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Review

The ups and downs of mitochondrial calcium signalling in the heart

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ABSTRACT

Regulation of intramitochondrial free calcium ($[Ca^{2+}]_m$) is critical in both physiological and pathological functioning of the heart. The full extent and importance of the role of $[Ca^{2+}]_m$ is becoming apparent as evidenced by the increasing interest and work in this area over the last two decades. However, controversies remain, such as the existence of beat-to-beat mitochondrial Ca^{2+} transients; the role of $[Ca^{2+}]_m$ in modulating whole-cell Ca^{2+} signalling; whether or not an increase in $[Ca^{2+}]_m$ is essential to couple ATP supply and demand; and the role of $[Ca^{2+}]_m$ in cell death by both necrosis and apoptosis, especially in formation of the mitochondrial permeability transition pore. The role of $[Ca^{2+}]_m$ in heart failure is an area that has also recently been highlighted. $[Ca^{2+}]_m$ can now be measured reasonably specifically in intact cells and hearts thanks to developments in fluorescent indicators and targeted proteins and more sensitive imaging technology. This has revealed interactions of the mitochondrial Ca^{2+} transporters with those of the sarcolemma and sarcoplasmic reticulum, and has gone a long way to bringing the mitochondrial Ca^{2+} transporters to the forefront of cardiac research. Mitochondrial Ca^{2+} uptake occurs via the ruthenium red sensitive Ca^{2+} uniporter (mCU), and efflux via an Na^+/Ca^{2+} exchanger (mNCX). The purification and cloning of the transporters, and development of more specific inhibitors, would produce a step-change in our understanding of the role of these apparently critical but still elusive proteins. In this article we will summarise the key physiological roles of $[Ca^{2+}]_m$ in ATP production and cell Ca^{2+} signalling in both adult and neonatal hearts, as well as highlighting some of the controversies in these areas. We will also briefly discuss recent ideas on the interactions of nitric oxide with $[Ca^{2+}]_m$.

1. Introduction

Mitochondria were first shown to be capable of taking up Ca^{2+} in the 1960's [1,2], and the pathway for Ca^{2+} efflux was discovered by Carafoli in 1974 as an Na^+-dependent Ca^{2+} release mechanism in isolated heart mitochondria[3]. With considerable insight, Carafoli and Lehninger wrote in 1971: "…Ca^{2+} accumulation plays a general and fundamental role in vertebrate cell physiology"[4]. It has taken us almost forty years to begin to appreciate the full extent and importance of this role.

Under physiological conditions in the heart the roles of intramitochondrial free calcium ($[Ca^{2+}]_m$) include coupling of ATP supply and demand by activation of the Ca^{2+}-sensitive dehydrogenases [5] and possibly the ATP synthase [6], modulating whole-cell Ca^{2+} signalling via interactions with endo/sarcoplasmic reticulum Ca^{2+}-release pathways [7] and plasma membrane Ca^{2+} channels [8], and more recently mitochondrial fission/fusion [9]. An increase in $[Ca^{2+}]_m$ has been associated with the transition from reversible to irreversible cell injury in ischaemic/reperfused myocardium for many years [10], and we now know that a major contributing factor to this injury is Ca^{2+}-induced opening of the mitochondrial permeability transition pore [11,12]. A role for $[Ca^{2+}]_m$ in heart failure, and for the mitochondrial Ca^{2+} efflux pathway in the treatment of this condition has also been suggested [13,14]. Ca^{2+} can activate nitric oxide synthase (NOS) under both physiological and pathological conditions, which may impact on mitochondrial function especially in view of the current albeit controversial evidence for the existence of a mitochondrial form of NOS (mNOS). In this review we will discuss the physiological roles of $[Ca^{2+}]_m$, highlighting recent controversies. The role of $[Ca^{2+}]_m$ in ischaemia/reperfusion injury can be found in other recent reviews [15,16].

2. Mitochondrial Ca^{2+} transport in the heart

2.1. Mitochondrial Ca^{2+} transport pathways

Mitochondria from all vertebrate sources tested can accumulate Ca^{2+} in a respiration-dependent manner via the ruthenium red (RuR)-sensitive Ca^{2+}-uniporter (mCU). The Na^+/Ca^{2+} exchanger (mNCX) is the major efflux pathway in the heart [3], although an Na^+-independent pathway exists in other tissues such as liver[17]. The properties of these transporters were initially derived from studies on isolated mitochondria (reviewed in [17,18]) and their kinetics indicated that they were too slow to play any role in intracellular

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Ca^{2+} signalling during excitation-contraction (EC) coupling of the heart. These pathways are outlined in Fig. 1. The mCU exhibits low affinity, high capacity transport of Ca^{2+}, whereas the mNCX has a much lower Vmax, saturating at $[Ca^{2+}]_m$ below 1 μM. The mNCX was initially thought to be electroneutral [19], but later evidence pointed to an electrogenic exchanger, of $3Na^+$:Ca^{2+}[20,21]. A recent theoretical model of the properties of the mNCX also found that 3:1 stoichiometry gave best fit to available experimental data [22]. Thus the stoichiometry of Na^+/Ca^{2+} exchange is still in dispute.

It was predicted that net Ca^{2+} influx would occur only when external $[Ca^{2+}]$ rose above about 500 nM, which is much higher than the resting, diastolic cytosolic free $[Ca^{2+}]$ ($[Ca^{2+}]_c$) of 100-200 nM [23]. This, together with the sigmoidal nature of mitochondrial Ca^{2+} uptake (in presence of physiological $[Mg^{2+}]$) suggested that the mCU responded to a "weighted average" of cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$), so systolic increases in $[Ca^{2+}]_c$ (typically about 1 μM) would contribute much more to mitochondrial Ca^{2+} uptake than diastolic $[Ca^{2+}]_c$ [23]. Parallel measurements of $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ in rat myocytes revealed that resting $[Ca^{2+}]_m$ was about 80 nM compared with resting $[Ca^{2+}]_c$ of 150 nM whereas in mitochondria isolated from beating hearts the $[Ca^{2+}]_m$ was estimated to be slightly higher, about 170 nM [24]. In rat myocytes $[Ca^{2+}]_m$ remained less than $[Ca^{2+}]_c$ until the latter rose above 500 nM [23] agreeing with studies on isolated mitochondria [25]. However, a seminal paper by Rizzuto and Pozzan in 1992 using aequorin targeted to mitochondria revealed that these organelles were capable of taking up Ca^{2+} on a fast timescale, due to their proximity to intracellular Ca^{2+} stores [26]. The ability of mitochondria to respond to single fast cytosolic Ca^{2+} transients in the heart is discussed in below.

A major step forward in research into control of $[Ca^{2+}]_m$ would be provided by knowing the molecular identity of the transporters. However, although there have been a few sporadic attempts to purify the transporters, they have yet to be fully purified or cloned (reviewed in [27]).

2.2. Time-averaged integration of cytosolic Ca^{2+} transients or beat-to-beat changes?

The question of whether mitochondrial Ca^{2+} transients exist during normal excitation contraction coupling is still somewhat controversial, although a consensus seems to be slowly emerging. This topic, together with methods of measuring $[Ca^{2+}]_m$ in intact cells or hearts has been covered in several recent reviews [7,28,29]. One inescapable conclusion is that whether or not beat-to-beat changes occur is species dependent[7]. For example, in a direct comparison study, we found that mitochondrial Ca^{2+} transients could be detected in guinea-pig but not rat cardiomyocytes [30]; see Fig. 2. It appears that single transients do not occur in adult rat, mice, hamster, ferret or cat cardiomyocytes, whereas mitochondrial Ca^{2+} transients can be detected in neonatal rat, and adult guinea-pig and rabbit cardiomyocytes (see [7] and references therein). In a recent study, changes in $[Ca^{2+}]_m$ during individual $[Ca^{2+}]_c$ transients were found to occur in adult rat myocytes, but these were very small, approximately 2-10 nM [31], and they integrated gradually to a steady-state. This paper also reported changes in mitochondrial Ca^{2+} uptake in response to cytosolic Ca^{2+} waves, but which varied in mitochondria in different parts of the cell; interfibrillar mitochondria giving a more consistent rise than subsarcolemmal mitochondria [31].

We did observe beat-to-beat changes in $[Ca^{2+}]_m$ in adult rat myocytes using targeted aequorin [32], as opposed to our previous results using indo-1, which showed no transients in these cells [23,33]); however, this required high external $[Ca^{2+}]$ plus presence of an adrenergic agonist, which may have pushed up $[Ca^{2+}]_c$ to levels capable of activating the mCU during a single systolic transient, and which would not occur in absence of the agonist. The other difference between the studies is that in order to express aequorin in mitochondria, the cells had to be cultured for 2-3 days. It is known that adult cells in culture start to de-differentiate, and eventually revert back to a neonatal phenotype [34] although whether sufficient changes occurred over the short culture period we used (1-3 days) is something we did not investigate.

The questions of how and why Ca^{2+} transport should differ between species, or indeed during development from neonate to adult, have not been addressed fully. It may be that the proximity of mitochondrial Ca^{2+} transporters to Ca^{2+} channels of the SR or sarcolemma are different between species, and/or that there are populations of mitochondria with different Ca^{2+} transport properties. One study found that in guinea-pig myocytes, Ca^{2+} transients occurred in peripheral but not central mitochondria [35]. This is one

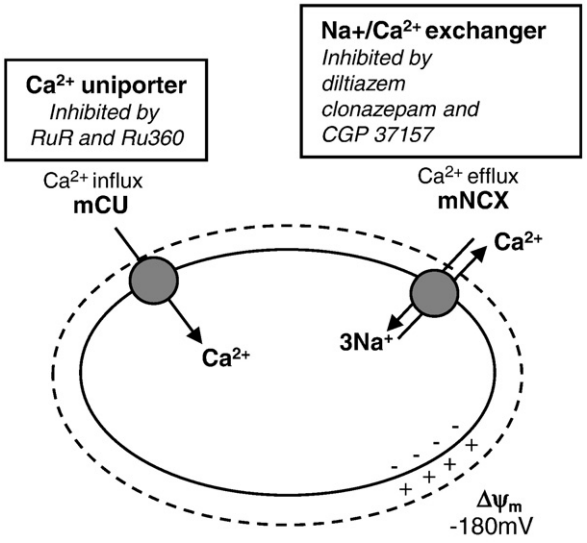

Fig. 1. Ca^{2+} transport pathways in isolated mitochondria under physiological conditions. Ca^{2+} influx is via the Ca^{2+}-uniporter (mCU), and Ca^{2+} efflux via the Na^+/Ca^{2+} exchanger (mNCX). The solid oval depicts the inner membrane and the dashed oval the permeable outer membrane. $\Delta\psi_m$ – mitochondrial membrane potential, approximately -180 mV in actively respiring mitochondria (inside negative).

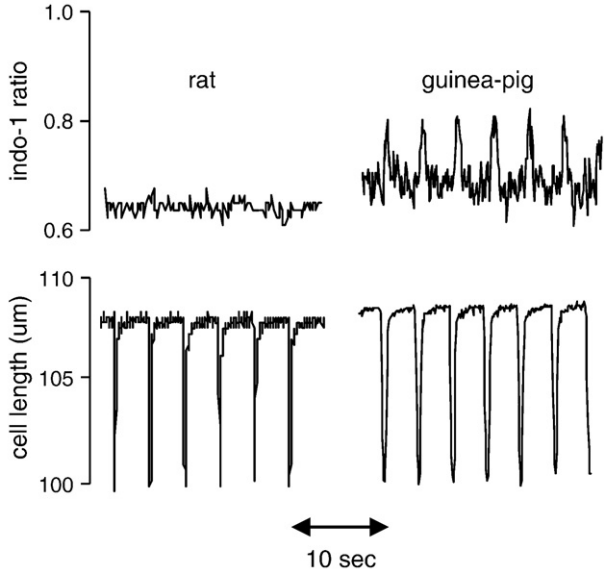

Fig. 2. Mitochondrial Ca^{2+} transients in guinea-pig but not rat cardiomyocytes. $[Ca^{2+}]_m$ was measured in myocytes isolated from rat or guinea-pig hearts, and indo-1 loaded under conditions that promote a mitochondrial localisation. Cells were stimulated to beat at 0.2 Hz, and cell length monitored simultaneously with indo-1 fluorescence. Figure adapted from data first presented in [30].

of the only proposed explanations for differences in Ca^{2+} handling between different populations of mitochondria in a single cell, and could possibly be extrapolated to account for differences between species.

As discussed above, the low affinity of the mCU for Ca^{2+} may be overcome by being juxtaposed to the SR ryanodine receptor (RyR), although there is some evidence for other possible pathways of Ca^{2+} transport: A rapid uptake mode (RAM) has been proposed [36,37] which, as its name suggests, is much faster than the mCU. However, the RAM was inactivated for over 1 min following a pulse of Ca^{2+}, which means that it could not take part in any rapid mitochondrial Ca^{2+} transients [37]. Neither is it obvious how this pathway is reconciled with original studies on isolated mitochondria, indicating that, although the mCU can take up large amounts of Ca^{2+}, uptake is relatively slow. A recent study in mitoplasts isolated from human myocytes provided evidence for 2 distinct types of voltage-dependent Ca^{2+} uptake pathways, differing in their sensitivity to Ru360. In mitoplasts from failing hearts both channels showed reduced activity [38].

In guinea-pig cardiomyocytes, simultaneous measurement of $[Ca^{2+}]_m$ (using rhod-2) and $[Ca^{2+}]_c$ (indo-1 dialysed into the cytosol), the upstroke of the mitochondrial Ca^{2+} transient preceded that of the cytosolic transient, whereas the decay lagged behind slightly [39]. Dialysing Ru360 into the cells to inhibit mCU prevented the rise in $[Ca^{2+}]_m$, but increased the amplitude of the cytosolic transient. Inhibiting mNCX with CGP37157 caused an increase in $[Ca^{2+}]_m$, but also reduced the amplitude of the cytosolic transient – an effect ascribed to increased buffering of Ca^{2+} by the mitochondria [39]. Another explanation, however, is that the mNCX contributes to SR refilling following the action potential, and that by preventing this pathway, there is less Ca^{2+} available for release upon subsequent contractions. We also found a slightly reduced $[Ca^{2+}]_c$ transient in the presence of the mNCX inhibitor clonazepam [32], but were unable to detect any changes in SR Ca^{2+} load, as measured by a caffeine-induced transient. However, this method may not be the most accurate, and it would be interesting to repeat the experiments using probes targeted to the SR to more accurately measure $[Ca^{2+}]$ in this compartment in presence of mNCX inhibitors.

The existence of a ryanodine receptor in the mitochondrial membrane has also been proposed [40–42]; this appears to allow rapid uptake of Ca^{2+} at relatively low concentrations, and so could account for physiological beat-to-beat Ca^{2+} uptake. However, this data relies on the purity of the mitochondrial preparation - which was not specifically addressed in the studies [40,42]. We were unable to detect presence of a RyR in purified heart mitochondria (Cheng and Griffiths, unpublished data). This observation, plus work showing that it is possible to obtain functional mitochondrial/SR membrane units under certain isolation conditions [43], argues against the existence of a mitochondrial RyR.

2.3. Modulation of myocyte Ca^{2+} signalling

Initial studies on non-cardiac cells found that mitochondria located in close proximity to Ca^{2+}-release channels on the endoplasmic reticulum (ER) are exposed to a much higher level of $[Ca^{2+}]$ than those elsewhere in the cytosol [26,44]. In these localised regions $[Ca^{2+}]_c$ has been estimated to be between 20-50 μM [45,46]. Evidence has emerged over the last few years that such focal release of Ca^{2+} is also likely to occur in the heart. Sharma et al. [45] found that in skinned myocytes application of caffeine to the cells caused a large release of Ca^{2+} from the SR that was taken up by mitochondria, as well as entering the cytosol, and the mitochondrial uptake was inhibited by RuR. Thus it is conceivable that mitochondria located in close proximity to Ca^{2+} channels on the SR are exposed to a much higher level of $[Ca^{2+}]$ than those elsewhere in the cytosol [47] accounting for the ability of at least a subpopulation of mitochondria to take up Ca^{2+} on a beat-to-beat basis; this topic has been the subject of several recent reviews [7,27,48]. A recent paper found that inhibition SR Ca^{2+} handling by thapsigargin, tetracaine or caffeine prevented the progressive rise in $[Ca^{2+}]_m$ in response to $[Ca^{2+}]_c$ waves, confirming that Ca^{2+} released from the SR was responsible for the rise in $[Ca^{2+}]_m$ .Fig. 3 gives an outline of how mitochondrial Ca^{2+} transporters may interact with those of the SR and sarcolemma during excitation contraction coupling.

There is evidence that the mCU may also interact with L-type Ca^{2+} channels on the sarcolemma, and may therefore play a role in controlling Ca^{2+} fluxes: Ru360 reduced the amplitude of cytosolic Ca^{2+} transients when cells were stimulated at a frequency of 3 Hz, but not at 0.1 Hz [8]. The authors suggested that mitochondria played a role in sequestering Ca^{2+}, and so removed Ca^{2+} -dependent inactivation of L-type Ca^{2+} channels. However, another study found that in guinea-pig cardiomyocytes Ru360 increased cytosolic Ca^{2+} transients at 10 nM, but caused a decrease in the amplitude of $[Ca^{2+}]_c$ at 100 nM [39].

There is now evidence for a direct physical coupling between mitochondria and the SR that persists during isolation [43,49]): mitochondrial fractions were obtained with associated SR components, and these particles were highly resistant to further purification [43]. The “SR appendices” were capable of transferring Ca^{2+} directly to mitochondria (measured with rhod-2 fluorescence) upon stimulation with caffeine, and of producing an increasing in the NADH autofluorescence signal, suggesting activation of the mitochondrial dehydrogenases. This is the first direct evidence that Ca^{2+} transfer directly from the SR to mitochondria is capable of activating oxidative metabolism [43].

A large proportion of sarcolemmal transporters are within the transverse tubules (t-tubules) – about 50% of L-type Ca^{2+} channels and the majority of the sarcolemmal Na^+/Ca^{2+} exchanger (NCX) [50]. T-tubule membranes are located in close proximity (about 15 nm) to the junctional cisterns of the SR, regions termed dyadic clefts. Connections between the two have been observed by electron microscopy – so-called “feet” - which are thought to be RyRs. A recent study used electron tomography of mouse ventricular myocytes to obtain a more detailed picture of the dyadic clefts, and this also revealed electron dense structures – “bridges” - linking mitochondrial outer membranes with SR or t-tubules, although there were far fewer bridges between t-tubules and mitochondria than SR and mitochondria [51]. The authors speculated that mitochondria play and additional role in preventing “catastrophic runaway Ca^{2+} release in normal hearts” [51]. The identity of the mitochondrial bridges is not yet known.

Whereas there has been much work done in both non-cardiac and now cardiac cell types on interactions of the SR and mCU, the possibility of similar interactions with the SR and mNCX seems to have been somewhat overlooked. However, there is evidence from non-cardiac cells that this may occur: In HeLa cells challenged with histamine (which releases Ca^{2+} from the ER via IP_3 signalling) there was a decline in $[Ca^{2+}]_{ER}$ (measured with a targeted cameleon), but this decline was greater in presence of the mNCX inhibitor CGP37157, suggesting that efflux of Ca^{2+} from mitochondria normally contributes to maintaining $[Ca^{2+}]_{ER}$ [52]. A similar result was found in an endothelial cell line, namely that inhibition of mitochondrial Ca^{2+} efflux prevented ER re-filling following a histamine challenge [53]. In aortic smooth muscle cells, inhibiting the mNCX with CGP37157 impaired SR re-filling upon stimulation with ATP to activate purinergic receptors [54]. A recent review has considered whether the mNCX is also situated close enough to the ER to allow return of mitochondrial Ca^{2+} directly into the ER and contribute to store-refilling in neuronal cells [55]. Whether the mNCX interacts with the SR in the heart remains to be investigated.

2.4. Role of $[Ca^{2+}]_m$ in energy production

In the heart there needs to be a way of rapidly producing ATP to meet changes in energy demand, such as upon increased workload or hormonal stimulation. Early studies in isolated mitochondria found

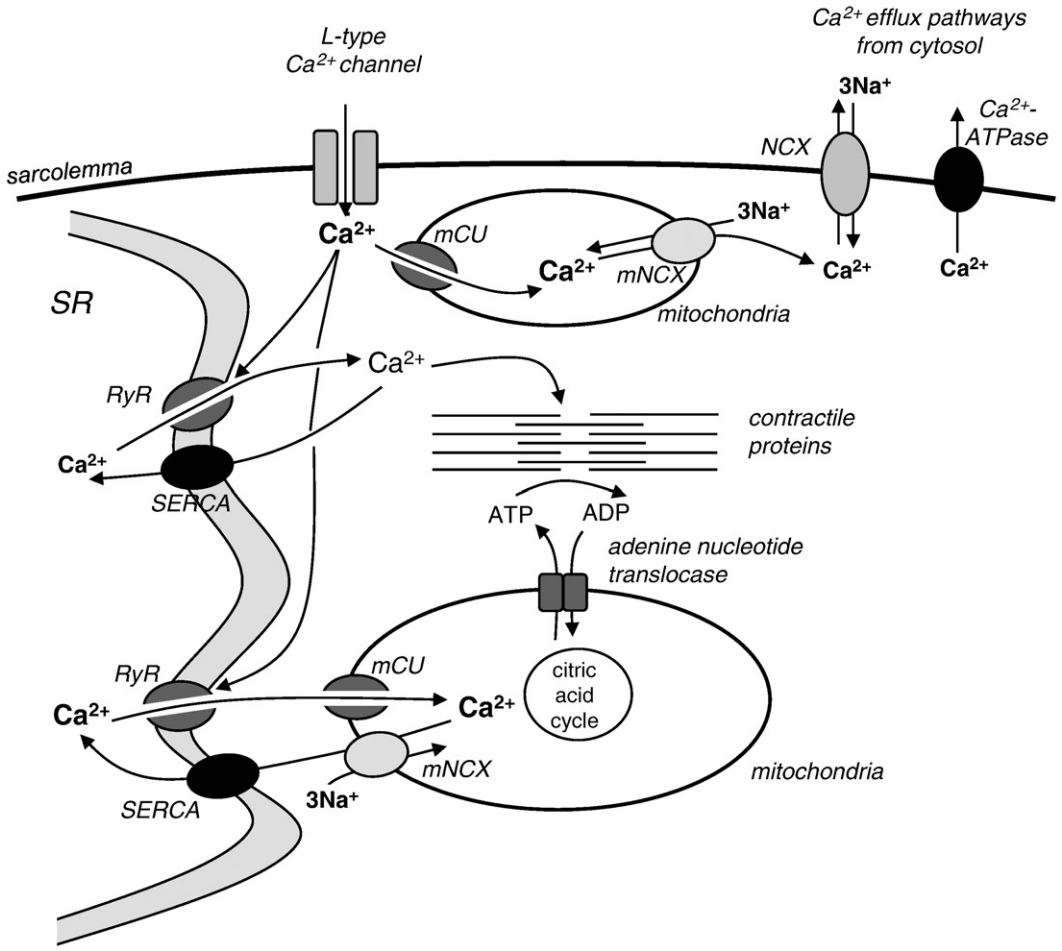

Fig. 3. Mitochondrial Ca^{2+} transport in excitation-contraction coupling. The routes of Ca^{2+} influx and efflux during excitation-contraction coupling are shown, together with possible interactions of the mitochondrial Ca^{2+} transport pathways with those of the SR and sarcolemma. In this model Ca^{2+} is shown entering mitochondria directly from the RyRs or L-type Ca^{2+} channels via mCU, and efflux from mNCX contributes to SR re-filling during relaxation. Efflux of Ca^{2+} from mitochondria near the sarcolemmal NCX is also shown, although there is no experimental evidence as yet for such a mechanism. There is evidence for all the other interactions shown – see text for details. mCU - Ca^{2+}-uniporter; mNCX – mitochondrial Na^+/Ca^{2+} exchanger; NCX – sarcolemmal Na^+/Ca^{2+} exchanger; RyR – ryanodine receptor; SERCA – sarcoplasmic/endoplasmic reticular Ca^{2+}-ATPase.

that the ADP/ATP ratio was the main regulator of ATP production [56], however later studies in beating hearts found that the ratio does not change in well-oxygenated hearts even during large increases in workload [57,58]. In support of this, we found recently, using targeted luciferase, that ATP levels in beating cardiomyocytes were remarkably constant in both cytosolic and mitochondrial compartments [32].

In the 1970's and 1980's Denton and McCormack found that Ca^{2+} could activate mitochondrial dehydrogenases in the physiological range; $K_{0.5}$ for both pyruvate dehydrogenase (PDH) and oxoglutarate dehydrogenase (OGDH) was around 1 μM, whereas that of isocitrate dehydrogenase (ICDH) was higher, 5-50 μM, depending on the ATP/ADP ratio [59]. This lead them to propose that an increase in the supply of reducing equivalents in the form of NADH and FADH would increase ATP production; thus suggesting a parallel activation model of stimulation of contraction and ATP synthesis by Ca^{2+} [5,59]; see Fig. 4 for a summary of the model. The mitochondrial F_1F_0ATP synthase (ATPase) may also be stimulated by Ca^{2+} [6,60]; binding of Ca^{2+} to an ATPase inhibitor protein – IF1 – has been proposed[61,62], although there is little direct evidence as to how this occurs. Recent evidence indicates a role for IF1 in regulation of mitochondrial structure, for example IF1 overexpression increases the density of cristae in the mitochondria, increases ATP synthase activity, and protects against ischaemia/reperfusion injury [63,64]. It therefore presents an attractive control point; however, how, and indeed whether, it is regulated by Ca^{2+} is far from clear.

Balaban [65] 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. To illustrate this he quoted evidence showing that in a canine heart the entire ATP pool is turned over in 1 min under normal conditions, and in about 10 sec under conditions of high workload [65–67].

Upon increases in pacing frequency in freshly isolated rat cardiomyocytes, form 0.2 Hz to 4 Hz (12 to 240 bpm), $[Ca^{2+}]_m$ did not increase, even though ATP demand would have increased substantially under these conditions [23,33], and see Fig. 5. However, upon addition of an adrenergic agonist, $[Ca^{2+}]_m$ increased gradually, over tens of seconds (Fig. 5). Similarly in beating guinea-pig hearts perfused with 10 μM Ru360, which should inhibit Ca^{2+} uptake into mitochondria, no effect on contraction was observed [68]. This implies that ATP levels were maintained even though no increase in $[Ca^{2+}]_m$ should have occurred. This again suggests that, whereas increases in $[Ca^{2+}]_m$ may play a role under conditions of increased workload, under basal conditions there are alternative mechanisms coupling ATP supply to demand. We found that in myocytes stimulated to beat suddenly from rest, and under conditions of high external $[Ca^{2+}]$, there was a drop in $[ATP]_m$ before it started to rise again, and this exactly matched the time over which an increase in $[Ca^{2+}]_m$ occurred [32], and see Fig. 6. Very little change in $[ATP]_c$ occurred during this time, confirming that it is extremely well buffered [32].

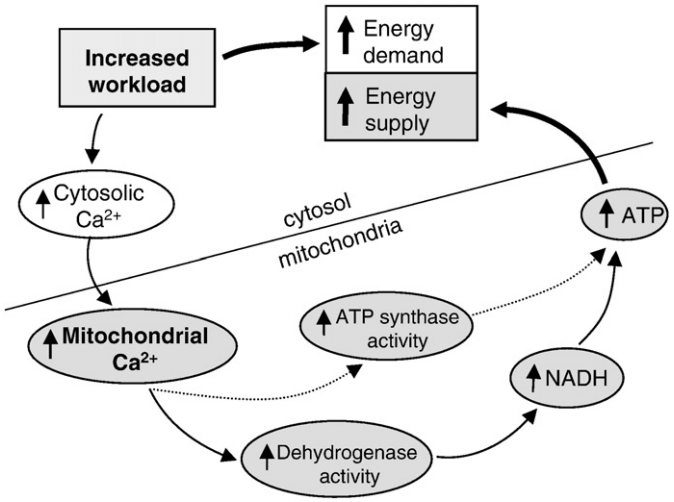

Fig. 4. Mitochondrial Ca^{2+} - key role in regulation of energy supply and demand. Increases in cytosolic Ca^{2+} are relayed to the mitochondrial matrix via the mitochondrial Ca^{2+} uniporter (mCU). $[Ca^{2+}]_m$ activates dehydrogenase of the citric acid cycle and possibly the ATP synthase to increase ATP supply in line with the increased demand. See text for further details.

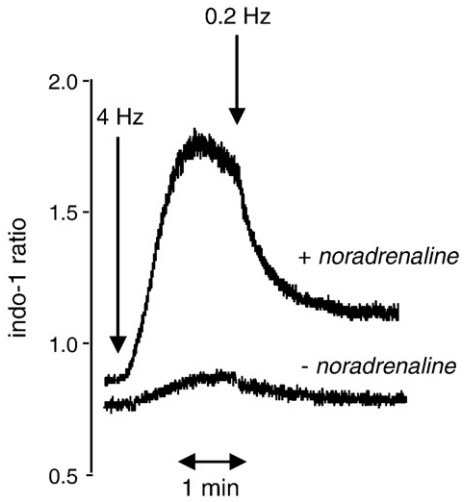

Fig. 5. Changes in mitochondrial $[Ca^{2+}]$ during rapid stimulation of adult rat myocytes. Myocytes were loaded with indo-1 under conditions that promote mitochondrial localisation of the dye, and stimulated to contract rapidly (4 Hz) in absence or presence of noradrenaline. Only a small increase in $[Ca^{2+}]_m$ occurs upon rapid stimulation alone, but a much larger increase occurs in presence of the adrenergic agonist. See text for discussion; figure adapted form data first presented in [33].

Since ADP/ATP does not change in beating hearts or myocytes, Saks and colleagues have put forward the idea that the subcellular architecture changes during contraction, causing a decrease in the Km of ADP for stimulating respiration, and removing diffusion limits of ADP that occur in resting cells [69,70]. 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" - where channelling of ADP occurs [71–73].

3. Developmental changes in mitochondria and Ca^{2+} handling

Changes in $[Ca^{2+}]_m$ during development present an interesting study since mitochondrial Ca^{2+} transients occur in neonatal but not adult rat myocytes. How and why this occurs is not known, but there are major metabolic and ionic changes occurring over this period. During the first weeks following birth the SR and surface membrane system are underdeveloped. During the 7 to 14 day old period (in rats), the surface membrane system rapidly increases in complexity and surface area and the SR develops a honeycomb network around the myofibrils. By 3 weeks, all these structures have reached their adult form [74].

In adults the crucial tight spatial and temporal coupling between the RyRs and the L-type Ca^{2+} channels allows for Ca^{2+}-induced Ca^{2+}-release, and the majority of Ca^{2+} used for contraction comes form the SR. Neonates, however, are more reliant upon Ca^{2+} entry across the sarcolemma for contraction [75,76], but the exact changes are species dependent since some animals are more mature at birth than others [77]. In adult heart muscle ryanodine completely abolished contractile force, proving that the SR plays a major role in E-C coupling, but in neonates ryanodine failed to abolish contraction completely [78]. Although the SR is less developed in neonates, it does appear to contain a large store of Ca^{2+}: caffeine-induced Ca^{2+} release in the neonates was actually quite robust, suggesting that the SR is primed with Ca^{2+} in the neonatal ventricular myocytes, but, paradoxically it is not released in response to electrical stimuli [79,80]. So although the SR at birth may be capable of releasing Ca^{2+} in response to caffeine, the scarcity of t-tubules functionally isolates the SR from taking part in E-C coupling.

In neonatal myocytes, $[Ca^{2+}]_c$ increased in the subcellular space during each transient, followed by a smaller rise in the cell centre. This is in contrast to the uniform elevation and decline of whole cell Ca^{2+} transients that occurs in adult myocytes. The transition from the neonatal to the adult pattern of Ca^{2+} transients coincides with the appearance of a mature t-tubular network [79]. Immature myocytes also relax more slowly than adult myocytes; disabling the SR did not slow relaxation in the younger ages, as it did in the adults, suggesting that the sarcolemmal NCX is the predominant pathway for the removal of Ca^{2+} [79]. Mitochondria in neonatal myocytes appear to be connected into a mitochondrial reticulum, rather than the punctated appearance in adult cells (see Fig. 7). We do not yet know the implications of this for control of energy metabolism in neonates, neither have there been any studies on the interactions of

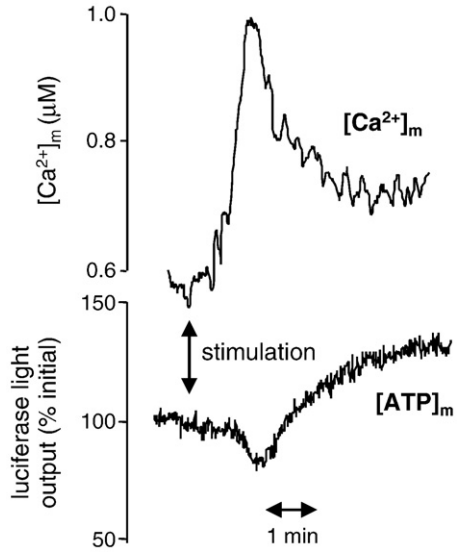

Fig. 6. Changes in $[ATP]_m$ and average $[Ca^{2+}]_m$ in adult cardiomyocytes stimulated to beat form rest. $[Ca^{2+}]_m$ and $[ATP]_m$ were measured using targeted aequorin and luciferase, respectively, in parallel experiments on small populations of cells stimulated to contract at 2 Hz from rest in presence of isoproterenol. In this figure the average $[Ca^{2+}]_m$ is shown – see text for discussion. Figure based on data first published in [32].

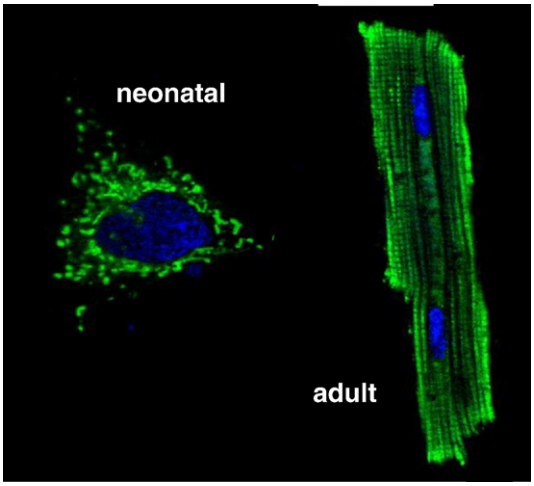

Fig. 7. Adult and neonatal rat cardiomyocytes showing distribution of mitochondria. The image shows developmental changes in the distribution of mitochondria (using mitotracker green) and also cell nuclei (stained with DAPI) in a neonatal (left) and adult (right) cardiomyocyte (Balaska and Griffiths, unpublished data).

mitochondrial transport pathways with those of the sarcolemma or SR in these cells.

There are also fewer mature mitochondria in the neonatal myocytes and reduced facility for aerobic ATP production compared with the adult; a dependence that lessens with age as mitochondrial function matures and oxidative metabolism increases. The mitochondrial cristae of young myocytes are sparse and widely separated, which indicates a lower amount of aerobic metabolism and the number, volume and density of mitochondria is also lower in the younger animals [81]. Mitochondrial volume occupies about 20% of the cell volume in a one day old rat versus about 40% in an adult rat [82]. The postnatal development of enzyme activities depends on the degree of maturation of individual species at birth, for example, in the rat (which is highly immature at birth) mitochondrial enzymes such as citrate synthase and cytochrome c oxidase increase steadily from the end of prenatal life up to adulthood [77]. The activity of creatine kinase was also increased during development [83].

The question of whether Ca^{2+} plays a role in stimulating respiration in neonatal hearts has not been addressed previously to the best of our knowledge. In preliminary experiments we found that respiration is stimulated by Ca^{2+} at non-saturating substrate levels in mitochondria from neonatal hearts, as for adult hearts [84]. We have also studied one of the dehydrogenases, OGDH, and found that there are significant differences compared with the adult: Km for its substrate is reduced, and although Ca^{2+} acts to reduce Km, as in the adult, this effect is even greater in the neonatal mitochondria [84]. How and why this occurs, and whether it also occurs for the other dehydrogenases, is yet to be investigated.

4. Nitric oxide synthase and mitochondria

NO produced endogenously from nitric oxide synthases (eNOS, iNOS and nNOS) is crucial for many physiological and pathological processes in the heart [85–87]. Increasing evidence indicates that mitochondria may be one site of action for the protective effects of NO (reviewed in [88]); NO has been reported to inhibit the mitochondrial permeability transition pore leading to less necrotic death in ischaemia/reperfusion injury, and to prevent cytochrome c leading to an inhibition of apoptosis [88]. Furthermore studies on isolated mitochondria have revealed a potential role for NO in the regulation of respiration and ATP synthesis [89,90].

Given the possible control of mitochondrial function by NO, the existence of an isoform of NOS in mitochondria (mNOS) has generated a lot of interest since it was first proposed in 1995 [90,91]. However, there is significant controversy in this area, especially with regard to the likelihood of contamination of "mitochondria" with other membrane fractions, such as sarcolemma or SR, giving rise to false positive results for either presence of NOS, or detection of NO (reviewed in [92]). Indeed in one study using mitochondria isolated from human heart papillary muscle or from mouse heart, western blotting with seven different NOS antibodies failed to detect NOS in mitochondria [93]. We have also found no evidence for the existence of NOS in purified heart or liver mitochondria, using a variety antibodies against all known forms of NOS (Cheng and Griffiths, unpublished data), and therefore conclude that any regulation of mitochondrial function by NO must be arising from NO released from a non-mitochondrial form of NOS. In myocytes, nNOS has been detected in the SR [94–96] and reported to regulate RyR function, probably by S-nitrosylation, and co-precipitates with the RyR [95]. nNOS may therefore also be located in close proximity to mitochondria in cardiomyocytes *in situ*, and so be able to regulate mitochondrial function, particularly in view of evidence (discussed above) indicating a close association between mitochondria and the SR in membrane particles after isolation [43].

One proposed role of having NOS localised in or near mitochondria is in modulation of mitochondrial Ca^{2+} uptake: Adding SNAP (an NO-donor) to neonatal cardiomyocytes caused rapid inhibition of respiration (as indicated by an increase in NADH fluorescence), and decreases in mitochondrial membrane potential ($\Delta\psi_m$) and Ca^{2+} uptake [97]. In endothelial cells loaded with fura-2 and then permeabilised to releases the cytosolic dye so that the signal originated from mitochondria only, the NO-donor spermine-NONOate decreased $\Delta\psi_m$ (TMRE fluorescence) and also reduced accumulation of added Ca^{2+} by mitochondria [98]. This reduction of Ca^{2+} accumulation by NO is the mechanism whereby NO has been reported to delay opening of the MPTP, as seen in a model of oxidative stress in isolated cardiomyocytes [99], and see Fig. 8.

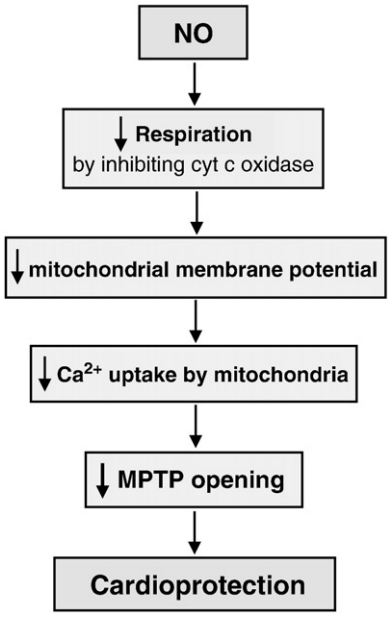

Fig. 8. Mechanism of NO-induced inhibition of MPTP opening in cardiomyocytes. There is evidence from studies on single myocytes that NO form either exogenous or endogenous sources can inhibit respiration, which would depolarise the mitochondrial membrane potential. This in turn inhibits Ca^{2+} uptake by the Ca^{2+}-uniporter, and therefore reduces likelihood of the MPTP opening, resulting in cardioprotection. Scheme based on data published in [98,107,108].

A physiological role of NO in modulating mitochondrial Ca^{2+} is less obvious, however, since normally O_2 would out-compete NO for binding to cytochrome c oxidase. Increases in $[Ca^{2+}]$ in the physiological range (about 0.2 μM) can activate eNOS and nNOS, located in or near mitochondria. This would then lower $[Ca^{2+}]_m$ by decreasing $\Delta\psi_m$, and tend to inhibit activation of the mitochondrial dehydrogenases by Ca^{2+} [59]. Ca^{2+} would therefore have dual and opposing effects on ATP synthesis, as suggested by Giulivi et al. [100], giving a complex interplay whereby Ca^{2+}both stimulates and inhibits respiration. Under conditions such as hypoxia, or high altitude adaptation the role of NO may become more important since it will be able to out-compete oxygen for binding to cytochrome c oxidase, and therefore be able to limit mitochondrial Ca^{2+} uptake and MPTP opening[99].

5. Conclusions and implications for cardiac disease

$[Ca^{2+}]_m$ plays an important role in both myocardial energy production and intracellular Ca^{2+} signalling. However, there is likely to be more than one mechanism coupling ATP supply and demand in the heart; and the alternative mechanisms may operate under different conditions: for example under basal conditions regulation of respiration by channelling of ADP, the metabolic compartmentation model, may be the dominant one, but upon large changes in workload, when systolic $[Ca^{2+}]_c$ increases, parallel activation of mitochondrial metabolism ensures a greater, rapid, production of ATP. These processes may have evolved to ensure that three are sufficient backup mechanism to ensure that the ATP supply does not fail.

Both mCU and mNCX have been proposed as possible targets for cardioprotective drugs. A full discussion is beyond the scope of this review. However, protective effects of RuR have been found in ischaemia reperfusion injury [101,102], although it is not certain that the drug was acting on mCU since it is not specific and also affects SR Ca^{2+} flux [103]. Indeed we found, using a myocyte model of hypoxia/reoxygenation injury, that RuR was not protective[104], however, in the same model inhibiting the mNCX with clonazepam was protective. We concluded that the route of Ca^{2+} entry into mitochondria under hypoxic conditions is likely to be the mNCX rather than mCU [27,104].

The mNCX has also been considered a target for cardioprotection in other recent studies: $[Na^+]_i$ is increased during ischaemia/reperfusion injury, and in heart failure (see [105] for a recent review on regulation of $[Na^+]_i$). Recent work from O'Rourke's group has shown that dysregulation of 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 $[Na^+]$ was 16 mM compared with 5 mM in control cells [13]. 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 mNCX, was able to prevent the decrease in NADH in the failing myocytes. It is thus likely to restore ATP levels: earlier work showed that the mNCX is capable of regulating $[Ca^{2+}]_m$ and dehydrogenase activity since adding Na^+ to isolated mitochondria shifts the activation curves for PDH and OGDH by Ca^{2+} to the right [106].

A great deal of progress has been made in elucidating the roles of $[Ca^{2+}]_m$ in the heart with the development of fluorescent indicators and now fluorescent proteins targeted to the mitochondrial matrix, together with advances in imaging technology. There is now evidence for interactions between mitochondria and SR from both imaging studies of subcellular architecture plus dynamic studies using inhibitors of the SR and mitochondrial transport pathways, and the precise role of $[Ca^{2+}]_m$ in cell signalling and energy production is becoming clearer, although not yet resolved. However, the biggest limitation currently is the lack of identification of the mitochondrial Ca^{2+} transporters.

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