



## Regulation of ATP production by mitochondrial $\text{Ca}^{2+}$

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### ABSTRACT

Stimulation of mitochondrial oxidative metabolism by  $\text{Ca}^{2+}$  is now generally recognised as important for the control of cellular ATP homeostasis. Here, we review the mechanisms through which  $\text{Ca}^{2+}$  regulates mitochondrial ATP synthesis. We focus on cardiac myocytes and pancreatic  $\beta$ -cells, where tight control of this process is likely to play an important role in the response to rapid changes in workload and to nutrient stimulation, respectively. We also describe a novel approach for imaging the  $\text{Ca}^{2+}$ -dependent regulation of ATP levels dynamically in single cells.

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### 1. Introduction

Mitochondria are the major site of oxidative metabolism, and hence ATP synthesis, in eukaryotic cells. Thus, whereas the glycolytic metabolism of glucose generates 2 ATP molecules, 36–38 are generated by the reactions of the citrate cycle and the oxidation of the resulting NADH and  $\text{FADH}_2$  by the respiratory chain [1]. The latter reactions are tightly controlled by intramitochondrial  $\text{Ca}^{2+}$  in a mechanism designed to ensure that ATP synthesis is closely coupled to the cell's energetic needs. We discuss this form of regulation in detail here, taking as examples two cell types with contrasting energetic needs, the cardiac myocyte and the pancreatic  $\beta$  cell.

### 2. $\text{Ca}^{2+}$ regulation of intramitochondrial oxidative metabolism: a brief historical perspective

Mitochondrial oxidative metabolism has long been recognised as subject to complex regulation by several factors, notably the

*Abbreviations:*  $[\text{ATP}/\text{ADP}]_c$ , cytosolic ATP/ADP ratio;  $[\text{Ca}^{2+}]_c$ ,  $[\text{Ca}^{2+}]_m$ , cytosolic and mitochondrial free concentrations of calcium; EC, excitation-contraction; ICEU, intracellular energy unit;  $K_{\text{ATP}}$ , ATP-sensitive  $\text{K}^+$  channel; MCU, mitochondrial uniporter; mNCX/NCLX, mitochondrial sodium-calcium exchanger; Pi, PPI, inorganic phosphate and pyrophosphate, respectively; RuR, ruthenium red.

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concentrations of ADP and substrate(s) [2]. However, regulation by  $\text{Ca}^{2+}$  has emerged in recent years as a further important means of controlling this vital aspect of cell function. Mammalian mitochondria have a huge capacity for  $\text{Ca}^{2+}$  uptake and for many years it was thought that these organelles served as mobilisable intracellular reservoirs of  $\text{Ca}^{2+}$  ions [3]. Studies in the 1970s and 80s by Denton and McCormack [4] as well as Hansford and Castro [5] and Crompton [6] indicated instead that under basal conditions total mitochondrial  $\text{Ca}^{2+}$  content was low and that increases in cytosolic free  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_c$ ) in response to extrinsic agents (nutrients, hormones, neurotransmitters, etc.), were likely to provoke increases in intramitochondrial free  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_m$ ) concentrations. Thus, based on the properties of a group of key intramitochondrial dehydrogenases (later characterised in some detail – see the following sections) [7,8], it was proposed that the uptake of  $\text{Ca}^{2+}$  ions from the cytosol increased  $[\text{Ca}^{2+}]_m$  from  $\sim 0.1$  to  $10 \mu\text{M}$  or more. The consequent activation of oxidative metabolism would then provide an increased supply of reducing equivalents to drive respiratory chain activity and ATP synthesis. Export of mitochondrial ATP in exchange for ADP was anticipated then to meet an increased ATP demand to fuel energy-requiring processes in the cytosol such as ion pumping, contraction, exocytosis etc. The biochemical analyses (e.g. [9]) upon which the above model was based were subsequently complemented and reinforced in the 1990s by highly selective measurements of intramitochondrial free  $\text{Ca}^{2+}$  in living cells by Rizzuto, Pozzan, Rutter and others [10,11]. These experiments used recombinant expression of the  $\text{Ca}^{2+}$ -sensitive

photoprotein aequorin, targeted to the mitochondrial matrix by in-frame fusion with the signal peptide of cytochrome c subunit VIII. Later extended by Rutter et al. to measurements at the level of single cells [12], this approach provided evidence that basal intramitochondrial  $\text{Ca}^{2+}$  levels were indeed low (similar to or lower than those in the cytosol, i.e.  $\leq 100$  nM) and that the free concentrations of the ion increased within mitochondria in response to  $\text{Ca}^{2+}$ -mobilising agonists. Subsequent refinements of these approaches, including the use of GFP-based probes, highlighted the importance of close contacts between the endoplasmic reticulum and mitochondria in the uptake of  $\text{Ca}^{2+}$  by the latter [13] and demonstrated that mitochondrial  $\text{Ca}^{2+}$  increases stably elevate mitochondrial [ATP] [14].

### 3. Mitochondrial $\text{Ca}^{2+}$ transport: roles of the mitochondrial uniporter MCU and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, mNCX/NCLX

An impediment to progress in this field has been that the mitochondrial  $\text{Ca}^{2+}$  transport proteins were not purified or cloned, and that use of the inhibitors of these pathways is problematic in living cells. However, recent work has very likely identified the proteins responsible for both the  $\text{Ca}^{2+}$  influx and  $\text{Ca}^{2+}$  efflux pathways in mitochondria (see below).

Increases in  $[\text{Ca}^{2+}]_c$  are relayed to the mitochondria by the mitochondrial  $\text{Ca}^{2+}$  uniporter (MCU), resulting in activation of mitochondrial dehydrogenases and stimulation of ATP synthesis. Average  $[\text{Ca}^{2+}]_m$  is controlled by the activities of the MCU and the efflux pathway, the mitochondrial  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (mNCX).  $\text{Ca}^{2+}$  uptake is respiration-dependent, thus high rates of uptake can effectively lower the mitochondrial membrane potential ( $\Delta\Psi_m$ ) (reviewed in [15]). This phenomenon can be observed clearly in isolated mitochondria at supraphysiological concentrations of extramitochondrial  $\text{Ca}^{2+}$ ; however, under physiological conditions of  $\text{Ca}^{2+}$  uptake, a change in  $\Delta\Psi_m$  has not been detected in isolated cardiomyocytes using fluorescent indicators. Thus,  $\text{Ca}^{2+}$  uptake would not normally be expected to lower the membrane potential, at least for a sustained period, or to such an extent that it would inhibit ATP synthesis. Nonetheless, transient and small decreases in mitochondrial membrane potential may occur during cytosolic  $\text{Ca}^{2+}$  peaks in other systems, and can be observed in pancreatic  $\beta$  cells following rhodamine-123 accumulation [16] or sensitive fluorescent ATP probes (Tarasov and Rutter, unpublished and see below).

The  $\text{Ca}^{2+}$  transport pathways of mitochondria are covered in more detail by other reviews in this issue. However, a brief discussion of their properties specifically with regard to energy production will be given here.

Early studies on isolated mitochondria characterised the MCU as a low affinity, high capacity transporter of  $\text{Ca}^{2+}$ , whereas the mNCX had a much lower  $V_{\text{max}}$ , saturating at  $[\text{Ca}^{2+}]_m$  below  $1 \mu\text{M}$  (reviewed in [17,15]). Thus, it was predicted that net  $\text{Ca}^{2+}$  influx would occur only when external  $[\text{Ca}^{2+}]_c$  rose above about 500 nM, much higher than normal resting  $[\text{Ca}^{2+}]_c$  of 100–200 nM [18]. Studies in living adult rat myocytes  $[\text{Ca}^{2+}]_m$  confirmed this prediction, since  $[\text{Ca}^{2+}]_m$  remained less than  $[\text{Ca}^{2+}]_c$  until the latter rose above 500 nM [18]. However, the seminal paper by Rizzuto, Pozzan and colleagues in 1992, using aequorin targeted to mitochondria, revealed that these organelles were capable of taking up  $\text{Ca}^{2+}$  on a fast timescale, due to their proximity to intracellular  $\text{Ca}^{2+}$  stores [10] so they would effectively “see” much higher  $[\text{Ca}^{2+}]_c$  than that of the bulk cytosol. In some cells and species  $[\text{Ca}^{2+}]_m$  has been reported to change in response to physiological, submicromolar, changes in  $[\text{Ca}^{2+}]_c$ ; but, in cardiomyocytes for example, the reflected changes in  $[\text{Ca}^{2+}]_m$  were small, 10–20 nM [19].

Research into the mitochondrial  $\text{Ca}^{2+}$  transporters is now likely to undergo another step change, with the identification of

proteins corresponding to both MCU and mNCX. Two papers published simultaneously in 2011 identified a  $\text{Ca}^{2+}$  uptake pathway in mitochondria, inhibited by ruthenium red: Baughman et al. [20] identified the MCU as a novel predicted transmembrane protein (CCD109A) that formed oligomers in the inner membrane, and interacted with their previously identified regulator protein, MICU1. Silencing MCU in cultured cells or in vivo in mouse liver abrogated  $\text{Ca}^{2+}$  uptake, but membrane potential and respiration remain intact. A point mutation removed the sensitivity to ruthenium red (RuR). The other paper [21] identified MCU as a 40 kD protein with 2 transmembrane regions. RNA silencing of MCU in HeLa cells reduced mitochondrial  $\text{Ca}^{2+}$  uptake, and purified MCU reconstituted in bilayers exhibited  $\text{Ca}^{2+}$  channel activity that was sensitive to RuR.

In 2010, Palty et al. [22] identified NCLX in mitochondria – this protein is a novel NCX, lying in an ancestral branch of the NCX superfamily. The protein occurred as 50, 70 and 100 kD forms in mitochondrial fractions of mouse heart and brain; the 100 kD form likely representing dimers. The authors demonstrated that mitochondrial  $\text{Ca}^{2+}$  efflux was enhanced upon over-expression of NCLX in HEK 293 cells and inhibited by CGP 371457, and that  $\text{Ca}^{2+}$  efflux was greatly reduced using an anti-NCLX siRNA.

### 4. $\text{Ca}^{2+}$ sensitive mitochondrial enzymes

#### 4.1. FAD-glycerol phosphate dehydrogenase (FAD-GPDH)

Physiological levels of  $[\text{Ca}^{2+}]_c$  regulate FAD-GPDH ( $K_{0.5} \simeq 0.1 \mu\text{M}$  in rat liver mitochondrial extracts [23]), via a cytosol-facing EF-hand binding site for  $\text{Ca}^{2+}$  [24]. Increases in extramitochondrial  $\text{Ca}^{2+}$  lowered the  $K_M$  for DL-glycerolphosphate ( $K_{0.5} = 30\text{--}100$  nM) in permeabilized INS1  $\beta$  cells [25].

#### 4.2. Pyruvate dehydrogenase phosphatase (PDHP)

The 50 MDa pyruvate dehydrogenase (PDH) multi-enzyme complex catalyses the irreversible reaction:  $\text{pyruvate} + \text{CoA} + \text{NAD} \rightarrow \text{acetyl-CoA} + \text{NADH}_2 + \text{CO}_2$ , with the product, acetyl-CoA, then entering the citrate cycle or fatty acid synthesis. The complex comprises multiple copies of three enzymes: E1 (that decarboxylates pyruvate); E2 (forms acetyl-CoA) and E3 (reduces  $\text{NAD}^+$  to NADH).

The activity of the PDH complex is a rate-limiting step for glucose oxidation and is therefore reflected in the rate of respiration and ATP synthesis in mammalian tissues [26]. The regulation of the PDH complex activity is achieved via end-product inhibition and reversible phosphorylation by a PDH kinase, which inhibits the activity of E1 subunit via phosphorylation at three sites around S293 [27,28]. A  $\text{Mg}^{2+}$ -dependent PDH phosphatase, in turn, dephosphorylates the E1 subunit.

The activity of the  $\text{Ca}^{2+}$ -regulated phosphatase depends on the association of the catalytic subunit of the predominating isoform 1 of PDH phosphatase (PDHP1c) and the L2 domain of the E2 subunit of the PDH complex, which is potentiated by  $\text{Ca}^{2+}$  [29]. PDHP1c and the L2 domain of the E2 subunit lack  $\text{Ca}^{2+}$  binding motifs so the binding site for the cation is likely to be formed jointly by residues of both interacting proteins [30].  $\text{Ca}^{2+}$  activates the phosphatase in heart mitochondrial extracts with  $K_{0.5}$  of  $\sim 1 \mu\text{M}$  [31] and extramitochondrial  $\text{Ca}^{2+}$  stimulates phosphatase activity over the physiological range (0.1–1.0  $\mu\text{M}$ ), in rat heart [32] liver [33] and adipose tissue [34] mitochondria.

#### 4.3. NAD<sup>+</sup>-isocitrate dehydrogenase (NAD-ICDH)

NAD-ICDH is a hetero-octamer consisting (in eukaryotes) of  $2 \times (2\alpha, \beta, \gamma)$  subunits [35]. The enzyme binds  $\sim 2\text{Ca}^{2+}$ /octamer [7]

but the binding site is as yet unknown. In the presence of ADP,  $\text{Ca}^{2+}$  decreased the  $K_M$  for isocitrate  $\sim 8$ -fold [36]) with a  $K_{0.5}$  for  $\text{Ca}^{2+}$  of  $1.2 \mu\text{M}$  in mitochondrial extracts [37] and  $5\text{--}43 \mu\text{M}$  (increasing with ATP/ADP ratio), in toluene-permeabilised mitochondria [38]. ADP (or ATP) and  $\text{Mg}^{2+}$ -isocitrate are required for  $\text{Ca}^{2+}$  binding [7]. The lower sensitivity of this enzyme to  $\text{Ca}^{2+}$  compared to other intramitochondrial dehydrogenases may extend the range over which  $\text{Ca}^{2+}$  is able to modulate oxidative metabolism by these organelles [8].

#### 4.4. 2-Oxoglutarate dehydrogenase (OGDH)

The OGDH complex, similarly to the PDH complex, consists of multiple copies of three subunits: E1 ( $\alpha$ -ketoacid decarboxylase), E2 (dihydrolipoyl transacetylase) and E3 (dihydrolipoamide dehydrogenase) [39]. The activity of the complex is directly regulated by  $\text{Ca}^{2+}$ , most likely via the E1 subunit [40] ( $K_{0.5} = 1\text{--}7 \mu\text{M}$  in mitochondrial extracts) [7]. Each enzyme complex binds  $\sim 3.5\text{Ca}^{2+}$  ions, suggestive of a  $\text{Ca}^{2+}$  binding site at E1 dimer interfaces [7].  $\text{Ca}^{2+}$  decreases the  $K_M$  for 2-oxoglutarate with little effect on  $V_{\text{max}}$  [41]. The  $K_{0.5}$  for extramitochondrial  $\text{Ca}^{2+}$  is  $\sim 0.15 \mu\text{M}$  in intact heart mitochondria [37,42].

#### 4.5. $F_1\text{--}F_0$ ATP synthase

The possibility that the rate of ATP production can be regulated independently from the respiration rate or mitochondrial membrane potential ( $\Delta\psi_m$ ) has been demonstrated in intact mitochondria [43] and confirmed using control theory analysis [44]. Early in vitro [45] and in vivo [46] studies reported  $F_1\text{--}F_0$  synthase as a potential target of this regulation. Although shown to bind  $\text{Ca}^{2+}$  directly [47], the  $F_1\text{--}F_0$  ATP synthase is likely to be regulated via post-translational modifications and phosphorylation of the  $\gamma$ -subunit was sensitive to mitochondrial  $\text{Ca}^{2+}$  [48] with a  $K_{0.5}$  in the  $\mu\text{M}$  range. Recent work has identified a protein that binds to the  $F_1\text{--}F_0$  synthase in  $\text{Ca}^{2+}$ -dependent manner, resulting in an increased capacity for ATP production [49].

#### 4.6. Other potential mitochondrial targets for $\text{Ca}^{2+}$

*Cytochrome oxidase (COX)* is allosterically inhibited by ATP/ADP (binding to the matrix domain of subunit IV). This inhibition is switched on by the cAMP-dependent phosphorylation of subunits II (and/or) III and Vb by protein kinase A (PKA), and results in an enhanced H<sup>+</sup>/e<sup>-</sup> stoichiometry and therefore more efficient energy transduction.  $\text{Ca}^{2+}$ -dependent phosphatase reverses the cAMP-mediated effect (in vitro or in isolated mitochondria) [50]. cAMP is likely to stimulate the phosphorylation at Tyr304 of subunit I, which decreases the  $V_{\text{max}}$  and increases the  $K_M$ . COX binds  $\text{Ca}^{2+}$  with a  $K_d$  of around  $1 \mu\text{M}$ , competitively with  $\text{Na}^+$  [51].

*Malate–aspartate shuttle activity.*  $\text{Ca}^{2+}$  activates this shuttle in brain mitochondria ( $K_{0.5} = 0.3 \mu\text{M}$ ) [52]. The aspartate–glutamate transporter has a cytosolic EF domain and is also regulated by  $[\text{Ca}^{2+}]_c$ , albeit in the supraphysiological range [53].

*Pyrophosphatase activity in liver and heart mitochondria* is inhibited by  $\text{Ca}^{2+}$ , due to competition of  $\text{CaPPi}$  with  $\text{MgPPi}$  ( $K_{0.5} = 12 \mu\text{M}$ ) [54]. The accumulation of  $\text{PPi}$  is expected to result in an increase in mitochondrial volume and activation of the respiratory chain.

*The ATP-Mg<sup>2+</sup>/P<sub>i</sub> transporter (SCaMC1).* This transporter is stimulated by extramitochondrial  $\text{Ca}^{2+}$  and may regulated the total content of adenine nucleotides inside mitochondria, and hence alter  $\text{Ca}^{2+}$  buffering. SCaMC1 is overexpressed in some cancer cell lines, and this has been proposed to contribute to the ability of the cells to withstand  $\text{Ca}^{2+}$  loads that would otherwise trigger opening of the mitochondrial permeability transition pore [56].

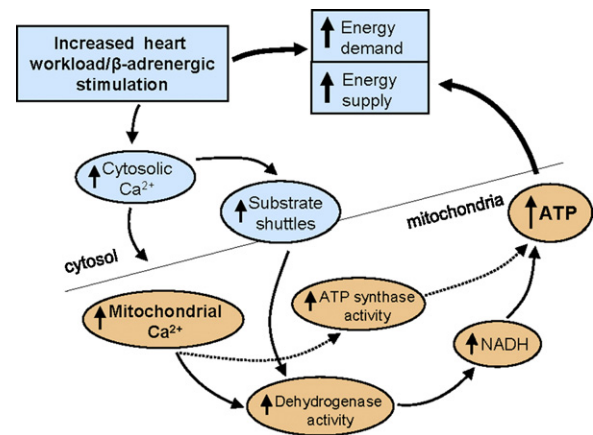


Fig. 1. Role of  $\text{Ca}^{2+}$  uptake by mitochondria in the heart.

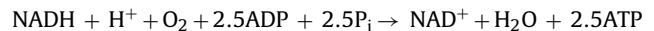
$\text{Ca}^{2+}$  reportedly potentiates fatty acid oxidation to  $\beta$ -hydroxybutyrate [55] though the underlying mechanisms are not understood.

## 5. Role of mitochondrial $\text{Ca}^{2+}$ transport in specific cell types

### 5.1. Cardiac myocyte

Fig. 1 presents a summary of the model proposed for mitochondrial  $\text{Ca}^{2+}$  uptake in the control of ATP synthesis in the heart.

Oxidative phosphorylation in mitochondria provides over 90% of ATP production in the heart [45]. The overall equation is given below, although estimates of the number of ATP molecules synthesised per molecule of NADH vary:



ATP production can potentially be regulated by several different mechanisms, such as increases in NADH/NAD<sup>+</sup> and ADP/ATP. It was originally proposed that changes in the ADP/ATP ratio were the main regulator of ATP synthesis [2], and this was easily demonstrated in isolated mitochondria. However, later experiments using isolated beating rat hearts found that high energy phosphate levels did not change under conditions that imposed a large increase in energy demand, e.g. increases in workload of the heart [57,58]. In fact utilisation of ATP under these conditions was exactly matched by an increase in ATP production. Changes in ATP are initially buffered by the phosphocreatine system but this only lasts for a few seconds, implying the existence of alternative mechanisms for rapidly increasing ATP synthesis.

This role was later found to be fulfilled by intramitochondrial  $\text{Ca}^{2+}$ , following the discovery of the activation by  $\text{Ca}^{2+}$  of mitochondrial enzymes, as discussed above, namely the matrix dehydrogenases PDH [31], NAD-ICDH [37], OGDH [9], and possibly the ATP synthase [59]. The observations led Denton, McCormack and colleagues to propose that an increase in the supply of reducing equivalents in the form of NADH and  $\text{FADH}_2$  resulting from  $\text{Ca}^{2+}$  activation of the dehydrogenases would increase ATP production; thus suggesting a parallel activation model of stimulation of contraction and ATP synthesis by  $\text{Ca}^{2+}$  [60,61] (and see Fig. 1). The mitochondrial  $F_1F_0$ ATP synthase (ATPase) may also be stimulated by  $\text{Ca}^{2+}$  in this setting [43,47,62,63].

Whether  $\text{Ca}^{2+}$  transport into mitochondria occurs in the heart, and whether or not  $[\text{Ca}^{2+}]_m$  changes on a beat-to-beat basis in this organ, has remained controversial for some time. Based on work in isolated mitochondria, the MCU and mNCX pathways could certainly not respond quickly enough to the very rapid (ms) changes in  $[\text{Ca}^{2+}]_c$  that occur during excitation-contraction (EC) coupling.

However, more recent work using isolated myocytes has suggested that mitochondrial  $\text{Ca}^{2+}$  transients do occur during EC coupling in neonatal and adult cardiac myocytes [64–66]. There is conflicting data on this in the literature since other studies reported that mitochondrial  $\text{Ca}^{2+}$  accumulation occurred over tens of seconds in cardiac myocytes [18,67,68]. Part of the conflict may be due to different species or stage of development (neonate versus adult), although why and how mitochondrial  $\text{Ca}^{2+}$  spiking occurs in some species but not others remains unknown. However, there is agreement that the main role of MCU and mNXC under physiological conditions is to translate or decode cytosolic  $\text{Ca}^{2+}$  signals in the mitochondria, so that  $\text{Ca}^{2+}$  activation of mitochondrial metabolism occurs.

In addition to the known proximity of mitochondria to the ER in a variety of cell types, there is now evidence for a direct physical coupling that persists during isolation of mitochondria (reviewed in [69]). This evidence was provided in heart by isolation of mitochondrial fractions with associated SR components that were highly resistant to purification [70]. These “SR appendices” were capable of transferring  $\text{Ca}^{2+}$  directly to mitochondria (measured with rhod-2 fluorescence) upon stimulation with caffeine (which activated the ryanodine receptor, thus triggering release of  $\text{Ca}^{2+}$  from the SR), and of increasing the NADH autofluorescence signal, suggesting activation of the dehydrogenases. This was the first direct evidence that  $\text{Ca}^{2+}$  transfer directly from the SR to mitochondria is capable of activating oxidative metabolism [70].

Activation of ATP synthesis in the heart by mitochondrial  $\text{Ca}^{2+}$ . Parallel measurements of  $[\text{Ca}^{2+}]_m$  and  $[\text{Ca}^{2+}]_c$  in rat myocytes revealed that resting  $[\text{Ca}^{2+}]_m$  was about 80 nM compared with resting  $[\text{Ca}^{2+}]_c$  of 150 nM [18]. Whereas in mitochondria isolated from beating hearts the  $[\text{Ca}^{2+}]_m$  was estimated to be slightly higher, about 170 nM [71]. Upon rapid stimulation in presence of an adrenergic agonist (myocytes), or increased workload (isolated hearts), the value of  $[\text{Ca}^{2+}]_m$  increased to 500–1000 nM [18,71]. These values of  $[\text{Ca}^{2+}]_m$  under physiological conditions are within the range for activation of the dehydrogenases (see above).

There is also evidence to suggest that extramitochondrial  $\text{Ca}^{2+}$  regulates substrate supply into mitochondria via the aspartate–glutamate carrier (aralar) and the malate–aspartate shuttle (see above). This newer mechanism has been proposed to act as a “gas pedal, supplying... substrate on demand” [72].

A problem in elucidating the relationship between  $[\text{Ca}^{2+}]_m$  and [ATP] was that although ATP has been measured in whole hearts, and now in animals and humans using non-invasive  $^{31}\text{P}$ -NMR (reviewed in [73]), only relatively slow responses were measured, and so it could not be determined whether ATP was varying beat-to-beat, or during the time taken for mitochondria to accumulate sufficient  $\text{Ca}^{2+}$  to activate the dehydrogenases.

We directly measured ATP in mitochondria and cytosol,  $[\text{ATP}]_m$  and  $[\text{ATP}]_c$ , respectively, using targeted luciferase [64], and  $[\text{Ca}^{2+}]_m$  and  $[\text{Ca}^{2+}]_c$  using targeted aequorin: in adult cardiac myocytes stimulated to contract at 2 Hz, there was no change in [ATP] in either compartment on addition of isoproterenol, despite an increase in contractile force and increases in both  $[\text{Ca}^{2+}]_m$  and  $[\text{Ca}^{2+}]_c$ . This indicated that  $[\text{ATP}]_c$  was extremely well-buffered in myocytes, i.e. that the rate of ATP supply and/or synthesis can keep pace with that of ATP breakdown, even under conditions that imposed a large energy demand on the cell. This lack of change in [ATP] is in agreement of studies using whole hearts [74].

However, when we allowed cells to rest, then suddenly stimulated to contract in the presence of isoproterenol (“standing-start” experiments),  $[\text{ATP}]_m$  showed a significant transient drop (up to 22% in some cells) followed by recovery to higher than initial levels [64]. These changes likely reflect an initial activation of ATP-requiring processes in the cytosol, such as ion pumps and contractile proteins, which would cause a drop in  $[\text{ATP}]_m$  before a

time-dependent activation of mitochondrial oxidative metabolism by  $\text{Ca}^{2+}$  stimulated ATP synthesis [75]. The changes in  $[\text{ATP}]_c$  were much smaller than those in the mitochondrial matrix, again suggesting that even when  $[\text{ATP}]_m$  does change, the cytosolic energy supply is very rapidly matched to the increased demand. The lag phase where  $[\text{ATP}]_m$  falls before recovering, exactly matches the time course for  $[\text{Ca}^{2+}]_m$  to increase [64,76].

Similarly it has been generally observed that under conditions of adequate oxygen consumption, NAD(P)H levels remain constant in beating hearts and myocytes, again highlighting that ATP supply and demand are extremely well-coupled. The only time NAD(P)H was observed to decrease was in similar experiments to the “standing start” experiments described above, where there was an initial drop in NAD(P)H levels before recovery, again being slightly preceded by an increase in  $[\text{Ca}^{2+}]_m$  [77].

Do ADP and  $\text{Ca}^{2+}$  have joint role in regulating ATP production in myocytes? Balaban [73] has argued that, given the importance of ensuring a rapid response system of ATP synthesis in the myocardium, more than one mechanism is likely to operate to coordinate ATP supply and demand. Whether  $[\text{Ca}^{2+}]_m$  is absolutely required to maintain ATP levels during continuous or large increases in energy demand is not yet known and the idea of  $[\text{Ca}^{2+}]_m$  being the only factor that is important physiologically in regulating ATP supply and demand is not universally accepted. Our own studies show that in rat cardiac myocytes when stimulation rate is increased from 0.2 Hz to 4 Hz, which presumably causes a large increase in ATP demand, there is very little change in  $[\text{Ca}^{2+}]_m$  unless an adrenergic agonist is also present [67]; presumably this may increase the systolic  $[\text{Ca}^{2+}]_c$  sufficiently that MCU is activated to increase  $[\text{Ca}^{2+}]_m$ .

One possibility is again that of the subcellular arrangement of mitochondria and proximity to other cell compartments: Saks and colleagues have put forward the idea that the subcellular architecture changes during contraction, causing a decrease in the apparent  $K_M$  of ADP for stimulating respiration, and removing diffusion limits of ADP that occur in resting cells (reviewed in [78]). This requires the mitochondria to be organised in structural units with ATP consuming processes in the cell, such as myofibrils, SR and sarcolemmal ATPases, so-called “intracellular energy units (ICEUs)” – where channelling of ADP occurs.

This group also argues that  $[\text{Ca}^{2+}]_m$  may not be the sole factor, or possibly not a major factor, in regulating ATP supply and demand in the heart, since modelling studies have predicted that the increase in  $[\text{Ca}^{2+}]_m$  in the heart would only be enough to stimulate respiration 2–3 fold, whereas increases of 10–20-fold are seen in vivo [43,79]. Therefore localised regulation of [ADP] in the ICEUs would present a method of stimulating ATP synthesis by mitochondria without changes in bulk [ADP] or [ATP] [80]. However, it is not clear how far these studies can be extrapolated to the physiological situation of rapidly beating cardiac myocytes, either cells or whole hearts that are continually shortening and re-lengthening. If the authors are correct, the theory also implies that control of respiration by ADP is operating on a millisecond timescale (in rat cardiac myocytes, for example). Alternatively, respiration could be sensitive to the time-averaged local [ADP], in a similar manner as we suggested above for rapid changes in  $[\text{Ca}^{2+}]_m$ .

*Implications for cardiac disease.* Pathologically, an increase in  $[\text{Ca}^{2+}]_m$  has been associated with the transition from reversible to irreversible cell injury in ischaemia/reperfusion injury of the heart, and a major contributing factor to this injury is  $\text{Ca}^{2+}$ -induced opening of the mitochondrial permeability transition pore [81,82]. A role for  $[\text{Ca}^{2+}]_m$  in heart failure, and for the mitochondrial  $\text{Ca}^{2+}$  efflux pathway in the treatment of this condition has also been suggested [83].

Both MCU and mNXC have been proposed as possible targets for cardioprotective drugs (reviewed in [76]). However, protective

effects of RuR have been found in ischaemia reperfusion injury [84,85], although it is not certain that the drug was acting on MCU since it is not specific and also affects SR  $\text{Ca}^{2+}$  flux [86]. Indeed we found, using a myocyte model of hypoxia/reoxygenation injury, that RuR was not protective [87], but that inhibiting the mNCCX with clonazepam was. We concluded that the route of  $\text{Ca}^{2+}$  entry into mitochondria under hypoxic conditions is likely to be the mNCCX rather than MCU [87,88].

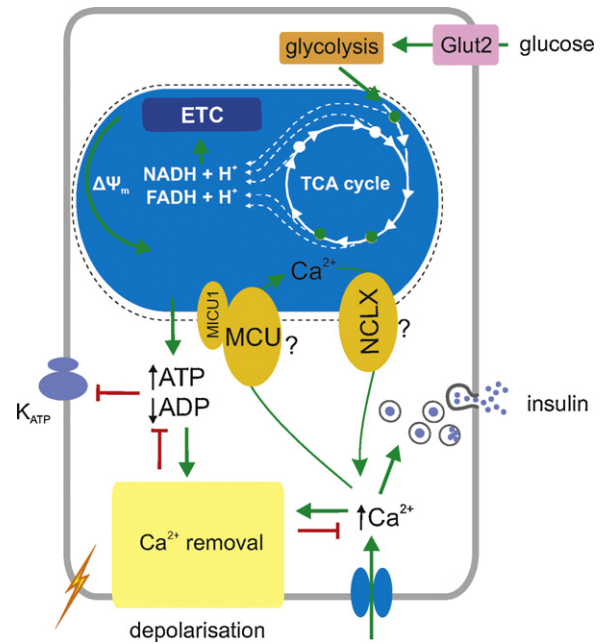
The mNCCX has also been considered a target for cardioprotection in heart failure. Recent work from O'Rourke's group has shown that dysregulation of  $\text{Na}^+$  homeostasis in heart failure may be a primary cause of mitochondrial dysfunction: in a guinea-pig model of heart failure (induced by aortic constriction), intracellular  $[\text{Na}^+]$  was 16 mM compared with 5 mM in control cells [89]. Rapid pacing of the cells induced a decrease in NADH fluorescence, an indirect indicator of respiratory chain activity, whereas this was maintained in controls. CGP 37157, an inhibitor of mNCCX, was able to prevent the decrease in NADH in the failing myocytes. It is thus likely to restore ATP levels although this has yet to be shown directly: earlier work showed that the mNCCX is capable of regulating  $[\text{Ca}^{2+}]_m$  and dehydrogenase activity since adding  $\text{Na}^+$  to isolated mitochondria shifts the activation curves for PDH and OGDH by  $\text{Ca}^{2+}$  to the right [32].

### 5.2. The pancreatic islet $\beta$ -cell

Insulin secretion from pancreatic  $\beta$ -cells is prompted by increases in blood glucose over the physiological range (4–8 mM), thanks to the expression of high  $K_M$  glucose transporters (Glut2 in rodents) and glucokinase [90]. Defective  $\beta$ -cell glucose sensitivity [91,92] as well as a decrease in numbers of these fuel-sensing cells [93,94] result in hyperglycaemia and eventually type 2 diabetes.

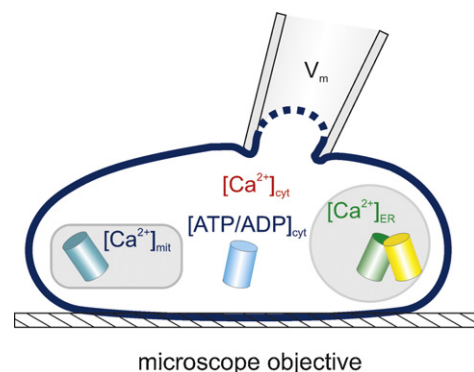
The metabolic configuration of  $\beta$ -cells is adapted to favour the complete oxidation of glucose by mitochondria [95] through the suppression of genes involved in the production of lactate (LDHA and the plasma membrane monocarboxylate transporter, SLC16A1/MCT1) [96–99], and the expression at high levels of FAD-GPDH [24,25,100]. Consequently, increases in extracellular glucose are obligatorily linked to increased flux through the citrate cycle [100] and lead to clear elevations in cytosolic ATP/ADP ratio [101], which block ATP-sensitive  $\text{K}^+$  ( $\text{K}_{\text{ATP}}$ ) channels on the plasma membrane [102]. This triggers plasma membrane depolarisation, electrical activity and  $\text{Ca}^{2+}$  influx into the cytosol via voltage-gated  $\text{Ca}^{2+}$  channels [103]. The elevated  $[\text{Ca}^{2+}]_c$  then triggers exocytosis of insulin granules [90,104] (Fig. 2). Other, more poorly defined “ $\text{K}_{\text{ATP}}$ -independent” effects of glucose, possibly involving the inhibition of AMP-activated protein kinase [105,106], also contribute to “amplifying” effects of the sugar on secretion [107]. Changes in ATP/ADP ratio also regulate exocytosis directly [108], modulating the effects of cAMP [109,110].

In contrast to most tissues including the heart (see above), cytosolic ATP changes over a relatively wide range in  $\beta$ -cells [111] and this is likely to play a key-signalling role. Our early measurements of glucose-induced ATP dynamics in MIN6 and primary islet  $\beta$  cells [75,112,113], using the recombinant bioluminescent reporter firefly luciferase (an approach whose sensitivity is relatively poor), suggested a monophasic elevation of ATP occurs in response to high glucose, although evidence for oscillations was also obtained [113,114]. Significant differences between the glucose-induced changes in the bulk cytosol and a sub-plasma membrane “microdomain”, as well as the mitochondrial influx, were also demonstrated [75]. Importantly, blockade of  $\text{Ca}^{2+}$  influx with EGTA or through the inhibition of voltage-gated  $\text{Ca}^{2+}$  channels substantially inhibited glucose-induced ATP/ADP increases, implicating a role for  $\text{Ca}^{2+}$  influx in metabolic control [113].

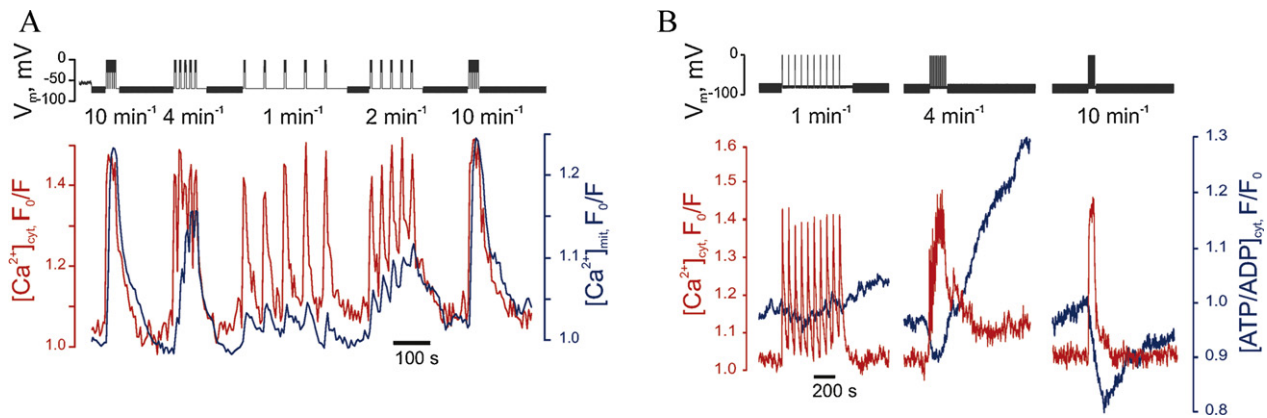


**Fig. 2.** Potential role of  $\text{Ca}^{2+}$  uptake by mitochondria in the pancreatic  $\beta$ -cell. ETC, electron transport chain. See the text for further details of the  $\text{Ca}^{2+}$  sensitive intramitochondrial dehydrogenases.

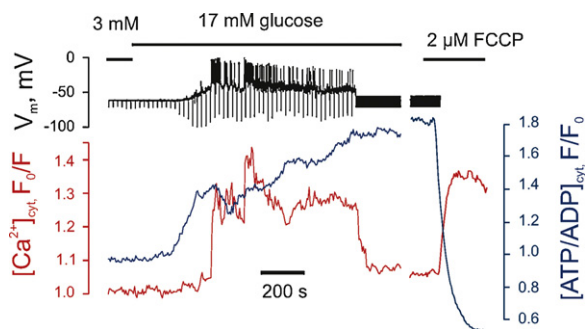
In light of the above limitations, we have recently established a technique whereby  $[\text{Ca}^{2+}]_m$  can be imaged in real time in single primary living  $\beta$ -cells using a mitochondrially targeted, adenovirally-delivered and GFP-based reporter (“Pericam”; 2mt8RP) simultaneously with  $[\text{Ca}^{2+}]_c$  measured with the trappable small molecule, Fura Red (Fig. 3). Importantly, after the formation of a perforated patch, imaging of the two probes can be performed simultaneously with recordings or manipulation (at will) of plasma membrane potential. This approach demonstrates that  $[\text{Ca}^{2+}]_m$  is unable to track fast oscillations of  $[\text{Ca}^{2+}]_c$ , imposed by single action potentials that have the duration of 50–200 ms in  $\beta$ -cells [115]. However,  $[\text{Ca}^{2+}]_m$  does follow slow changes in  $[\text{Ca}^{2+}]_c$  similar to those associated with slow electrical bursting [115]. This “accumulation” mechanism makes  $[\text{Ca}^{2+}]_m$  highly sensitive to the frequency of  $\text{Ca}^{2+}$  oscillations [115] (Fig. 4), such that low frequency spiking barely affects  $[\text{Ca}^{2+}]_m$ . This “Frequency-Amplitude” demodulation may thus allow filtering of low level stimulation (for example at low glucose concentrations) whilst amplifying the response as glucose levels rise – thus contributing to the well-known highly sigmoidal response of insulin secretion to blood glucose levels [95]. This positive feedback may play an important role in coordinating insulin



**Fig. 3.** Principle of simultaneous patch-clamp recording and multiparameter imaging of compartmentalised  $\text{Ca}^{2+}$  and ATP/ADP in single cells.



**Fig. 4.** Mitochondrial  $\text{Ca}^{2+}$  (A) and  $[\text{ATP/ADP}]_{\text{cyt}}$  (B) increases respond to the frequency of electrical bursting and  $\text{Ca}^{2+}$  oscillations in a single primary mouse  $\beta$ -cell. Depolarisations of the plasma membrane were applied at different frequency from  $10 \text{ min}^{-1}$  to  $1 \text{ min}^{-1}$  [115].



**Fig. 5.** Multiphasic increases in  $[\text{ATP/ADP}]_{\text{c}}$  are prompted by high glucose in the  $\beta$ -cell. Recordings and image collection were as in Fig. 4; note the second phase of ATP/ADP increase  $\sim 400 \text{ s}$  after the increase in glucose [115].

secretion in pancreatic islets where  $\beta$ -cells are electrically coupled [116]. Using the same approach to combine imaging of  $[\text{Ca}^{2+}]_{\text{c}}$  and  $[\text{ATP/ADP}]_{\text{c}}$ , using the genetically encoded fluorescent sensor Perceval [117], we have been able to demonstrate a biphasic increase in  $[\text{ATP/ADP}]_{\text{c}}$  in response to high glucose, with  $\text{Ca}^{2+}$  entry into cytosol being the key factor for the second phase [115] (Fig. 5).

These observations are consistent with a model for the  $\beta$ -cell in which elevation of  $[\text{Ca}^{2+}]_{\text{c}}$  leads firstly to the activation of a number of processes (e.g.  $\text{Ca}^{2+}$  pumping [118,119]) that elevate ATP consumption [101,113,115]. This is then matched by a  $\text{Ca}^{2+}$ -dependent potentiation of mitochondrial metabolism [115,120]. The existence of these two phases thus generates a biphasic increase in  $[\text{ATP/ADP}]_{\text{c}}$  (Fig. 5). Whilst the role in stimulus-secretion coupling of the second phase is unclear, an intriguing possibility is that it is involved in the mobilisation of secretory granules from a “reserve pool” in the cell [95].

Consistent with these findings, silencing of MCU [115], or deployment of an intramitochondrial  $\text{Ca}^{2+}$  buffer [120], blocks both mitochondrial  $\text{Ca}^{2+}$  uptake and the second phase of ATP/ADP increase. Interestingly, glucolipotoxic conditions mimicking the diabetic milieu and known to affect mitochondrial integrity [121], suppress  $[\text{Ca}^{2+}]_{\text{m}}$  changes and selectively reduce the second phase of  $[\text{ATP/ADP}]_{\text{c}}$  increase [115]. Defective mitochondrial  $\text{Ca}^{2+}$  handling may thus play a part in the defects in insulin secretion in type 2 diabetes.

## 6. Conclusions

There is no doubt that  $[\text{Ca}^{2+}]_{\text{m}}$  plays an important role in both myocardial energy production and in intracellular  $\text{Ca}^{2+}$  signalling in many other systems including the pancreatic  $\beta$ -cell.

Future challenges will involve further dissection of the molecular mechanisms involved (including identification of the  $\text{Ca}^{2+}$  binding sites on the mitochondrial dehydrogenases) and exploration of the potential therapeutic use of new information flowing from the identification of the molecular players in  $\text{Ca}^{2+}$  transport.

## Conflict of interest

None of the authors declares any conflict of interest.

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