment with 500 \(\mu\)M charbacol in the presence or absence of 50 \(\mu\)M kaempferol. The results in Fig. 7A show that in the total absence of MICU1, the Ca\(^{2+}\) uptake rate produced by kaempferol treatment was clearly reduced from a 2.5 ± 1.5-fold increase (control HEK-293T cells) to a 1.4 ± 0.3-fold increase (MICU1 KO HEK-293T cells). Given that we did not have MICU2 KO available, in the experiments presented in Fig. 7B, HeLa cells treated with MICU2 siRNA were used. The mitochondria Ca\(^{2+}\) uptake rate elicited by 10 \(\mu\)M charbacol in the presence of 50 \(\mu\)M kaempferol in this case was stimulated by a 2.6 ± 1.3-fold increase in controls compared with a 1.9 ± 0.4-fold increase in siRNA MICU2 cells.

Ca\(^{2+}\) affects the composition of the mitochondrial Ca\(^{2+}\) uptake machinery

Given the sensitivity of CAMCU to changes in MCU complex composition, we next investigated whether Ca\(^{2+}\) preincubation affects the protein composition of the complex itself. To this end, we performed protein extraction from isolated mouse liver mitochondria or HeLa cells overexpressing MCU, MICU1, and MICU2, using mild detergent conditions, which should preserve protein interactions, in the presence of 500 \(\mu\)M EGTA, 3 \(\mu\)M free Ca\(^{2+}\), or 50 \(\mu\)M kaempferol, respectively. In the experiment presented in Fig. 8A, HeLa cells overexpressing MCU-Myc (or MCU-FLAG as control), MICU1-HA, and MICU2-FLAG were solubilized using mild detergent conditions, and the extracts were immunoprecipitated using magnetic beads.
conjugated to antibodies against Myc. The samples were eluted from the beads with nonreducing sample buffer and subjected to 10% SDS-PAGE. In these experimental conditions, a MICU1–MICU2 protein complex of about 100 kDa co-immunoprecipitated with MICU. Elution conditions and SDS-PAGE were the same as with HeLa cell extracts. Also, in this case, we observed about a 30% decrease in the amount of a 100 kDa band, co-immunoprecipitated with MICU when the incubation was performed in the presence of 3 μM free Ca²⁺.

To determine whether uniporter high-molecular weight complexes were influenced by the presence or absence of Ca²⁺, we performed blue native experiments. Isolated mouse liver mitochondria were incubated in the presence of about 3 μM free Ca²⁺ or 50 μM EGTA, solubilized with 6% digitonin, and subsequently centrifuged at 100,000 × g for 30 min. The supernatants were loaded onto 3–12% bis-tris native gel and separated by electrophoresis. Part of the gel was stained with Coomassie Blue, and the rest was transferred onto a polyvinylidene difluoride membrane, which was probed with anti-MCU antibody and subsequently with antibody against complex II for protein-loading comparison. As shown in Fig. 8C, we observed an MCU-positive, high-molecular weight complex at about 480 kDa, and the amount of the complex extracted in the presence of Ca²⁺ was about 55 ± 18% of the complex extracted in the presence of EGTA (mean of 4 independent extractions).

Discussion

Ca²⁺ transport across the inner mitochondrial membrane is a tightly regulated phenomenon supervised by at least two independent pathways: one for Ca²⁺ uptake, represented mainly by the Ca²⁺ uniporter, and one for the efflux carried out mostly by the sodium calcium exchanger.

The existence of CAMCU was described over 30 years ago by H. Kröner (17, 35) when he defined a condition able to modify the kinetics of mitochondrial Ca²⁺ uptake at low physiological Ca²⁺ concentrations, by means of an apparent increased Ca²⁺ affinity. The remarkable features of CAMCU are its time course (a few minutes to be completed) and its efficacy: more than 10-fold increase (in permeabilized cells) of the rate of Ca²⁺ uptake for extramitochondrial Ca²⁺ levels in the low-micromolar range. To the best of our knowledge, no other pathophysiological event or genetic treatment can modify in such a dramatic way the speed of mitochondrial Ca²⁺ accumulation, and the slow time necessary for completion of its effect strongly argues against a classical conformational modification of some protein involved in the MCU complex. In fact, protein conformational changes usually occur in a time scale of micro- or milliseconds and not of minutes. Accordingly, the molecular mechanism of CAMCU most likely involves some more complex phenomena, such as protein–protein interactions or enzyme-dependent covalent modifications. As to enzymatic activity, the present data exclude the possibility of a kinase as the trigger, given that the phenomenon occurs in the total absence of added ATP and in the presence of oligomycin, an inhibitor of the mitochondrial ATPase. In addition, CAMCU is reversible upon removal of extramitochondrial Ca²⁺ in dozens of seconds, again in the absence of ATP, thus excluding the possibility of rephosphorylation as the mechanism for CAMCU inhibition under these conditions. The effect of extramitochondrial Ca²⁺ (and thus of its concentration in the intermembrane space) appears, on the contrary, consistent with an effect on the uniporter, and one for the efflux carried out mostly by the sodium calcium exchanger.

Figure 7. Kaempferol treatment of intact cells. A, mitochondrial Ca²⁺ uptake speed normalized to untreated samples in intact control HEK-293T and MICU1-KO cells treated (closed symbols) or not treated (open symbols) with 50 μM kaempferol; Ca²⁺ uptake was induced with 500 μM charbacol. B, mitochondrial Ca²⁺ uptake speed normalized to untreated samples, in control and MICU2 siRNA HeLa cells, untreated (open symbols) or treated (closed symbols) with 50 μM kaempferol; Ca²⁺ uptake induced with 10 μM histamine. Data are shown plus mean quantification and S.D.; Mann–Whitney test; *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; ****, p < 0.0001.
Time-dependent $\text{Ca}^{2+}$ modulation of mitochondrial $\text{Ca}^{2+}$ uptake

A

| Input | Co-IP MCU-Myc |
|-------|---------------|
| control | EGTA | $\text{Ca}^{2+}$ | kaemp | control | EGTA | $\text{Ca}^{2+}$ | kaemp |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |

$k$Da 250

IB MICU 1

IB MCU

complex

B

| Input | Co-IP MCU |
|-------|-----------|
| EGTA | $\text{Ca}^{2+}$ | control |
| 1 | 2 | 3 | 4 | 5 | 6 |

$k$Da 100

IB-MICU1

complex

MICU1

IB MCU

C

| EGTA | $\text{Ca}^{2+}$ |
|------|-----------------|
| 1 | 2 | 3 | 4 | 5 | 6 |

$k$Da 1236

IB MCU

IB complex II

Blue Coomassie

normalized band intensity

MCU

MICU1 100 kDa complex

normalized band intensity

MCU

MICU1 100 kDa complex
Time-dependent Ca\textsuperscript{2+} modulation of mitochondrial Ca\textsuperscript{2+} uptake

Reduction in MICU2 levels is expected and consistent with the inhibitory action of this protein on the MCU complex activity (29) and with the effect of its overexpression (Fig. 3). Less obvious is why MICU1 down-regulation results in an acceleration of CAMCU. We propose that the speed of CAMCU activation depends on the MICU1/MICU2 ratio. This explanation is consistent with the effect of MICU1 overexpression, as observed in the experiments presented in Fig. 3, and also with the paradoxical result that MICU1 down-regulation has the same effect on CAMCU as MICU1 overexpression. It has been observed, in fact, that reduction in MICU1 levels by a specific siRNA causes an even more dramatic decrease in MICU2 protein levels, without any effect on MICU2 mRNA. It has been proposed that a reduced level of MICU1 impairs the stability of MICU2 and thus causes a drastic reduction of the expression also of the latter protein (28, 29). Thus, paradoxically, the treatment with a MICU1-specific siRNA results in a reduction in the levels of both proteins but could produce a net increase in the MICU1/MICU2 ratio and thus an acceleration of the CAMCU process.

Clearly, given the slow development of the process, CAMCU cannot be due solely to the conformational change of individual proteins, but rather to a more complex mechanism. Recently, Petrungaro et al. (31), while studying the effect of the oxidoreductase MIA-40 on the formation of MICU1–MICU2 dimers, demonstrated that these latter two proteins are capable of forming a complex with MCU in a Ca\textsuperscript{2+}-dependent way. In particular, they showed that the MICU1–MICU2 complex co-precipitates with MCU at low Ca\textsuperscript{2+} levels, whereas it dissociates at high Ca\textsuperscript{2+} concentrations. Multiple experimental data suggest that MICU1 and/or MICU2 act as gatekeepers of MCU and that they control the affinity of the channel for Ca\textsuperscript{2+}. This process occurs rapidly, as activation of mitochondrial Ca\textsuperscript{2+} influx follows by a few milliseconds the increase in cytosolic Ca\textsuperscript{2+}. CAMCU, on the contrary, depends on the medium (cytosolic) Ca\textsuperscript{2+} levels, but it takes minutes to reach completion. It is therefore compatible with a slow dissociation of the complex from MCU, as described (31). Our data confirm and extend the observations of Petrungaro et al. (31), as we observed a reduction in the association of MICU1-2 with MCU in the presence of 3 \(\mu\)M Ca\textsuperscript{2+} and a reduction in the high-molecular weight complexes in the presence of Ca\textsuperscript{2+}. In other words, the simplest explanation regarding the molecular mechanism of CAMCU is that the presence of micromolar levels of Ca\textsuperscript{2+} for a few minutes in the intermembrane space results in the binding of Ca\textsuperscript{2+} to the EF-hand domains of the MICUs that causes a conformational change of the two proteins. This, in turn, might cause a slow dissociation of the proteins from the MCU, with a consequent increase in the affinity of the channel for Ca\textsuperscript{2+}. The effect of drugs such as kaempferol and SB202190 (previously shown to increase the apparent affinity for Ca\textsuperscript{2+} of the uniporter (i.e. similar to the effect observed in CAMCU)) is particularly interesting. Indeed, these drugs and CAMCU appear not to be additive, as the effect of kaempferol is strong at time 0, it is reduced after 2 min of incubation with Ca\textsuperscript{2+}, and it is null at 5 min when CAMCU has reached its maximum. The stimulatory effect of kaempferol on Ca\textsuperscript{2+} uptake rate is markedly reduced in MICU1-KO cells or, although less effectively, when MICU2 is down-regulated. Both drugs increase the apparent Ca\textsuperscript{2+} affinity of the Ca\textsuperscript{2+} uptake system, and in cells incubated with kaempferol (in the absence of Ca\textsuperscript{2+}), we observe a reduction of the MICU1-MICU2 complex associated with MCU. In other words, kaempferol, and possibly SB202190, mimic both functionally and at the molecular level the effects of CAMCU. It remains unclear whether the target(s) of these drugs is MICU1 and/or MICU2 or MCU itself. The latter possibility appears unlikely, given the strong inhibition by MICUs down-regulation on kaempferol activation of Ca\textsuperscript{2+} uptake rate.

The key and final question concerns the physiological role of CAMCU. It is easy to speculate that CAMCU will be maximally activated by ischemic conditions, where nonrespiring mitochondria will be exposed for prolonged periods (minutes to hours) to high Ca\textsuperscript{2+}. Upon reperfusion, and thus reactivation of the respiratory chain activity and repolarization of membrane potential, CAMCU should be maximally activated and may thus contribute to the mitochondrial Ca\textsuperscript{2+} overload characteristic of ischemia–reperfusion in different tissues, the heart in particular. Prolonged, smaller increases in cytosolic Ca\textsuperscript{2+} are known to occur in several pathophysiological conditions, and CAMCU can therefore be activated under those conditions and contribute to increasing the efficacy of mitochondrial Ca\textsuperscript{2+} uptake. Clearly, CAMCU is a double-faced tool. For relatively small increases in [Ca\textsuperscript{2+}], it can be beneficial, priming the mitochondria Ca\textsuperscript{2+} uptake machinery and thus making it more efficient. On the other hand, however, it can be disastrous; it can increase the probability of mitochondrial Ca\textsuperscript{2+} overload, leading to cell death.

**Experimental procedures**

Procedures involving the use of animals were carried out in strict adherence to the Italian regulations on animal protection and care and with the explicit approval of the Italian animal welfare regulations: authorization number 287/2015–PR from
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the ethical committee (OPBA) of the Padua University and Italian Ministry of Health.

Isolated mitochondria calcium uptake

Mitochondria were isolated from the liver of C57Bl/6 mice by standard differential centrifugation (38). Protein concentration was measured using the bicinchoninic acid assay.

The Ca\textsuperscript{2+} uptake measurements were carried out at 37 °C, and the incubation medium contained 120 mM KCl, 10 mM MOPS, 1 mM P\textsubscript{i}-Tris, 5 μM EGTA, 1 μM Calcium Green-5N (Molecular Probes\textsuperscript{TM}), 5 mM succinate, 1 μM rotenone or, alternatively, 400 μM TMPD, 10 mM ascorbate, 20 mM antimycin A, pH 7.4, and with 0.5 mg/ml mitochondrial protein concentration.

Mitochondrial calcium uptake was measured in 2-ml stirred cuvettes with a PerkinElmer Life Sciences LS50B spectrofluorometer (excitation and emission wavelengths 505 and 535 nm, respectively). Mitochondria were incubated for 2 min in the medium without substrates and Ca\textsuperscript{2+} to equilibrate, and then 8 μM Ca\textsuperscript{2+} was added together with substrate specific for the respiratory complex examined; alternatively, substrate addition was delayed 1, 2, 5, or up to 10 min. Free-Ca\textsuperscript{2+} concentration was determined with MaxChelator version 2.1 (39, 40). Calcium Green-5N fluorescence was converted into [Ca\textsuperscript{2+}] after careful titration with Ca\textsuperscript{2+} additions of known concentration.

Cell culture and transfection/silencing

HeLa cells (ATCC\textsuperscript{®} CCL-2\textsuperscript{TM}) and HEK-293T cells (for MICU1 KO HEK-293T cell generation; see Sancak et al. (22)) were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 100 IU/ml streptomycin and 100 μg/ml penicillin. Cells were maintained in an incubator at 37 °C and 5% CO\textsubscript{2} and controlled humidity. 1 month in culture plus penicillin. Cells were maintained in an incubator at 37 °C and 5% CO\textsubscript{2} and controlled humidity. After 1 month in culture supplemented with 5% fetal bovine serum, 100 IU/ml streptomycin and 100 μg/ml penicillin. Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 100 IU/ml streptomycin and 100 μg/ml penicillin. Cells were maintained in an incubator at 37 °C and 5% CO\textsubscript{2} and controlled humidity.

The Cu\textsuperscript{2+} uptake measurements were performed 24 h after transfection. For RNAi experiments, the growth medium was replaced 1 h before transfection with antibiotic-free medium. Cells were transfected using TransIT-LT1 (Mirus Bio) as per the manufacturer’s instructions. Cells were transfected with low-affinity mitochondrial aequorin probe as described. Ca\textsuperscript{2+} uptake measurements were performed 48 h after silencing.

Ca\textsuperscript{2+} uptake measurements

The coverslip with the cells was incubated with 5 μM coelenterazine for 1–2 h in Krebs-Ringer–modified buffer (KRB: 135 mM NaCl, 5 mM KCl, 0.4 mM KH\textsubscript{2}PO\textsubscript{4}, 1 mM MgSO\textsubscript{4}, 1 mM MgCl\textsubscript{2}, 5.5 mM glucose, 20 mM HEPES, pH 7.4, at 37 °C) supplemented with 1 mM CaCl\textsubscript{2}, and then transferred to the perfusion chamber. After a 3-min equilibration with KRB, cells were permeabilized using a buffer mimicking the cytosolic ionic composition: 130 mM KCl, 10 mM NaCl, 1 mM KH\textsubscript{2}PO\textsubscript{4}, 1 mM MgCl\textsubscript{2}, 20 mM HEPES, supplemented with 50 μM EGTA and 50 μM digitonin. Cells were perfused for 1 min with digitonin and washed for 2 min with cytosolic-like buffer; subsequently, cells were perfused with cytosolic-like buffer devoid of EGTA and supplemented with 3 μM Ca\textsuperscript{2+} together with 5 mM glutamate, 2.5 mM malate, 1 μM oligomycin, or 5 mM succinate plus oligomycin and 1 μM rotenone; alternatively, the addition of the respiratory chain substrates followed 1 or 5 min after Ca\textsuperscript{2+} addition. The experiments were terminated by lysing the cells with 100 μM digitonin in a hypotonic Ca\textsuperscript{2+}-rich solution (10 mM CaCl\textsubscript{2} in H\textsubscript{2}O\textsubscript{2}), thus discharging the remaining aequorin pool. The light signal was collected and calibrated into [Ca\textsuperscript{2+}] using an algorithm based on the Ca\textsuperscript{2+} response curve of aequorin at physiological conditions of pH, [Mg\textsuperscript{2+}], and ionic strength, as described previously (41). Mitochondrial Ca\textsuperscript{2+} uptake speed was calculated as the first derivative by using the Origin differentiate function and averaging for three time points.

Western blotting and immunoprecipitation

For protein extraction, about 10\textsuperscript{6} HeLa cells were grown and transfected with the indicated constructs or siRNA as described above. 24 or 48 h after transfection/silencing, cells were solubilized in radioimmune precipitation buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, pH 7.5), with cOmplete\textsuperscript{TM}, mini, EDTA-free protease inhibitor mixture (Roche Applied Science) and incubated on ice for 30 min. Crude extracts were centrifuged at 10,000 \( \times \) g for 20 min at 4 °C to remove debris; proteins in the supernatant were quantified using the BCA protein assay kit (Pierce\textsuperscript{TM}). 40 μg of proteins were dissolved in reducing Laemmli sample buffer and heated for 5 min at 85 °C.

For native mild IPs, 10\textsuperscript{6} HeLa cells grown on a 10-cm Petri dish were transiently transfected as described above with pcDNA3.1-MCU-FLAG, pcDNA3.1-MCU-Myc, pcDNA3.1-MICU2-FLAG, and pcDNA3.1-MICU1-HA. After 24 h of expression, cells were washed three times with cold PBS and then lysed with an appropriate volume of native mild lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.2% n-dodecyl \( \beta \)-d-maltopyranoside (DDM), 1.7 mM MgCl\textsubscript{2}, and 0.5 mM EGTA, cOmplete\textsuperscript{TM}, mini, EDTA-free protease inhibitor mixture (Roche Applied Science), in the presence or absence of 3 μM free Ca\textsuperscript{2+} or 50 μM kaempferol, incubated for 10 min at 4 °C, and centrifuged for 1 h at 25,000 \( \times \) g. The supernatants
were quantified for protein content and immunoprecipitated using magnetic beads conjugated to antibodies against Myc as per the manufacturer’s instructions. The samples were washed three times using native mild lysis buffer and once with lysis buffer without DDM and then eluted from the beads with 2× concentrated nonreducing sample buffer and subjected to 10% SDS-PAGE. Alternatively, 1 mg of mouse liver crude mitochondria was also used and lysed using the same protocol. The supernatants were preclarified by adding 1 μg of irrelevant rabbit IgG together with 20 μl of protein A/G PLUS-agarose (sc-2003, Santa Cruz Biotechnology, Inc.) and incubated at 4 °C for 1 h on a rotating mixer. Cleared extracts were then incubated overnight with an antibody against MCU (HPA016480, Sigma-Aldrich) and then adsorbed on 20 μl of protein A/G PLUS-agarose at 4 °C for 3 h. Immunoprecipitates were collected by centrifugation at 2,000 rpm and washed three times using native mild lysis buffer and once with lysis buffer without DDM. Elution was performed with 40 μl of 2× nonreducing Laemmli sample buffer. Proteins were separated by SDS-PAGE and immunoblotted with the indicated antibody.

Blue native gel electrophoresis

Isolated mouse liver mitochondria were resuspended at 1 mg of protein/ml of buffer (250 mM mannitol, 20 mM Hepes, 1 mM P_{p}, 1 mM MgCl_2, 2 μM TPEN, 1 μM rotenone, 1 μg/ml oligomycin) in the presence of 3 μM free Ca^{2+} (measured) or 50 μM EGTA; incubated for 5 min at room temperature; and subsequently centrifuged at 4 °C for 15 min in a microcentrifuge at maximal speed. All of the following procedures were performed at 4 °C. Pellets were resuspended with 80 μl of extraction buffer: 50 mM NaCl, 50 mM imidazole, 2 mM 6-aminocaproic acid, 1 mM EDTA, pH 7.6. Digitonin was added to each suspension, which was then incubated for 30 min and centrifuged at 100,000 × g for 30 min. Supernatants were treated with 5% glycerol (final concentration) and with 5% Coomassie G-250 to concentrate nonreducing sample buffer and subjected to 10% native PAGE; and Valeria Petronilli, Cristina Fasolato, Paola Pizzo, Riccardo Filadi, Diana Pendin, Elisa Greotti, and Paolo Magalhães for helpful advice and suggestions on this work. Maintenance of the instruments for imaging utilized in this work has been supported by the Euro-bioimaging European Strategy Forum on Research Infrastructures (ESFRI) platform (under funding project ID is 688945, Grant DSB.AD008.008).

Antibodies

Antibodies used were as follows: anti-MCU, Sigma-Aldrich HPA016480, lot CD114358, dilution 1:1,000; anti-MICU1, Sigma-Aldrich HPA037489, lot B90039, lot N107141, dilution 1:1000; anti-MICU2 AbClonal A12198, lot 0065620101, dilution 1:1,000; anti-HA Cell-Signaling C29F4, lot 6, dilution 1:1,000; anti-FLAG Sigma-Aldrich F7425, lot 111M4809, dilution 1:1,000; anti-SHDA Abcam ab147175, dilution 1:10,000; anti-cyclophilin D ThermoFisher 45590 mouse monoclonal, 1:1,000; anti-HSP60 ThermoFisher PA1-41400, 1:5,000; anti-HSP90 Abcam ab13495, 1:3,000; anti-Tom20 Santa Cruz Biotechnology, sc-11415, rabbit polyclonal, 1:2,000.

Statistical analyses

Data were analyzed using Origin version 8 SR2 (OriginLab, Northampton, MA) or with GraphPad Prism software. Averages are expressed as mean ± S.D. For samples with normal distribution, a two-sample t test was used. When comparing more than two samples, one-way analysis of variance and Bonferroni post hoc tests were used. For samples without normal distribution, nonparametric Wilcoxon–Mann–Whitney test and Kruskal–Wallis plus Dunn’s multiple-comparison test were used. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.

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