Voltage-energized calcium-sensitive ATP production by mitochondria

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The regulation of ATP production by mitochondria, crucial for multicellular life, is poorly understood. Here, we investigate the molecular controls of this process in the heart and provide a framework for its Ca²⁺-dependent regulation. We find that the entry of Ca²⁺ into the matrix through the mitochondrial calcium uniporter (MCU) in the heart has neither an apparent cytosolic Ca²⁺ threshold nor a gating function, and guides ATP production by its influence on the inner mitochondrial membrane (IMM) potential, ∆Ψm. This regulation occurs through matrix Ca²⁺-dependent modulation of pyruvate and glutamate dehydrogenase activity and not through any effect of Ca²⁺ on ATP synthase or on electron transport chain complexes II, III or IV. Examining the ∆Ψm dependence of ATP production over the range of −60 mV to −170 mV in detail reveals that cardiac ATP synthase has a voltage dependence that distinguishes it fundamentally from the previous standard, the bacterial ATP synthase. Cardiac ATP synthase operates with a different ∆Ψm threshold for ATP production than bacterial ATP synthase and reveals a concave-upward shape without saturation. Skeletal muscle MCU Ca²⁺ flux, while also having no apparent cytosolic Ca²⁺ threshold, is substantially different from the cardiac MCU, yet the ATP synthase voltage dependence in skeletal muscle is identical to that in the heart. These results suggest that, while the conduction of cytosolic Ca²⁺ signals through the MCU appears to be tissue dependent, as shown by earlier work, the control of ATP synthase by ∆Ψm appears to be broadly consistent among tissues but is clearly different from that in bacteria.

ATP consumption in the heart is the highest of any tissue and, even with the phosphocreatine regenerating system, the ATP reserve is minimal—it would last less than 1 min if ATP production were stopped instantaneously. It is thus not surprising that mitochondria, the primary source of ATP production, play an important role in cardiovascular physiology and pathophysiology. Mitochondria are similarly important in other high-energy-consuming tissues such as brain, kidney, liver and skeletal muscle. Despite the critical importance of mitochondria in producing ATP, there is little time-resolved quantitative data on how mitochondria work. Here we provide quantitative information on how cardiac mitochondria generate ATP, how ATP production is powered by ∆Ψm, and how ∆Ψm is regulated by Ca²⁺ concentration in the mitochondrial matrix ([Ca²⁺]m).

Figure 1a,b show surface plots of ATP production, measured as functions of [Ca²⁺]m and the added concentrations of ADP ([ADP]). Fluorescence [Ca²⁺]m and luminescence ATP measurements were performed together from isolated heart mitochondria and quantitatively calibrated in each test condition (Extended Data Figs. 1–3). These data show that elevated [ADP] and [Ca²⁺]m robustly stimulate ATP production (Fig. 1a–c), with a half-activation concentration of ~20 μM for [ADP] (Fig. 1g) and ~600 nM for [Ca²⁺]m (Fig. 1d–f).

ADP and [Ca²⁺]m work by different mechanisms. Elevated [ADP] increases the ADP flux into the mitochondrial matrix, mediated by the adenine nucleotide translocase (ANT), thus increasing substrate availability for the ATP synthase to augment ATP production⁹,¹⁰ (also see Supplementary Discussion 2.1). On the other hand, increasing the steady-state availability of inorganic phosphate (Pᵢ) from 1 mM (physiological) to 10 mM (super-physiological) diminished ATP production (Fig. 1b,f,g). Figure 1c shows the absolute value of the difference. These results are the first determination of the dependence of ATP production on the three key variables, [Ca²⁺]m, [ADP] and [Pᵢ], and provide an important context for additional physiologic investigations.

The above findings (Fig. 1a–g) demonstrate regulation of ATP production by [Ca²⁺]m but shed no light on the mechanisms. Figure 1h–l present experiments designed to identify the nodes of Ca²⁺ regulation: they probe the [Ca²⁺]m sensitivity of ATP production powered by diverse metabolic substrates. We examined different carbon sources, such as carbohydrates and amino acids that are metabolized through the tricarboxylic acid cycle (TCA cycle). We also examined lipids, which undergo catabolic steps in the mitochondrial matrix: they only enter the TCA cycle after β-oxidation, during which they regenerate abundant NADH and FADH₂ and are broken down to acetyl-CoA and succinyl-CoA. We have identified specific carbon substrates that enable the [Ca²⁺]m-sensitive ATP production and others that do not (Fig. 1j–l). The extensive mitochondrial literature makes no such differentiation between mitochondrial substrates, and regards mitochondrial ATP production as Ca²⁺-dependent in general (see Supplementary Discussion 2.2, 2.3). Our findings show that the entry of glutamate and pyruvate into the TCA cycle is regulated by [Ca²⁺]m, and it is through this regulation that increasing [Ca²⁺]m augments ATP production. Glutamate and pyruvate are the metabolic products of amino acids and carbohydrates. Our findings thus identify [Ca²⁺]m as a key in regulating mitochondrial utilization of carbohydrates and amino acids. These findings are consistent with predictions made by earlier work with purified mitochondrial dehydrogenases¹¹,¹². Our results, however, are supported by NADH measurements (Extended Data Fig. 1d) and contradict other published predictions. Our results indicate that neither β-oxidation (of lipids) nor complexes II, III, IV or V (ATP synthase) are substantially regulated by [Ca²⁺]m despite arguments to the contrary¹³,¹⁴ (see Supplementary Discussion 2.2, 2.3).

Sensitivity to [Ca²⁺]m enables ATP production to ramp up with elevated cellular workload. To test the physiological impact of the [Ca²⁺]m sensitivity on ATP production, we examined ∆Ψm in cardiac myocytes under conditions in which they are electrically and mechanically active (see Supplementary Video. 1). We provided specific carbon substrates that enable [Ca²⁺]m-sensitive ATP

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production (pyruvate), and also substrates that support high-rate ATP production that is insensitive to [Ca\(^{2+}\)]\(_{\text{m}}\) (that is, membrane-permeant di-AM-succinate in combination with rotenone to block electron transport chain (ETC) complex I; see Fig. 1k). We first examined the effect of pyruvate on changes in ΔΨ\(_{\text{m}}\) when a single cardiac cell is integrated into an optically clear but mechanically resistive poly(vinyl alcohol) (PVA) hydrogel (Fig. 1m–o)\(^\text{9}\). The cell was mechanically coupled to the hydrogel by attaching its surface carbohydrates (glycans) to the hydrogel through a 4-armed PEG-boronic acid crosslinker (Fig. 1o; chemical synthesis in Methods section and Extended Data Fig. 4). Attachment to the PVA gel increased the mechanical load on the cell. The cell-stimulation frequency was increased from 1 to 8Hz. Notably, despite increased ATP consumption at 8Hz, there was no change in ΔΨ\(_{\text{m}}\) when metabolic substrates that impart Ca\(^{2+}\)-sensitivity on ATP production were present (pyruvate). This suggests that when ΔΨ\(_{\text{m}}\) was maintained by NADH production through Ca\(^{2+}\)-sensitive metabolism, the ETC could keep ΔΨ\(_{\text{m}}\) hyperpolarized (also see Extended Data Fig. 1d). In stark contrast, ΔΨ\(_{\text{m}}\) depolarized during elevated workload when we supplied substrates that support ATP production that is not boosted by [Ca\(^{2+}\)]\(_{\text{i}}\) (diAM-succinate + rotenone). With these substrates present, upon return to low workload conditions, the rate of ΔΨ\(_{\text{m}}\) depolarization subsided (Fig. 1q,t). These findings indicate that when workload is elevated, only Ca\(^{2+}\)-dependent ATP production is sustainable and this does not result in a decline of ΔΨ\(_{\text{m}}\).

While these findings (Fig. 1m–o) suggest that [Ca\(^{2+}\)]\(_{\text{i}}\) has a major physiological role in regulating ΔΨ\(_{\text{m}}\), they shed no light on the quantitative relationship between the three critical components of ATP production—ADP (the substrate), Ca\(^{2+}\) (the regulator) and ΔΨ\(_{\text{m}}\) (the energy source). These relationships are investigated under conditions in which they can be controlled or measured quantitatively using isolated cardiac mitochondria. Our findings are shown in the surface plot in Fig. 2a. The value of ΔΨ\(_{\text{m}}\) mapped in the surface plot represents the points of balance between ETC proton efflux and proton influx via ATP synthase and any other proton-permeation pathway, such as the ANT. ATP synthase consumes energy stored in ΔΨ\(_{\text{m}}\) by coupling proton influx to the conversion of ADP to ATP in the absence of extra-mitochondrial ADP (and hence in the absence of mitochondrial ADP), ATP synthase does not produce ATP and does not consume energy (that is, there is no proton influx). Under this condition, ΔΨ\(_{\text{m}}\) is stable and energized at about ~170 mV. However, when extra-mitochondrial [ADP] (ADP) is elevated to 500μM, ATP production is strongly stimulated. Under the same condition, if [Ca\(^{2+}\)]\(_{\text{i}}\) is very low (<200 nM), then the ATP production leads to significant depolarization of ΔΨ\(_{\text{m}}\) to about ~120 mV. However, with an elevated [Ca\(^{2+}\)]\(_{\text{i}}\) (~3 μM) to stimulate NADH production, the same 500μM [ADP] increases ATP production with a more energetic ΔΨ\(_{\text{m}}\) of ~145 mV (Fig. 2a,b). These data show that higher [ADP] augments ATP production but causes depolarization of ΔΨ\(_{\text{m}}\) with half-maximal depolarization occurring at [ADP] = 12 μM (Fig. 2c). This depolarization is significantly counteracted by an increase in [Ca\(^{2+}\)]\(_{\text{i}}\), with a half-maximal activation at [Ca\(^{2+}\)]\(_{\text{i}}\) ~500 nM (Fig. 2d). Thus, our findings demonstrate that the [Ca\(^{2+}\)]\(_{\text{i}}\)-sensitive processes that support the utilization of energy in carbon sources to regenerate NADH[14 to 17] stimulate higher proton efflux by the ETC and produce a more hyperpolarized ΔΨ\(_{\text{m}}\).

We find that this effect of [Ca\(^{2+}\)]\(_{\text{i}}\) on ΔΨ\(_{\text{m}}\) develops gradually (in seconds) and does not follow fast changes in [Ca\(^{2+}\)]\(_{\text{i}}\) (see Extended Data Fig. 5). Importantly, over the full range of the [Ca\(^{2+}\)]\(_{\text{i}}\) and ADP-regulated ATP production as mapped in Fig. 1a and Fig. 2a, there is no significant change in mitochondrial pH (pH\(_{\text{m}}\)), as shown in Fig. 2e–g. Under these same conditions, there is an approximately 30-fold change of [Ca\(^{2+}\)]\(_{\text{i}}\) and a 20-fold change of [ADP] and a 1.5-fold change in ΔΨ\(_{\text{m}}\) which lead to a 3-fold change of ATP production. These observations suggest that, although proton movement across the inner membrane enables the ATP synthase to work, ΔΨ\(_{\text{m}}\) itself is the critical regulator of ATP production. Taken together, the data in Fig. 2 show the importance of [Ca\(^{2+}\)]\(_{\text{i}}\) in enhancing NADH generation, which is critical in keeping ΔΨ\(_{\text{m}}\) hyperpolarized without material influence on pH\(_{\text{m}}\).

Next, we investigate how physiological activity leads to changes of [Ca\(^{2+}\)]\(_{\text{m}}\). First, we examine the movement of cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{\text{c}}\)) into the mitochondrial matrix. [Ca\(^{2+}\)]\(_{\text{c}}\) increases when Ca\(^{2+}\) enters the mitochondria through the MCU channel in the inner mitochondrial membrane, the only known route of mitochondrial Ca\(^{2+}\) influx[18-22]. By this route, as [Ca\(^{2+}\)]\(_{\text{i}}\) increases to trigger heart contraction, it also enters the matrix through MCU and thereby elevates [Ca\(^{2+}\)]\(_{\text{m}}\) which, as shown in Fig. 1, boosts ATP production. Measurements of MCU-mediated Ca\(^{2+}\) influx into the cardiac mitochondrial matrix under physiological conditions are shown in Fig. 3a–d. Measuring Ca\(^{2+}\) influx quantitatively at high temporal resolution is challenging at the physiological [Ca\(^{2+}\)],...
because MCUs are sparse (15–65 per cardiac mitochondrion) and have low single-channel conductance (~0.1 fS at 500nM [Ca\(^{2+}\)], and Δ\(\Psi_m\) = −160 mV, see Methods and Williams et al.). Nevertheless, we were able to make these biophysical measurements for the first time using stopped-flow fluorometry, in which Ru360-inhibitable mitochondrial Ca\(^{2+}\) influx was quantified with...
millisecond resolution (Extended Data Fig. 6). Fluo-4 or fluo-4FF was used to measure extramitochondrial (that is, cytosolic) [Ca^{2+}], while [Ca^{2+}]_{i} and ΔΨ_{m} were measured with fura-2 and tetramethylrhodamine methyl ester perchlorate (TMRM), respectively; all measurements were performed under identical conditions (see Methods). Figure 3a shows MCU-mediated Ca^{2+} influx as a function of [Ca^{2+}], while Fig. 3b shows the relative MCU conductance as a function of [Ca^{2+}]. These measurements are used to obtain the number of open MCU channels as a function of [Ca^{2+}], (for more details, see Supplementary Discussion 2.4) Our data provide an unambiguous result — the number of open MCU channels remains essentially unchanged over the physiological range of [Ca^{2+}] in cardiac mitochondria, as shown in Fig. 3c. Furthermore, our data show a surprisingly simple result: as the availability of the conducting ion increases, so does its flux, and as ΔΨ_{m} becomes less negative, MCU-mediated Ca^{2+} flux decreases, following the electrochemical driving force for Ca^{2+} entry into the mitochondrial matrix (see Fig. 3d). The result is surprising because other investigators have reported that the MCU has a [Ca^{2+}]_{i} ‘threshold’ of ~1 μM for conducting Ca^{2+}, below which no Ca^{2+} flux is seen. In contrast, we see no threshold, a finding that suggests a threshold may not be a common feature of all tissues (Supplementary Discussion 2.5). Furthermore, the [Ca^{2+}]_{i}-gating functions of the MCU, that regulate the number of open MCU channels are not observed by us in cardiac mitochondria under the conditions of these experiments.

The putative purpose of the gating/threshold combination is to limit excessive mitochondrial Ca^{2+} loading. However, our quantitative measurements suggest that this can be achieved by the low number of MCU channels in the heart. Furthermore, the behaviour of cardiac myocyte MCUs shown here suggests that [Ca^{2+}]_{i} should track [Ca^{2+}], in the heart without excessive weight being given to elevated [Ca^{2+}]_{i} transients.

Part two of our examination of the movement of cytosolic Ca^{2+} into the mitochondrial matrix focuses on simultaneous measurements of [Ca^{2+}]_{i} and [Ca^{2+}]_{i} under physiological conditions in patch-clamped ventricular myocytes. The physiological context of cytosolic and mitochondrial Ca^{2+} is shown in Fig. 3e–j in myocytes patch-clamped and stimulated by 100-ms depolarizations at 0.5 Hz. [Ca^{2+}]_{i} was measured with a mitochondrially targeted fluorescent Ca^{2+} sensor, MityCam (characterized in Extended Data Fig. 7), while Rhod-2 salt loaded into the cytosol via the patch pipette was used to examine [Ca^{2+}]_{i}. The calibrated confocal fluorescence images and signals are shown in Fig. 3e–g. These data show that in the quiescent state, [Ca^{2+}]_{i} and [Ca^{2+}]_{i} are stable and [Ca^{2+}]_{i} is ~50–100 nM higher than [Ca^{2+}]_{i} (Fig. 3g,h). Every depolarization-triggered heartbeat evokes a [Ca^{2+}]_{i} transient (Fig. 3g,i). With repeated depolarizations, the [Ca^{2+}]_{i} transient peaks become larger (as sarcoplasmic reticulum Ca^{2+} content increases with stimulation), while the diastolic [Ca^{2+}]_{i} and [Ca^{2+}]_{i} both rise slowly (Fig. 3g,j). These findings demonstrate that rhythmic elevations of [Ca^{2+}]_{i} do not cause synchronized large
transients of \([\text{Ca}^{2+}]_{\text{m}}\) (Fig. 3i). Instead, a series of cytosolic \(\text{Ca}^{2+}\) transients causes a gradual beat-dependent elevation of \([\text{Ca}^{2+}]_{\text{m}}\). Thus, in a physiological context, a single heartbeat cannot materially change \([\text{Ca}^{2+}]_{\text{m}}\). Rather, a series of heartbeats and their pattern control \([\text{Ca}^{2+}]_{\text{i}}\) and ATP production.

The clear role of \(\Delta \Psi_{\text{m}}\) in regulating ATP production, as shown in Figs. 1 and 2, is examined quantitatively in Fig. 4. Isolated mitochondria were used in these experiments without high-energy substrate, and were depleted of \([\text{Ca}^{2+}]_{\text{m}}\) for the initial tests to minimize the \([\text{Ca}^{2+}]_{\text{m}}\)-dependent contributions. \(\Delta \Psi_{\text{m}}\) was controlled independently using valinomycin (a \(K^+\) ionophore) in combination with the \(K^+\) gradient across the inner mitochondrial membrane; \(\Delta \Psi_{\text{m}}\) was measured and calibrated with TMRM. With all other factors held constant, ATP production was measured as a function of \(\Delta \Psi_{\text{m}}\) (see Methods). The resulting findings were unexpected. The curve is concave upward and shows ATP production accelerating as \(\Delta \Psi_{\text{m}}\) is increasingly hyperpolarized. To examine further the role of \([\text{Ca}^{2+}]_{\text{m}}\), the experiment was repeated at high \([\text{Ca}^{2+}]_{\text{m}}\) and in the presence of high-energy substrate—with all other conditions held constant. Figure 4a shows that the relationship was virtually identical under high and low \([\text{Ca}^{2+}]_{\text{m}}\)—confirming our earlier findings that \([\text{Ca}^{2+}]_{\text{m}}\) does not directly regulate the ATP synthase (Fig. 1f, i).

The IMM voltage dependence of ATP production by ATP synthase (complex V) in cardiac mitochondria reveals how protons in the intermembrane space use \(\Delta \Psi_{\text{m}}\) to power the synthase. Up to now, the known voltage dependence of bacterial ATP synthase has been used as the model system for predicting the behaviour of the mammalian ATP synthase\(^{27-29}\). The bacterial ATP synthase produces 0 ATP at 0 mV, and ATP production rises sigmoidally, saturating at \(-120\) mV, with half-saturation at \(-70\) mV (refs. \(27-29\)).

Our data in Fig. 4a are the first report of the voltage dependence of the mammalian ATP synthase. The shape of the curve and its quantitative characteristics should provide clues into the inner workings of the mammalian ATP synthase. The shape of the curve is a surprise—it is concave upward over the range of \(\Delta \Psi_{\text{m}}\) examined and does not suggest saturation. Additionally, in sharp contrast with the bacterial ATP synthase, which reaches \(37\%\) of its maximal rate at \(-65\) mV (ref. \(29\)), the mammalian enzyme produces essentially no ATP until an apparent ‘threshold’ voltage of \(-65\) mV is reached. Thermodynamically, the \(-65\) mV threshold suggests that when \(\Delta \Psi_{\text{m}}\) is more positive than about \(-65\) mV, there is insufficient energy when protons are bound to the synthase to enable ATP to be synthesized. Furthermore, Fig. 4a shows the increase in ATP production from \(-70\) to \(-120\) mV. Over this voltage range, the rates of ATP production are consistent with the need for three protons to be transported across the IMM by ATP synthase for every ATP molecule synthesized from ADP (red line in Fig. 4a).

This follows a ‘standard model’ of harnessing the energy of protons moving through the voltage field across the inner membrane to produce ATP\(^{30}\). On the other hand, at potentials more negative than \(-120\) mV, the measured rates of ATP production exceed the red line. The slope of the data over the range \(-130\) to \(-170\) mV rises to approximately 4 times that predicted by the red line. This finding is thus completely unexpected and has not been seen before. Mechanistically, we do not know why ATP production increases so quickly with \(\Delta \Psi_{\text{m}}\). We propose that the number of protons that move across the voltage field of the IMM via a single ATP synthase cycle can increase as \(\Delta \Psi_{\text{m}}\) hyperpolarizes negative to \(-120\) mV. In which case, this high rate of ATP production at more hyperpolarized IMM voltages is due to an increase in the stoichiometry of the ATP synthase (more protons moved per ATP produced), which we call ‘adaptive stoichiometry’. A second possibility is a nonlinear voltage-dependent increase in the ‘rotation’ of the C-ring of ATP synthase with unchanged stoichiometry, which may be termed ‘adaptive kinetics’. While one or more of these hypothesized voltage-sensitive mechanisms may apply, we do not yet have experimental techniques that enable us to determine which. Nevertheless, it is clear from the data that the voltage dependence shown in Fig. 4a differs radically from the measured voltage dependence of the bacterial ATP synthase. This feature may represent an improvement of metabolic adaptation in mammalian systems. It enables a higher rate of ATP production when it is needed and when the energy is available in the form of \(\Delta \Psi_{\text{m}}\). Additional discussion of several probable important features of the mammalian ATP synthase is presented in the Supplementary Discussion 2.6 and in Supplementary Information 1.1.

Our examination of ATP production and its regulation in the heart is shown in Fig. 4b. We found that mitochondrial production of ATP is controlled by \(\Delta \Psi_{\text{m}}\), which is sensitive to \([\text{ADP}]\) and to \([\text{Ca}^{2+}]_{\text{m}}\). We propose that the \(\Delta \Psi_{\text{m}}\) dependence of ATP production that we show here in cardiac mitochondria is a general feature of mammalian mitochondria. In support of this view, we carried out similar experiments with skeletal muscle mitochondria and found the same \(\Delta \Psi_{\text{m}}\) dependence of ATP production (see Extended Data Fig. 8). Nevertheless, these and other and tissue-specific mitochondrial features need further study. For example, in practically all tissues, the basal and dynamic ATP consumption rates are likely to be different\(^{25}\). Furthermore, the dynamic \([\text{Ca}^{2+}]_{\text{m}}\) signals are also generally cell-type and tissue specific\(^{30,30}\). Moreover, we and others show that MCU properties vary substantially in different tissues.

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**Fig. 3** | \([\text{Ca}^{2+}]_{\text{m}}\) dynamics and MCU \(\text{Ca}^{2+}\) conductance. a. Stopped-flow measurement of the MCU-dependent \(\text{Ca}^{2+}\) influx \(J_{\text{m}}\) (scaled to a litre of cytosol (see Methods and ref. \(26\))) \(J_{\text{m}}\) (\(\mu\text{M s}^{-1}\)) is plotted as a function of measured \([\text{Ca}^{2+}]_{\text{i}}\) (n=63 independent experiments, each with \([\text{Ca}^{2+}]_{\text{i}}\), \([\text{Ca}^{2+}]_{\text{m}}\), and \(\Delta \Psi_{\text{m}}\) measured). The inset shows the zoomed-in region between 0 and 3 \(\mu\text{M} \text{[Ca}^{2+}]_{\text{i}}\). Linear least-squares fit to the filled circles is shown (slope = 1.2.). Stopped-flow data are shown in Extended Data Fig. 6. b, MCU conductance \(G_{\text{MCU}}\) for each of 63 experiments shown in a normalized to the minimal conductance \(G_{\text{G}}\) of each dataset \(G_{\text{G}}\) plotted as a function of \([\text{Ca}^{2+}]_{\text{i}}\) (see Methods). The inset shows zoomed-in region between 0 and 3 \(\mu\text{M} \text{[Ca}^{2+}]_{\text{i}}\). Linear least-squares fit line to the filled circles is shown (slope = 6.1.). c, Number of open MCUs per mitochondrion \(N_{\text{p}}\) (NP) plotted as a function of \([\text{Ca}^{2+}]_{\text{i}}\). Taken from b after division by the number of mitochondria per litre cytosol (see Methods) and dividing by the \([\text{Ca}^{2+}]_{\text{i}}\)-dependent unitary conductance of MCU\(^{30}\). Linear least-squares fit to the filled circles is shown (slope = 0.116, intercept = 7.48). d, MCU conductance \(J_{\text{m}}\) \(J_{\text{m}}\) (\(\mu\text{M s}^{-1}\)) as a function of \(\Delta \Psi_{\text{m}}\) (\([\text{Ca}^{2+}]_{\text{i}}\), and \(\Delta \Psi_{\text{m}}\) measured as in a but using a multi-well plate reader. \(\Delta \Psi_{\text{m}}\) was set by using a \(K^+\) gradient and the \(K^+\) ionophore valinomycin (see Methods and Fig. 4). \(\Delta \Psi_{\text{m}}\) was set to 35 \(\mu\text{M}\) (n = 4, 7, 4 independent experiments for \(\Delta \Psi_{\text{m}}\) = 155, –122, –92 mV groups, respectively). MCU blocker Ru360 (1 \(\mu\text{M}\)) reduced \(J_{\text{m}}\) to near zero (n = 6 independent experiments). Data are mean ± s.e.m. e, Deconvolved Aaryian confocal image showing the fluorescence of the mitochondrially targeted \(\text{Ca}^{2+}\)-sensor MityCam expressed in a cardiomyocyte. Note distinct MityCam localization in individual mitochondria. f, Confocal line-scan images from a cardiomyocyte-expressing MityCam; top panels show the fluorescence of Rhod-2 (tripotassium salt, loaded via the patch-clamp pipette); lower panels show MityCam fluorescence. To stimulate \([\text{Ca}^{2+}]_{\text{m}}\), transients the membrane potential is stepped repeatedly from a holding level of –80 mV to 0 mV every 2 s. Isoproterenol (500 nM) is applied at the times indicated. Note that \([\text{Ca}^{2+}]_{\text{m}}\) binding reduces the fluorescence of MityCam. g, The time-course of changes in \([\text{Ca}^{2+}]_{\text{i}}\), and \([\text{Ca}^{2+}]_{\text{m}}\), from the respective fluorescence measurements shown in f. The experiments shown in panels a–f were repeated independently with similar results (n = 9 cells). h, Time-averaged \([\text{Ca}^{2+}]_{\text{i}}\), versus time-averaged \([\text{Ca}^{2+}]_{\text{m}}\), (n = 9 cells). Data are mean ± s.e.m.
eukaryotic species and tissues (see Extended Data Fig. 8 that compares our measurements of cardiac MCU to skeletal muscle MCU). Taken together, our findings and the developed quantitative tools lay the foundation to reshape our thinking and approach to energy utilization under physiological and pathophysiological conditions and in mitochondrial diseases.
Methods

Mitochondrial isolation. We anaesthetized 6- to 10-week-old Sprague-Dawley male rats (250–300 g, from ENVIGO, USA, stain code no. 002) with isoflurane (10 min) and administered heparin intraperitoneally (720 U per kg body weight, 5 min). A thoracotomy and fast excision of the heart was performed, with removal of the atria. The ventricles were minced in ice-cold isolation buffer (IB) containing: KCl 100 mM, MOPS 50 mM, MgSO4 5 mM, EGTA 2 mM, Na pyruvate 10 mM, and K2HPO4 10 mM. The minced tissue was washed repeatedly with IB until clear of blood. The remainder of the preparation was conducted in a cold room (4°C). 20 ml of IB-containing tissue was transferred to a Potter Elvehjem grinder and homogenized at high speed for 2 s, followed by 4 repetitive homogenizations at 600 g. The final supernatant was transferred to a clean centrifuge tube and spun for 8 min. The supernatant was transferred to a new centrifuge tube.

Isolation of adult cardiomyocytes. Isolated ventricular myocytes were obtained from adult male Sprague-Dawley rats (250–300 g, from ENVIGO, stain code no. 002). Rats were deeply anaesthetized by inhalation of vaporized isoflurane and heparinized (720 U per kg body weight). Ten min after heparin was injected, the heart was rapidly excised and rinsed with ice-cold 500µM EGTA isolation buffer containing 130 mM NaCl, 5.4 mM KCl, 0.5 mM MgCl2, 0.33 mM NaH2PO4, 10 mM t-glucose, 10 mM taurine, 25 mM HEPES and 0.01 unit ml–1 insulin (pH 7.4) (adjusted with NaOH). The aorta was quickly cannulated for Langendorff perfusion. The heart coronary arteries were perfused at 37 °C for 2 min with EGTA isolation buffer and then perfused for 7 min with isolation buffer supplemented with 1 mg ml–1 collagenase (type II; Worthington Bio-chemical), 0.06 mg ml–1 protease (XIV), 0.06 mg ml–1 trypsin, and 0.3 mM CaCl2. The ventricles were cut down, minced and kept in the same buffer for an additional 6 min at 36 °C. The myocardium was dispersed to form a cell suspension, which was then filtered through a Nylon mesh filter (300μm). The filtrate was spun at 180g, and the cell-containing pellet was resuspended in isolation buffer supplemented with 2 mg ml–1 BSA. Ca2+ was gradually added at 4 increments of 0.4 mM every 12 min. Cells were allowed to pellet by sedimentation and resuspended in NT solution, and were used within 4h of isolation. All procedures and protocols involving animal use were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine.

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Measuring the dissociation constants of Ca2+ indicators. Fluorescence titration curves with Ca2+ were done to measure the [Ca2+] dissociation constants (Kd) of the indicators used (see Extended Data Fig. 2). The Kd values of Fluo-4, Fluo-4FF and Rhod-2 are measured in the relevant experimental buffers using the method by Eberhard and Erne1, as shown in Extended Data Fig. 2a–c. The Kd of Fura-2 and Rhod-2, loaded via their acetoxyethyl (AM) form into the matrix of isolated mitochondria, was measured using the Ca2+ ionophore ionomycin to equilibrate the free mitochondrial Ca2+([Ca2+]mitochondria) with the free extra mitochondrial Ca2+(Ca2+[extra]), (see Extended Data Fig. 2d).

Measurements of mitochondrial ATP production and Ca2+ transients. Measurements of mitochondrial ATP production rate and [Ca2+] were carried out using a BMG LABTECH CLARIOstar plate reader. Rhod-2 AM loaded mitochondria (0.1 mg per ml) were mixed in ATP production assay buffer (AB) consisting of: K glutamate 130 mM, KCl 5 mM, K2HPO4, 1 or 10 mM, MgCl2 1 mM, EGTA 0.04 mM, BSA 0.5 mg ml–1, d-luciferin (Sigma) 0.005 mM, luciferase (Roche) 0.001 mg ml–1. A luminescence standard curve was performed daily over a range of 100 nM to 1 mM ATP with oligomycin A (15 μM)-treated mitochondria (see Extended Data Fig. 1a–c). The mitochondria were incubated for 2 min prior to the start of the assay with Ca2+ (0–50 μM added) and metabolic substrates. Assays were initiated by injection of 100 μl of ADP (50–500 μM) and Luciferin/luciferase in AB to bring the final volume to 200μl. Luminescence signal was recorded for
Measurements of $\Delta F_{\text{Ca}}$ and $[\text{Ca}^{2+}]_i$. Measurements of $\Delta F_{\text{Ca}}$ and $[\text{Ca}^{2+}]_i$ were carried out using either a BMG LABTECH CLARIOstar plate reader or stopped-flow instrument (SF-300X, KinTek). In these experiments, Fura-2 AM loaded mitochondria (0.25 mg per ml) were mixed in ATP production assay buffer (AB) without BSA and supplemented with 0.5 mM TMRM (2 mM TMRM per 1 mg per ml mitochondrial protein). The CLARIOstar was used for experiments testing $AP_{\text{Ca}}$, depolarization over a range of both $Ca^{2+}$ (0–50 μM added) and ADP (0–500 μM) using pyruvate (1 mM) and malate (0.5 mM) for substrate. After 2 min of incubation with substrate and $Ca^{2+}$, assays were initiated by injection of 100 μl of AB to bring the final volume to 200 μl. TMRM signal (excitation: 546–4 nm and 573–5 nm, emission: 619 ± 15 nm) and Fura-2 (excitation: 335 ± 6 nm and 380 ± 6 nm, emission: 490 ± 15 nm) fluorescence were measured within the same well for 200. Stopped flow measurements were done using the same buffers and three distinct protocols. Protocol 1: mitochondria were pre-incubated for 2 min with $Ca^{2+}$ then stimulated with 500 μM ADP. Protocol 2: mitochondria with $[\text{Ca}^{2+}]_i$ of less than 50 μM were pre-incubated with 500 μM ADP, followed by mixing with high $Ca^{2+}$ while keeping constant 500 μM ADP. All 3 protocols were executed for 20 s. TMRM (excitation: 546 nm and 573 nm, 593–643 nm emission) and Fura-2 (excited with 340 nm and 500 μM of EGTA. Since EGTA is saturated with $Ca^{2+}$ the $\Delta F_{\text{Ca}}$ and $[\text{Ca}^{2+}]_i$ are completely blocked by 1 μM of Ru360, and is therefore identified as MCU flux ($\Psi_{\text{mcu}}$) and scaled to a litre of cardiomycyte cytosol (μM s⁻¹ scaling is based on 80 g mitochondrial protein per L cardiomycyte cytosol). To measure $[\text{Ca}^{2+}]_i$ mitochondria were loaded with Fura-2 AM (calibration described above) and loaded with TMRM to measure $\Delta F_{\text{Ca}}$ in μV (calibration described above). The total MCU $\text{Ca}^{2+}$ conductance ($G_{\text{mcu}}$) was obtained from the measurements of $\Psi_{\text{mcu}}$ $[\text{Ca}^{2+}]_i$, $\Delta F_{\text{Ca}}$, and $\Delta F_{\text{Ca}}$ according to the following Eq. (6): $\Psi_{\text{mcu}} = I_{\text{mcu}}/(2FV)$, $I_{\text{mcu}} = G_{\text{mcu}}(\Delta F_{\text{Ca}} - \Delta F_{\text{Ca}})$, where $V$ is the myoplasm volume 18 ml and $F_{\text{Ca}}$ is the Nernst reversal potential for $Ca^{2+}$. The number of open MCU channels per mitochondrial are obtained from the measurements of $\Psi_{\text{mcu}}$, $[\text{Ca}^{2+}]_i$, $\Delta F_{\text{Ca}}$, and $\Delta F_{\text{Ca}}$ according to the following Eq. (7): $\text{Number of open MCU} = \frac{G_{\text{mcu}}}{I_{\text{mcu}}}$.

Valinomycin $\Delta F_{\text{Ca}}$ clamp: mitochondrial ATP production and mitochondrial Ca$^{2+}$ influx. $\Delta F_{\text{Ca}}$ clamp experiments were carried out using a BMG LABTECH CLARIOstar plate reader. The $\Delta F_{\text{Ca}}$ clamp was achieved using valinomycin and a $K^+$ gradient established between the mitochondria and the extra-mitochondrial solution. The extra-mitochondrial solution is varied from 0 to 70 mM while the mitochondrial matrix contained the same amount of $K^+$ at the beginning of each experiment (loaded to a steady-state level of $K^+$ during the 5-h isolation procedure in buffer with 100 mM K+). Two primary buffers were used: (1) $K^+$ free buffer: gluconic acid 130 mM, tetramethyl ammonium hydroxide 130 mM, NaH$_2$PO$_4$ 1 mM, MgCl$_2$ 1 mM, HEPES 20 mM and EGTA 0.04 mM, pH 7.2 with HCl; (2) high $K^+$ buffer: K gluconate 130 mM, KCl 5 mM, NaH$_2$PO$_4$ 1 mM, MgCl$_2$ 1 mM, HEPES 20 mM and EGTA 0.04 pH 7.2 with KOH. Buffers were supplemented with NaCl (Sigma) 0.055 mM, Luciferase (Roche) 0.001 mg ml$^{-1}$ and 2 μM TMRM per 1 ml medium. Valinomycin was used at a final concentration of 1 μM. For ATP production experiments, a 0.5 μl of 100 μg per ml mitochondrial stock was added to a well and re-suspended with a desired amount of high $K^+$ buffer. Three groups were assessed: (1) no substrate and [Ca$^{2+}$]$_{\text{extra,free}}$ = 200 mM, (2) 1 mM pyruvate and 0.5 mM malate with [Ca$^{2+}$]$_{\text{extra,free}}$ = 200 mM; and (3) 1 mM pyruvate and 0.5 mM malate with [Ca$^{2+}$]$_{\text{extra,free}}$ = 200 mM. The ATP production procedure was as follows: The injection was then used to add a desired amount of K$^+$ free buffer, bringing the volume of the well to 190 μl. An initial TMRM and Fura2 measurement was recorded for 30 s, allowing the mitochondrion to establish a steady state $\Delta F_{\text{Ca}}$, ATP production

\[ [\text{Ca}^{2+}]_{\text{extra,free}} = K_{\text{CaH+}} [\text{Fura}]_{\text{free}} - [\text{Fura}]_{\text{bound}} + [\text{Fura}]_{\text{bound}} - [\text{Fura}]_{\text{free}} \]

\[ \text{Fluo} - 4: \text{Ca}^{2+} = [\text{Fura}]_{\text{free}} - [\text{Fura}]_{\text{bound}} + [\text{Fura}]_{\text{bound}} - [\text{Fura}]_{\text{free}} \]

\[ \text{Fluo} - 4: \text{Ca}^{2+} = [\text{Fura}]_{\text{free}} - [\text{Fura}]_{\text{bound}} + [\text{Fura}]_{\text{bound}} - [\text{Fura}]_{\text{free}} \]
was initiated with an injection of 10 μl 10 mM ADP (final ADP, 500 μM) and 2 s mixing. Luminescence was measured for 15 s with 1 s-integration (calibrated daily). A final TMRM measurement was recorded for 15 s to ensure no change in ΔΨ. Confocal images from intracellular Ca\(^+\) in M. During the assay, the assay was conducted in (the absence of Na\(_2\)HPO\(_4\) and only 10 μM EGTA) with parallel measurement of TMRM and Ca\(^+\) influx using fluo-4F (as described above). Ca\(^+\) influx images were recorded at a [Ca\(^+\)]\(_{intracellular}\) of 15 ± 17 μM.

**Encapsulation of cardiomyocytes in a resistive hydrogel and measurements of ΔΨ and sarcomere length.** The 14% PVA hydrogel was prepared daily; 14 g PVA was dissolved in 100 mM Na\(_2\)SO\(_4\) solution by stirring at 90 °C, spun down at 200g to remove air, and kept at 25 °C. Equal volumes of isolated cardiomyocyte suspension and PVA hydrogel were mixed on the glass bottom of a plastic imaging chamber (Lab-Tek Chambered No. 1 Coverglass) and supplemented with cell-to-cell linker to a final concentration of 2% linker (the following section for synthesis details and Extended Data Fig. 4). Prior to mixing of cardiomyocytes with the hydrogel, the cells were incubated for 20 min in Na\(_2\)SO\(_4\) solution (36 °C) supplemented with 50 nM TMRM. This NT solution contained pyruvate (1 mM) and malate (0.5 mM), or diAB-muccinate (succinic acid diacetoxymethyl ester, 10 μM) and sodium acetate (250 mM). Incubations and subsequent experiments were done in solutions of the same composition. The hydrogel was given 4 min to circulate, after which the plastic imaging chamber was connected to electric stimulation wires and placed inside a microscope stage-top incubator (IN-TIWZ, TAKAI HIT, Japan). The system was given another 10 min of incubation at 36 °C before optical measurements of ΔΨ, (with TMRM) and simultaneous video-based myocyte sarcomere length measurements were then performed (see Supplementary Video). Line-scan confocal imaging was carried out, scanning every 10 ms along the traverse axis of a single cardiomyocyte. Line-scan confocal imaging was carried out using 900B:VSL system (Aurora Scientific). Field electrical stimulation (40 Volt cm\(^{-1}\)) to trigger contraction was performed [Ca\(^+\)]\(_{intracellular}\) was measured after a holding potential of ~80 mV to 0 mV for 100 ms every 2 s (0.5 Hz). Micro-electrode pipettes (Series Resistance 1.7–2.2 MΩ) were filled with an intracellular solution containing: KCl 5 mM, HEKES 5 mM, glucose 5.5 mM, MgCl\(_2\) 0.5 mM, NaCl 140 mM, Na\(_2\)HPO\(_4\) 0.33 mM, CaCl\(_2\) 1.8 mM, and Cytochalasin-D 0.08 mM, adjusted to pH 7.4 with NaOH. A prototype-cell voltage-clamp protocol was used to voltage-clamp triggering of [Ca\(^+\)]\(_{intracellular}\), transients (EPC10, HEKA Elektronik). The membrane potential of a patched cardiomyocyte was stepped from a holding potential of ~80 mV to 0 mV for 100 ms every 2 s (0.5 Hz). Micro-electrode pipettes (Series Resistance 1.7–2.2 MΩ) were filled with an intracellular solution containing: KCl 5 mM, HEKES 5 mM, glucose 5.5 mM, MgCl\(_2\) 0.5 mM, NaCl 140 mM, Na\(_2\)HPO\(_4\) 0.33 mM, CaCl\(_2\) 1.8 mM, and Cytochalasin-D 0.08 mM, adjusted to pH 7.4 with NaOH.

**Synthesis of 4-armed PEG-boroninic acid.** With stirring, 4-ArmPEG-NH\(_2\) (nominal MW 5,000, Biochempeg Scientific) 1 g, and 2 equivalents of tetraethylammonium boronic acid (DIPEA, 0.550 mL, 3.16 × 10\(^{-5}\) mol) and 4-carboxyphenylboronic acid (0.262 g, 1.58 × 10\(^{-3}\) mol) were added sequentially to the stirred solution. Thereafter, 1-[bis(dimethylamino)methylene]-1H-benzotriazolium hexafluorophosphate-3-oxide (HBTrU; 0.599 g, 1.58 × 10\(^{-3}\) mol) was added, and stirring of the reaction mixture was continued under argon. As the reaction progressed, the viscosity of the mixture increased. The data that support the findings of this study are available from the Data availability. Nature Research Reporting Summary linked to this article. Detailed statistical information is included in the Supplementary Tables linked to this article. Evaluation of the expected resonances (see Supplementary Fig. 1. The spectrum shows the expected resonances at (8) 5.75 (OCH\(_3\)), 2.71 (CH\(_2\)CH), and 2.12 (CH\(_2\)CO). High-resolution mass spectrometry (ESI+mode) showed a base peak at 263.07703, corresponding to [M+H]\(^{+}\): C\(_{7}\)H\(_{16}\)O\(_{5}\), which requires 263.076695.

**Cardiomyocyte [Ca\(^+\)]\(_{intracellular}\) and [Ca\(^+\)]\(_{extracellular}\) measurements.** Cardiomyocytes were perfused throughout experiments with a normal Tyrode’s (NT) bath solution containing: KCl 5 mM, HEKES 5 mM, glucose 5.5 mM, MgCl\(_2\) 0.5 mM, NaCl 140 mM, Na\(_2\)HPO\(_4\) 0.33 mM, CaCl\(_2\) 1.8 mM, and Cytochalasin-D 0.08 mM, adjusted to pH 7.4 with NaOH. A prototype-cell voltage-clamp protocol was used for voltage-clamp triggering of [Ca\(^+\)]\(_{intracellular}\), transients (EPC10, HEKA Elektronik). The membrane potential of a patched cardiomyocyte was stepped from a holding potential of ~80 mV to 0 mV for 100 ms every 2 s (0.5 Hz). Micro-electrode pipettes (Series Resistance 1.7–2.2 MΩ) were filled with an intracellular solution containing: KCl 20 mM, KCl 100 mM, tetraethylammonium chloride 20 mM, HEKES 10 mM, MgCl\(_2\) 4.5 mM, di-sodium EDTA 4 mM, potassium creatine phosphate 1 mM, Rhod-2, tripotassium salt, 0.05 mM, pH 7.2. To simultaneously measure [Ca\(^+\)]\(_{intracellular}\) and [Ca\(^+\)]\(_{extracellular}\), confocal line-scan imaging was carried out along the transverse axes of a patch-clamped cardiomyocytes. [Ca\(^+\)]\(_{intracellular}\) was measured using a mitochondrial targeted Ca\(^+\)-sensitive fluorescent protein-probe MityCam \(^{38}\) for adenosivational at 600 MOI (excited by the 488 nm Aragon laser line, emission 505–530 nm). [Ca\(^+\)]\(_{extracellular}\) was measured with the Ca\(^+\)-sensitive fluorescent indicator Rhod-2 (tripotassium salt) dialysed into the cytosol via the patch pipette (excited by the 536 nm Helium-neon laser line, emission 570–650 nm). For the calibration of the fluorescence signals, at the end of each trial the patched cardiomyocyte was perfused sequentially with two calibration solutions applied via a local micro-perfusion. The first solution was a NT solution devoid of Ca\(^+\) (chelated with 5 mM EGTA) and supplemented with the Ca\(^+\) ionophore, ionomycin (2 μM). The second was a NT solution with 10 mM Ca\(^+\) supplemented with ionomycin (2 μM). For more details about the calibration please see Boyman et al. \(^{38}\). [Ca\(^+\)]\(_{intracellular}\) in μM is obtained from the measured Rhod-2 fluorescence (F\(_{Rhod}\)) according to the following Eq. (8):

\[ [\text{Ca}^{2+}]_{\text{intracellular}} = \frac{F_{\text{Rhod}} - F_{\text{Rhod, min}}}{F_{\text{Rhod, Max}} - F_{\text{Rhod, min}}} \times F_{\text{Rhod, Max}} \]

where F\(_{\text{Rhod, Max}}\) is the fluorescence intensity of Rhod-2 in the absence of Ca\(^+\), F\(_{\text{Rhod, Min}}\) is the fluorescence of Rhod-2, dried on Ca\(^+\)-saturated MityCam. Rhod-2 dissociation constant of Rhod-2 (K\(_d\)) was taken as 2.5 μM (See Extended Data Fig. 2c).

\[ [\text{Ca}^{2+}]_{\text{intracellular}} = \frac{F_{\text{MityCam}} - F_{\text{MityCam, Min}}}{F_{\text{MityCam, Max}} - F_{\text{MityCam, Min}}} \]

where F\(_{\text{MityCam, Max}}\) is the fluorescence intensity of MityCam in the absence of Ca\(^+\), F\(_{\text{MityCam, Min}}\) is the fluorescence of the Ca\(^+\)-saturated MityCam. The Ca\(^+\) dissociation constant of MityCam (K\(_{d\text{MityCam}}\)) is taken as 0.2 μM (See Extended Data Fig. 7a).

**Luminescence measurements of NADH.** Rhod-2 AM loaded mitochondria (2 mg per ml) were mixed in assay buffer (AB) consisting of: K gluconate 130 mM, KCl 5 mM, K-HPO\(_4\) 1 mM or 10 mM, MgCl\(_2\) 1 mM, HEKES 10 mM, EGTA 0.04 mM, BSA 0.5 mg ml\(^{-1}\). The mitochondria were incubated for 2 min prior to the start of the assay experiment. For more information required. Statistical analysis was performed using either OriginPro 2018 or Matlab R2016a statistical package all with α = 0.05. Where appropriate, column analyses were performed using an unpaired, two-tailed t-test (for two groups) or one-way ANOVA with Bonferroni correction (for groups of three or more). Data fitting convergence was achieved with a minimal termination tolerance of 10\(^{-6}\). P values less than 0.05 (95% confidence interval) were considered significant. All data displayed a normal distribution and variance was similar between groups for each evaluation. Detailed statistical information is included in the Supplementary Tables linked to the online version of this article.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** The data that support the findings of this study are available from the corresponding author upon reasonable request.


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Author contributions

A.P.W. and L.B. were involved with all experiments, data collection and data analysis; A.P.W., L.B. and W.J.L. contributed to study design, data interpretation and writing the paper; J.P.Y.K. synthesized the four-armed PEG-boronic acid and the diAM-succinate. A.P.W. and L.B. were involved with all experiments, data collection and data analysis; J.P.Y.K. contributed to the study design, data interpretation and writing the paper; A.P.W., L.B. and W.J.L. contributed to study design, data interpretation and writing the paper; J.P.Y.K. synthesized the four-armed PEG-boronic acid and the diAM-succinate. All authors edited the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Extended Data Fig. 1 | Mitochondrial ATP production, NADH, voltage and pH. a, Timecourse of firefly luciferase luminescence signal measured from nine wells after addition of ATP to each well. In this calibration procedure, different amounts of [ATP] are added to each of the nine wells as indicated to the right of each line (μM). The inset shows the measured luminescence versus [ATP] added. Purple shaded area highlights the ‘Working Range’ in which the luminescence signal is a linear function of [ATP]. b, Measurements of [ATP] produced by isolated mitochondria. The calibration procedure shown in a is used to convert the measured luminescence signal to [ATP]. The inset shows the measured mitochondrial matrix free Ca2+ concentration ([Ca2+]m) associated with the ATP measurements in b, c, ATP production rate based on the linear fit to measurements in b and scaled to units of μmol per litre cytosol per second (μM s−1, scaling is based on 80 g mitochondrial protein per litre cardiomyocyte cytosol, for more details see main methods section and Williams et al.). d, The increase in [NADH] at high [Ca2+]m (>2 μM) relative to [NADH] at low [Ca2+]m (<200 nM) using the indicated combination of carbon substrates (P&M, pyruvate and malate; G&M, glutamate and malate; PC&M, palmitoylcarnitine and malate, n = 4 isolated mitochondria preparations per group, *P < 0.05 by two-sided t-test). Data are mean ± s.e.m. [Ca2+]m was measured with Rhod-2. [NADH] measurements were carried out with a luminescence assay kit (Promega, USA, for additional details see Supplementary Methods 1.4). e, Measured TMRM fluorescence ratio (F573/F46) over the maximal fluorescence ratio from the dataset. Mitochondria are exposed to 2,4-dinitrophenol ([DNP] in μM) as indicated. f, Measured extramitochondrial TMRM concentration. g, The mitochondrial inner membrane potential (ΔΨm) in mV is obtained from the measurements in f according to the method by Scaduto & Grotyohann. h, ΔΨm and its corresponding TMRM fluorescence ratio. Linear fit lines are as indicated in the inset. The calibration results shown in panels e–h were verified repeatedly on a daily basis with similar results obtained. i, Excitation and emission spectra of mitochondria loaded with BCECF (2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester). 15 independents measurements are shown at the indicated pH levels (1 μM FCCP is used to equilibrate pHm and the extra-mitochondrial buffer pH) with the similar spectrum shown. j, Measured fluorescence ratio from BCECF-loaded mitochondria at the indicated mitochondrial pH values (pHm). The calibration was verified in two mitochondrial preps, with similar results. k, The pHm measurements following exposure to sodium acetate at the indicated concentrations. Data are mean ± s.e.m. (results are from 3 independent experiments in each of the indicated 7 concentrations of sodium acetate.).
Extended Data Fig. 2 | Calibration of fluorescence measurements with Ca\(^{2+}\) indicators. **a**, Fluo-4 Ca\(^{2+}\) titration curve. Shown is the fraction of Ca\(^{2+}\)-bound fluo-4 bound at the indicated added [Ca\(^{2+}\)]. Each point is a triplicate average. Titration curves are carried out in the indicated buffers. **b**, Fluo-4FF Ca\(^{2+}\) titration curve. Shown is the fraction of Ca\(^{2+}\)-bound fluo-4FF at the indicated added [Ca\(^{2+}\)]. Each point is a triplicate average. **c**, Rhod-2 Ca\(^{2+}\) titration curve. Shown is the fraction of Ca\(^{2+}\)-bound rhod-2 at the indicated added [Ca\(^{2+}\)]. Each point is a triplicate average. Titration is done in the absence and presence of PVP (polyvinylpyrrolidone) in the assay buffer. **d**, Ca\(^{2+}\) titration curve for fura-2AM- or rhod-2AM-loaded mitochondria. Shown is the fraction of Ca\(^{2+}\)-bound fura-2 or rhod-2 at the indicated free Ca\(^{2+}\) concentration in the mitochondrial matrix ([Ca\(^{2+}\)]\(_{m}\)). Each point is a triplicate average. 1 μM FCCP and 1 μM of the Ca\(^{2+}\) ionophore ionomycin are used to equilibrate [Ca\(^{2+}\)]\(_{m}\) with the extra-mitochondrial free [Ca\(^{2+}\)] (that is, [Ca\(^{2+}\)]\(_{extra,free}\)). The [Ca\(^{2+}\)]\(_{extra,free}\) is measured with fluo-5N. Equations fit to the data for **a–d** are detailed in the main methods section.
Extended Data Fig. 3 | Measurements of mitochondrial ATP production when the MCU is blocked by RU360. **a**, Measurements of $[\text{Ca}^{2+}]_{\text{m}}$ in isolated cardiac mitochondria plotted as a function of the measured free extramitochondrial $[\text{Ca}^{2+}]$ (that is, $[\text{Ca}^{2+}]_{\text{extra,free}}$) in the presence of the selective MCU blocker RU360 (5 μM). Grey circles are measurements taken in the presence of pyruvate (1 mM) and malate (0.5 mM). n = 12 independent experiments. Orange circles are measurements done in the presence of glutamate (1 mM) and malate (0.5 mM). n = 12 independent experiments. $[\text{Ca}^{2+}]_{\text{m}}$ was measured with fura-2 loaded into the mitochondrial matrix via its acetoxymethyl (AM) ester, $[\text{Ca}^{2+}]_{\text{extra,free}}$ was measured with fluo-4 in the extra mitochondrial buffer. **b**, Measurements of ATP production plotted as a function of the measured $[\text{Ca}^{2+}]_{\text{extra,free}}$. The measurements of ATP production rates are normalized to the measurements at nominally zero $[\text{Ca}^{2+}]_{\text{extra,free}}$. **c**, ATP production at the indicated measured $[\text{Ca}^{2+}]_{\text{extra,free}}$ (from experiments in **a** and **b**). Grey bars (left) are ATP production with pyruvate and malate. Orange bars (right) are ATP production with glutamate and malate. Data are mean ± s.e.m. n = 3 isolated mitochondria preparations per group (* P < 0.05 by one-way two tailed ANOVA with Bonferroni correction).
Extended Data Fig. 4 | Synthesis of 4-armed PEG-boronic acid. Abbreviations used: HBTU, 1-[bis(dimethylamino)methylene]-1H-benzotriazolium hexafluorophosphate 3-oxide; DIPEA, diisopropylethylamine; DMF, N,N-dimethylformamide. The detailed description of the synthesis procedure is in the methods section.
Extended Data Fig. 5 | $\Delta \Psi_m$ kinetics during mitochondrial ATP production. **a**, Time-dependent stopped-flow measurement of $\Delta \Psi_m$ (upper panel) and of the corresponding $[\text{Ca}^{2+}]_{\text{m}}$ (lower panel). In this protocol (no. 1), mitochondria were incubated with increasing extra-mitochondrial free $\text{Ca}^{2+}$ and at $t = 0$, $500 \mu M [\text{ADP}]$ was added to the mitochondrial mix. Time-dependent depolarization of $\Delta \Psi_m$ is shown as is the near steady-state of $[\text{Ca}^{2+}]_{\text{m}}$. Black line: $[\text{Ca}^{2+}]_{\text{m}} = 50 \text{ nM} (n = 8)$; turquoise: $[\text{Ca}^{2+}]_{\text{m}} = 480 \text{ nM} (n = 3)$; light blue: $[\text{Ca}^{2+}]_{\text{m}} = 750 \text{ nM} (n = 6)$; grey-blue: $[\text{Ca}^{2+}]_{\text{m}} = 1.2 \mu M (n = 8)$; navy blue: $[\text{Ca}^{2+}]_{\text{m}} = 1.7 \mu M (n = 4)$. **b**, Same as **a**, but in this protocol (no. 2), $[\text{Ca}^{2+}]_{\text{m}}$ and $[\text{ADP}]$ ($500 \mu M$) were increased simultaneously at $t = 0$ (salmon-coloured line, $n = 7$). The injected $\text{Ca}^{2+}$ was set so that the $[\text{Ca}^{2+}]_{\text{m}}$ achieved a level between 1.5 and 2 $\mu M$ at 20 s. **c**, Same as **b**, but in this protocol (#3), $[\text{ADP}]$ ($500 \mu M$) rises 10 s before $[\text{Ca}^{2+}]_{\text{m}}$ was increased at $t = 0$ (grey line, $n = 6$). The injected $\text{Ca}^{2+}$ was set so that the $[\text{Ca}^{2+}]_{\text{m}}$ achieved a level between 1.5 and 2 $\mu M$ at 20 s, grey line. In **a**–**c**, the sample size ($n$) represents the number of independent experiments. **d**, The magnitude of $\Delta \Psi_m$ depolarization in experiments **a**–**c**. The sample size is the same as in panels **a**–**c**. (‘$^*$’ and ‘$^*$’ denote statistical comparisons to black and beige data, respectively). **e**, Exponential rate constant of $\Delta \Psi_m$ depolarization in experiments **a** and **b**. The sample size is the same as in panels **a** and **b**. In **d** and **e**, data are mean ± s.e.m. ‘$^P < 0.05$, ‘$^{**}P < 0.01$, ‘$^{***}P < 0.001$ by one-way two-tailed ANOVA with Bonferroni correction.
Extended Data Fig. 6 | Stopped-flow measurements of MCU Ca²⁺ flux and its driving force. 

**(a,e)**, Representative stopped-flow time-dependent measurements of extra-mitochondrial free Ca²⁺ (that is, \([\text{Ca}^{2+}]_{\text{extra,free}}\)). Mitochondria in uptake assay buffer (uAB) with low \([\text{Ca}^{2+}]_{\text{extra,free}}\) (<100 nM) are mixed with uAB buffer with added \([\text{Ca}^{2+}]\) 1 ms before fluorescence measurements begin. The levels of added \([\text{Ca}^{2+}]\) are set so that at the beginning of the measurements the \([\text{Ca}^{2+}]\) added will be as indicated in the inset. In **a**, \([\text{Ca}^{2+}]_{\text{extra,free}}\) is measured with fluo-4 in the uAB and in **e** with Fluo-4FF. **b,f**, The corresponding time-dependent measurements of matrix free Ca²⁺ (that is, \([\text{Ca}^{2+}]_{\text{m}}\)). Insets showing the corresponding time-dependent measurements of \(\Delta \Psi_m\). **c,g**, The corresponding time-dependent measurements of total extra-mitochondrial Ca²⁺ ([Ca²⁺]_{extra,total}). The [Ca²⁺]_{extra,total} is obtained from the fluo-4 or fluo-4FF signals (for more details see the Methods). **d,h**, MCU Ca²⁺ influx (\(J_{\text{mcu}}\) is the first derivative of the [Ca²⁺]_{extra,total}. The \(J_{\text{mcu}}\) is scaled to units of μmol per litre cytosol per second (μM s⁻¹, scaling is based on 80 g mitochondrial protein per litre cardiomyocyte cytosol, for more details see main methods section and Williams et. al.⁷). The shown stopped flow experiments were repeated independently 63 times with similar results at each [Ca²⁺]_{extra,free} as indicated in Fig. 3a.
Extended Data Fig. 7 | Characterization of MityCam, a mitochondrially targeted Ca\(^{2+}\)-sensitive fluorescent-protein probe expressed in heart muscle cells. a, MityCam fluorescence versus \([\text{Ca}^{2+}]_{\text{m}}\). Note that Ca\(^{2+}\) binding decreases MityCam fluorescence. Measurements are in saponin-permeabilized cardiomyocytes; \([\text{Ca}^{2+}]_{\text{m}}\) is set using the Ca\(^{2+}\) ionophore ionomycin (2 μM). The extracellular (bath) solution contains Rhod-2 (tripotassium salt, cell-impermeant) to measure the bath \([\text{Ca}^{2+}]_{\text{b}}\), a proton ionophore carbonyl cyanide m-chlorophenyl hydrazine (CCCP, 500 nM) to set the mitochondrial pH, rotenone (400 nM) to block the ETC and the production of reactive oxygen species, and oligomycin (5 mM) to block reverse-mode consumption of ATP by the ATP synthase, pH 7.8. Fit curve is a single-site binding model (n = 30 cells). b, Top: Confocal line-image from an intact cardiomyocyte expressing MityCam. Bottom: The time-course of changes in \([\text{Ca}^{2+}]_{\text{m}}\) from the confocal fluorescence measurements. Caffeine (10 mM) was applied for 10 s via a local micro-perfusion system to rapidly trigger Ca\(^{2+}\) release from the sarcoplasmic reticulum at the indicated times (highlighted with grey shading). The experiment was repeated independently with n = 10 cells with similar results. c, The average time-course of changes in \([\text{Ca}^{2+}]_{\text{m}}\) following caffeine applications. d, Confocal line-image from an intact cardiomyocyte expressing MityCam in a bath solution devoid of Ca\(^{2+}\) (chelated by 1 mM EGTA) and treated with thapsigargin (1 μM) for 10 min prior to imaging to deplete the sarcoplasmic reticulum of Ca\(^{2+}\). Top panel shows MityCam fluorescence; middle panel shows the fluorescence of sulforhodamine (sulforhodamine is included in the micro-perfusion solution to indicate the exact duration of 10 mM caffeine application). Lower panel shows the time-course of changes in \([\text{Ca}^{2+}]_{\text{m}}\) from the confocal fluorescence measurements. Note that throughout the entire time course of the experiment the extracellular solution is devoid of Ca\(^{2+}\) and also contains 1 μM thapsigargin. The experiment was repeated independently with n = 11 cells with similar results. e, Confocal images of cardiac mitochondria isolated from MityCam expressing cardiomyocytes. f, Measurements of \([\text{Ca}^{2+}]_{\text{m}}\) from isolated single mitochondria expressing MityCam. Top panel (i) shows MityCam fluorescence; lower panel (ii) shows sulforhodamine fluorescence, which indicates the duration of micro-perfusion of 100 μM Ca\(^{2+}\) (see bars above the top panel). Mitochondria isolated from MityCam-expressing cardiomyocytes are adhered to a glass coverslip for confocal microscopy measurements. Rapid step (2–3 ms rise time) of \([\text{Ca}^{2+}]_{\text{m}}\) from 0 (1 mM EGTA) to 100 μM is achieved via a micro-perfusion system. Sulforhodamine is included in the solution applied via the micro-perfusion system to indicate when a mitochondrion is exposed to the solution containing 100 μM Ca\(^{2+}\), \([\text{Ca}^{2+}]_{\text{m}}\) is raised to 1 mM, all solutions also contain ionomycin (5 μM), FCCP (5 μM), oligomycin (1 μM), pH = 7.8. Green trace for MityCam (n = 12 mitochondria) and red for Rhod-2 (n = 9 mitochondria). The time at which 1 μM \([\text{Ca}^{2+}]_{\text{m}}\) is measured is indicated by arrows, this time point is obtained by converting the shown fit lines to units of μM \([\text{Ca}^{2+}]_{\text{m}}\). For additional details see Boyman et al., 2014 (ref. 37).
Extended Data Fig. 8 | MCU conductance and voltage dependence of ATP production in heart and skeletal muscle. a, Measurement of the MCU-dependent Ca\(^{2+}\) influx (J_{mcu}) (nmole mg\(^{-1}\)s\(^{-1}\)) in cardiac mitochondria (green circles, from Fig. 3), skeletal muscle (black circles), and skeletal muscle with Ru360 (5 μM) (red circles) is plotted as a function of measured [Ca\(^{2+}\)]. (n = 63, n = 87, and n = 12 independent experiments, respectively, each with [Ca\(^{2+}\)], [Ca\(^{2+}\)]m and ΔΨm measured). Linear least-squares fit to the heart mitochondria data is shown (slope = 0.015). b, MCU conductance (G) for each measurement shown in a normalized to the minimal conductance (G_{min}) of the cardiac dataset (G/G_{min}) plotted as a function of [Ca\(^{2+}\)]. Linear least-squares fit line to the heart mitochondria data is shown (slope = 6.1). c, Relative number of open MCUs per mitochondrion plotted as a function of [Ca\(^{2+}\)], taken from b after dividing by the [Ca\(^{2+}\)]-dependent unitary conductance of MCU and normalized to the minimal number of open MCUs of the cardiac dataset. Linear least-squares fit line to the heart mitochondria data is shown (slope = 0.051, intercept = 3.3). For skeletal muscle data under [Ca\(^{2+}\)] of 1.5 μM the measurements were done using stopped flow as described in Extended Data Fig. 5. J_{mcu} at [Ca\(^{2+}\)] above 1.5 μM was collected using a multi-well-plate reader with ΔΨm set by using a fixed K\(^{+}\) gradient and the K\(^{+}\) ionophore valinomycin (see Methods). d, The dependence of ATP production on ΔΨm in the absence of carbon substrates and at [Ca\(^{2+}\)]m < 200 nM. The measurements of ATP production rates are normalized to the minimal production rate of each dataset. Measurements from heart mitochondria are shown in green circles (n = 77, replotted from Fig. 4a), the measurements from skeletal muscle mitochondria are in shown in black circles (n = 45 independent experiments). ΔΨm was set by using a fixed K\(^{+}\) gradient and the K\(^{+}\) ionophore valinomycin (see Methods).
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Sample size  All the experiments were performed using sample sizes based on standard protocols in the field. No statistical test was performed to predetermine sample size.

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Randomization  Randomization is not relevant for this study since all biological specimens are obtained from the same animal strain 6-10-week-old wild-type Sprague-Dawley rats (250-300 gr).

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