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Minireview

Calcium and mitochondria

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Abstract The literature suggests that the physiological functions for which mitochondria sequester Ca^{2+} are (1) to stimulate and control the rate of oxidative phosphorylation, (2) to induce the mitochondrial permeability transition (MPT) and perhaps apoptotic cell death, and (3) to modify the shape of cytosolic Ca^{2+} pulses or transients. There is strong evidence that intramitochondrial Ca^{2+} controls both the rate of ATP production by oxidative phosphorylation and induction of the MPT. Since the results of these processes are so divergent, the signals inducing them must not be ambiguous. Furthermore, as pointed out by Balaban [J. Mol. Cell. Cardiol. 34 (2002) 11259–11271], for any repetitive physiological process dependent on intramitochondrial free Ca^{2+} concentration ($[Ca^{2+}]_m$), a kind of intramitochondrial homeostasis must exist so that Ca^{2+} influx during the pulse is matched by Ca^{2+} efflux during the period between pulses to avoid either Ca^{2+} buildup or depletion. In addition, mitochondrial Ca^{2+} transport modifies both spatial and temporal aspects of cytosolic Ca^{2+} signaling. Here, we look at the amounts of Ca^{2+} necessary to mediate the functions of mitochondrial Ca^{2+} transport and at the mechanisms of transport themselves in order to set up a hypothesis about how the mechanisms carry out their roles. The emphasis here is on isolated mitochondria and on general mitochondrial properties in order to focus on how mitochondria alone may function to fulfill their physiological roles even though the interactions of mitochondria with other organelles, particularly with endoplasmic and sarcoplasmic reticulum [Sci. STKE re1 (2004) 1–9], may also influence this story.

Keywords: Mitochondrial Ca^{2+} transport; ATP production; Mitochondrial permeability transition

1. Intramitochondrial Ca^{2+} and stimulation of ATP production

An early hypothesis on the control of the rate of oxidative phosphorylation held that feedback of ADP and inorganic phosphate (Pi), from the cell's kinases to the mitochondria, controlled ATP production [3]. This represents a strong analogy to the economic law of supply and demand where ATP is the currency of biological energy. This view was supported by the observation that isolated mitochondria in suspension could increase their rate of ATP production manyfold upon addition of ADP and Pi supported only by substrate and oxygen. Today we understand that while this "supply and demand" process is real, it represents a default process and is not the usual mechanism by which ATP production rate is controlled. Careful studies of the relationship between the concentration of ADP and other adenosine phosphates, measured largely by ^{31}P NMR, and the rate of oxidative phosphorylation under physiological conditions in cells and tissue showed that increases of over a factor of four could occur in the rate of oxidative phosphorylation without any significant change in the concentrations of ADP, ATP, or Pi (for reviews, see [1,4]). Clearly, some other factor is used as the primary metabolic mediator under these conditions and it has been identified as $[Ca^{2+}]_m$. Extensive studies have shown that Ca^{2+} activates the rate of NADH production by pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (ICDH), and α-ketoglutarate dehydrogenase (α-KGDH) (for review, see [5]). Further studies have shown that Ca^{2+} activates the F_1F_0 ATP synthase [6]. Furthermore, intramitochondrial Ca^{2+} has been suggested to activate electron transport and cytosolic Ca^{2+} to activate the adenine nucleotide translocase (ANT) (see references in [7]). The studies by Territo et al. [6] showing that oxidative phosphorylation is stimulated more rapidly by pulses of Ca^{2+} than by creatine or even ADP leave little doubt that intramitochondrial Ca^{2+} is this metabolic mediator. Since cytosolic Ca^{2+} transients often induce energy usage as well as increases in ATP production, the use of $[Ca^{2+}]_m$ in this way allows the biological system to match supply to anticipated demand, the ultimate in "on time delivery" of ATP.

The literature suggests that ATP production can be activated quickly with a modest increase in intramitochondrial $[Ca^{2+}]$. Denton and coworkers measured the K_d's of Ca^{2+} activation of NADH production by isolated α-KGDH, PDH, and ICDH in toluene permeabilized mitochondria (see McCormack et al. [5] and references therein). For the isolated

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Abbreviations: Pi, inorganic phosphate; PDH, pyruvate dehydrogenase; ICDH, isocitrate dehydrogenase, α-KGDH, α-ketoglutarate dehydrogenase; MPT, mitochondrial permeability transition; PTP, permeability transition pore; cyp D, cyclophillin D; ROS, reactive oxygen species; Ca_c^{2+}, cytosolic Ca^{2+} transients; RaM, rapid mode of Ca^{2+} uptake; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; ROIs, regions of interest; CCCP, carbonyl cyanide mchlorophenyl hydrozone

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α-KGDH, the apparent K_m for Ca^{2+} activation was 1.2 μM [5]. For the toluene permeabilized mitochondria, the K_m for Ca^{2+} for PDH was 0.4 μM [5] and for α-KGDH varied from 0.19 to 1.5 μM in 0.75 mM ADP and 1.5 mM ATP, respectively. The K_m for Ca^{2+} activation of ICDH at the same ADP and ATP concentrations was 10.5 and 41 μM, respectively. In unpublished work with isolated α-KGDH, we have confirmed the earlier results of McCormack et al. [5] obtaining an apparent K_d below 1 μM. A conclusion of the Denton group regarding possible Ca^{2+} activation of PDH, α-KGDH, and ICDH was that both PDH and α-KGDH, which show K_m's in and below the low micromolar range, were very likely activated by intramitochondrial free calcium, while it was difficult to say whether ICDH with a K_m in the tens of micromolar range was also activated [5]. In work with intact mitochondria, it was estimated that PDH and α-KGDH could be activated by an uptake of about 4 nmol Ca^{2+}/mg mitochondrial protein [8].

Territo et al. [6] clearly demonstrated activation of ADP phosphorylation at the F_1F_0 ATP synthase following uptake of short pulses of Ca^{2+}. Furthermore, rapid measurements of intramitochondrial free calcium, NADH, and oxidation rate showed that Ca^{2+} uptake was very rapid (less than 100 ms), activation of NADH production by the Ca^{2+}-sensitive dehydrogenases was very rapid (within 200 ms), and establishment of an increased rate of oxidative consumption was also very rapid (within 270 ms). This rapid enhancement of oxidative phosphorylation showed maximal effects with a 535 nM pulse of Ca^{2+}, falling off both above and below this value. A 535 nM pulse is in the range of typical Ca^{2+} pulses measured over the cytosol of many types of cells using the common fluorescent probes. Taken together, work in these laboratories has shown that Ca^{2+} activates a set of intramitochondrial metabolic reactions that significantly increase the rate of ATP production. Increases of intramitochondrial free Ca^{2+} concentration ($[Ca^{2+}]_m$) only need to be in the low micromolar range to initiate this activation process, and isolated mitochondria are capable of showing this increase in the rate of oxidative phosphorylation after exposure to a brief 535 nM pulse of Ca^{2+}, typical of the average pulses of Ca^{2+} seen in the cytosol of many types of cells using fluorescent probes.

2. Intramitochondrial Ca^{2+} and the permeability transition

The mitochondrial Ca^{2+}-induced permeability transition (MPT) represents one of the major enigmas in bioenergetics. Mitochondria supply approximately 95% of the ATP used by the typical vertebrate cell. The MPT is caused by opening of a large pore in the mitochondrial inner membrane [9,10] that dissipates the electrochemical proton gradient, thereby obviating ATP production by oxidative phosphorylation. One would think that a process so threatening to the cell's vital energy supply as opening of the MPT would have been eliminated by natural selection long ago and yet the MPT behavior is conserved in all vertebrate and possibly all animal mitochondria [10]. Detailed properties of the MPT have been discussed in many reviews (see for example [10–14]) and need not be repeated in detail here. However, it will be useful to review the prevalent properties of the MPT briefly.

Permeability transition pore (PTP) opening is thought to be activated by binding of cyclophillin D (cyp D) to the matrix side of the pore protein [15]. This cyp D binding is potentiated by intramitochondrial Ca^{2+}. Cyp D has been reported to bind to the ANT [12,13,15]. While not everyone believes that the ANT is the pore protein [16], all agree that the ANT is closely associated with pore opening [11]. This pore is also often associated with a channel through both the inner and outer membranes set up by alignment of the PTP through the inner membrane with the VDAC pore in the outer membrane [15,17].

Opening of the PTP can be induced by intramitochondrial Ca^{2+} alone in sufficient amounts [10,11,14]; however, Ca^{2+} is usually aided by one or more "inducing agents". Some of these, like Pi, acetoacetate, and oxaloacetate, are present endogenously [10,11,14]. There are also many MPT inhibitors, some of which, like ADP, ATP, and Mg^{2+}, are also present endogenously [10,11,14]. The most commonly used inhibitor of the MPT, cyclosporin A, is thought to work by binding the cyp D in the mitochondrial matrix, thereby blocking cyp D binding to the pore protein [11–14].

While there is no consensus on the physiological role of the MPT, a number of feasible suggestions have been made. Among these are that the MPT provides a way of clearing the mitochondrial matrix of damaged or unneeded molecules [10], that the MPT provides an important pathway for inducing apoptosis in cells in which certain types of damage have occurred [18], and that the MPT provides a way of getting rid of damaged mitochondria [19,20]. The first of these suggestions could function under conditions in which the mitochondrial membrane potential flickers as has been observed in several types of mitochondria [21–23]. Clearly, induction of the MPT is sufficient to induce apoptosis [18], but it is not necessary because there are alternative pathways for apoptosis which do not involve the MPT [24,25]. Induction of the MPT causes rapid swelling of the inner membrane plus matrix, which can, in turn, cause rupture of the outer mitochondrial membrane. Such tearing of the outer mitochondrial membrane causes release of Cyt *c*, Smac/Diablo, AIF, and other inner membrane space factors into the cytosol. This can lead to caspase activation and apoptosis.

The question most vital to our considerations here is how large an amount of intramitochondrial Ca^{2+} will be tolerated over an extended period without any induction of the MPT. The literature would suggest that 300 nmol Ca^{2+}/mg protein without other inducing agents could usually induce the MPT within a few minutes, 80 nmol Ca^{2+}/mg protein could be tolerated perhaps for hours [11,14]. The amount which could be tolerated for a more extended period without inducing the MPT would vary considerably with Pi content and ROS production but amounts in the neighborhood of 20 nmol Ca^{2+}/mg protein would probably be safe (private discussion with Drs. Paolo Bernardi and Douglas Pfeiffer). Thus, while only small amounts of Ca^{2+} are necessary to activate ATP production, much greater amounts are necessary to induce the MPT.

3. Modulation of cytosolic Ca^{2+} transients

Mitochondrial Ca^{2+} uptake has profound consequences for specific characteristics of cytosolic Ca^{2+} (Ca_c^{2+}) transients. It is widely appreciated that the temporal characteristics of global Ca_c^{2+} signals can be influenced by mitochondrial Ca^{2+} uptake and that this is often manifested as a reduction in both the amplitude of the global Ca^{2+} signal and a diminished propagation rate of intracellular Ca^{2+} waves [26,27]. Perhaps the

most profound effect of mitochondrial Ca^{2+} uptake is to "shape" Ca_c^{2+} signals by *local* modulation of the cellular processes which underpin the increase in $[Ca^{2+}]_c$. The best characterized examples of this are probably the suppression by mitochondria of Ca^{2+} feedback activation of 1,4,5 inositol trisphosphate receptor-induced Ca^{2+} release [28] and the maintenance of store-operated Ca^{2+} influx by mitochondria which limit Ca^{2+}-induced desensitization [29]. In these cases, the intimate localization of mitochondria near these channels modulates the degree of Ca^{2+} feedback, leading to the fine tuning of the activity of the channels. The strategic sub-cellular localization of mitochondria can also markedly influence the spatial characteristics of Ca_c^{2+} signals. A striking example occurs in polarized pancreatic acinar cells. In these cells Ca^{2+} transients are effectively confined to the apical third of the cell by a belt of mitochondria which surround this region [30,31]. This localization acts as a "fire wall" to prevent continued propagation of the Ca_c^{2+} signal to the basal aspects of the cell. The physiological purpose of this extreme example of shaping the Ca_c^{2+} signal may be related to confining large Ca_c^{2+} signals (several μM) which are required for apical exocytosis of secretory granules while minimizing the potentially deleterious consequences of a large global Ca_c^{2+} signal. Spatial modulation of the Ca_c^{2+} signal is likely a general phenomenon, since many cell types have a preponderance of perinuclear mitochondria [32] and local Ca^{2+} uptake may serve to modulate the amplitude and shape of the nuclear Ca^{2+} signal with consequences for nuclear processes such as gene transcription. An alternative cellular rationale for localized mitochondrial Ca^{2+} uptake may simply be to provide local ATP for the energy consuming processes of the ER/SR and nuclear machinery.

We can make very rough estimates of the amount of Ca^{2+} which must be sequestered by mitochondria in order to cause changes in Ca_c^{2+} like those discussed in the literature. Basing a calculation on the approximately 1 μM changes in Ca_c^{2+}-induced by mitochondria in adrenal chromaffin cells with a diameter of around 13 μm [26] and assuming around 1000 mitochondria/cell, we would estimate that at a cytosolic calcium binding capacity of 1 the mitochondria would sequester around 0.025 nmol, Ca^{2+}/mg protein. However, the cytosolic calcium binding capacity for these and all other cells is much higher than 1 and ranges from around 10 for Xenopus oocytes, 10–100 for chromaffin cells, cardiac myocytes, and axons of some invertebrates, 100–1000 for neurons of invertebrates, between 1500 and 2000 for pancreatic acinar cells, Purkinje cells, and neutrophils ([33] and references therein). For mitochondrial sequestration of the order of 1 μm Ca^{2+} from the cytosol of these various cell types, this would suggest mitochondrial Ca^{2+} uptakes of the order of 0.25 nmol Ca^{2+}/mg protein for oocytes, 0.25–2.5 for chromaffin cells, 2.5–25 for invertebrate neurons, and 37.5–50 nmol Ca^{2+}/mg protein for pancreatic acinar cells, Purkinje cells, and neutrophils. The requirements for spatial and temporal modulation of cytosolic Ca^{2+} pulses seem to vary considerably with cell type and with the specific situation and probably often involves the mitochondrial Ca^{2+} uniporter.

4. Characteristics of the mechanisms mediating transport of Ca^{2+} across the mitochondrial inner membrane

Four mechanisms or modes of transport have been described in the literature over the past 43 years, two for influx and two for efflux. These mechanisms have been extensively reviewed in the recent years, so only a synopsis of their characteristics and a brief account of recent data and discussions will be given here [7,10,11,34]. Mitochondria may sequester Ca^{2+} via either the uniporter or the "rapid mode" or RaM. The RaM is called a "mode of transport" because current data cannot prove that it is molecularly distinct from the uniporter. It may represent the same protein complex in a different conformational form. Nevertheless, its kinetics are distinctly different from those of the uniporter, so the names "RaM" and "uniporter" describe the kinetics characteristic of these modes of transport as observed in isolated mitochondria. Mitochondria release Ca^{2+} via either the Na^+-dependent or the Na^+-independent efflux mechanisms or in special circumstances via the permeability transition pore.

5. The uniporter

Briefly, the uniporter may be described as a second order mechanism, although its measured Hill coefficient often appears to be a little less than 2 (for review, see [10] and references therein). The sigmoidicity of uniporter uptake kinetics is associated with the existence of separate activation and transport sites [10]. The membrane potential dependence of uptake follows that predicted by the Goldman Flux Equation $([e^{\Delta\phi/2}\Delta\phi/2]/[\sinh \Delta\phi/2])$ where $\Delta\phi = (b)(2F/RT)(\Delta\psi - \Delta\psi_0)$. F, R, and T are the Faraday constant, the gas constant, and the Kelvin temperature, respectively, while b is a fitting parameter accounting for the fraction of membrane potential spanned by the uniporter and $\Delta\psi_0$ is any possible offset of zero membrane potential across the transporter from that across the bulk

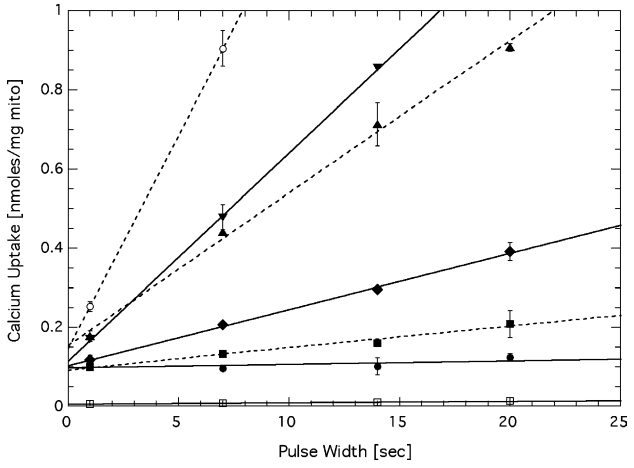

Fig. 1. Ca uptake vs. pulse width as a function of pulse height for rat liver mitochondria. Pulses were made using 2.4 mM EGTA and 2.7 mM $^{45}CaCl_2$-buffered with HEDTA to a free calcium in the pulse media of 1 μM. Experiment was run in HEPES-buffered 150 mM KCl (pH 7.2) with 5 mM Ksuccinate present with mitochondria at a concentration of 0.5 mg/ml. The pulse heights are as follows: □, 474 ± 14 nM with ruthenium red; ●, 165 ± 12 nM; ■, 307 ± 7 nM; ◆, 408 ± 8 nM; ▲, 567 ± 8 nM; ▼, 719 ± 2 nM; ○, 877 ± 9 nM. Error bars are one standard deviation. (Reprinted from Gunter, T.E., Buntinas, L., Sparagna, G.C. and Gunter, K.K. (1998) The Ca^{2+} transport mechanisms of mitochondria and Ca^{2+} uptake from physiological type Ca^{2+} transients. Biochim. Biophys. Acta 1366, 5–15 with permission from Elsevier Science.)

membrane (caused primarily by fixed charges). This mathematical form fits the data for Ca^{2+} uptake via the uniporter in liver mitochondria where $b = 1.0$ and $\Delta\psi_0 = 91$ mV [10]. These results and observations, showing that Ca^{2+} transport via this mechanism is not directly coupled to the transport of any other ion or molecule [10], strongly support the identification of this mechanism as a uniporter. They also support the driving force for uptake being the electrochemical Ca^{2+} gradient. Published values for $K_{0.5}$ for the uniporter range from 1 to 189 μM and are undoubtedly influenced by the fall in membrane potential caused by rapid Ca^{2+} uptake (for review, see [10] and references therein). Nevertheless, it is fair to say that the uniporter is not sensitive to low cytosolic free Ca^{2+} concentration, since it will not transport Ca^{2+} below a concentration of around 200–300 nM as seen in Fig. 1. Calcium uptake via the uniporter can be inhibited by ruthenium red, ruthenium 360, lanthanides and other agents [10,35]. At low Ca^{2+} concentration, uptake can be activated by spermine and other polyamines [10].

6. The RaM

Primarily because of studies using aequorin and fluorescent Ca^{2+} probes, it has become widely recognized that Ca^{2+} is an important second messenger in almost all cell types and that Ca^{2+} signaling is through Ca^{2+} transients or pulses in the cytosols of cells. However, prior to the mid 1990s mitochondrial Ca^{2+} uptake had only been studied using buffered or slowly varying pools of external Ca^{2+}. Exposure of isolated mitochondria to artificially created Ca^{2+} pulses, like those seen in the cytosol of cells, led to the discovery of Ca^{2+} uptake having very different kinetics from those of the uniporter [36]. The initial studies were carried out in rat liver mitochondria, but these were followed by studies in chicken heart mitochondria and a small amount of work with rat brain mitochondria [7,37]. Measurements of calcium uptake from pulses into all these mitochondria indicated very rapid uptake of small amounts of Ca^{2+} at the beginning of each pulse. The characteristics of this novel type of uptake in mitochondria from the different tissues not only showed similarities but also differences.

The initial experiments were carried out by rapidly injecting and mixing buffered Ca^{2+} followed by a Ca^{2+} chelator into a mitochondrial suspension in a cylindrical cuvette [36]. Fura-2 was used to determine the $[Ca^{2+}]$ in the medium vs time, while uptake was determined by pelleting the mitochondria after the uptake experiment and measuring $^{45}Ca^{2+}$. Uptake in these experiments was measured in the same way, as it has often been measured in older studies of uniporter uptake. Results from an early experiment are shown in Fig. 1. Control experiments showed that the apparent rapid uptake prior to the first data point was rapid net uptake and was at least hundreds of times faster than uniporter uptake under the same conditions [7,36,37]. This type of uptake was therefore called the "rapid mode" or RaM for short [36]. Control experiments also showed that mitochondrial uncouplers destroy this rapid uptake and that it is insensitive to thapsigargin, the inhibitor of Ca^{2+} uptake into ER and SR vesicles [7]. Spermine, ATP and GTP were found to increase the amounts of uptake via this rapid mode in liver mitochondria manyfold so that the maximum Ca^{2+} uptake via the RaM measured following a single pulse was around 7–8 nmol/mg protein [36]. Similarities between the uptake characteristics in the liver, heart and brain mitochondria studied were: (1) In all samples the uptake at the beginning of the Ca^{2+} pulse was completely finished by the end of the shortest pulse which could be made by the technique (0.3 s) [36,37]. (2) This rapid uptake was not observed in cases where the $[Ca^{2+}]$ prior to the pulse exceeded approximately 150–160 nM. It became apparent that this form of uptake was inhibited by Ca^{2+} binding to an external binding site with an affinity in this range. (3) To reestablish rapid Ca^{2+} uptake, all that was necessary was to lower the $[Ca^{2+}]$ of the medium to near or below 100 nM for a period to allow removal of Ca^{2+} from this external binding site [7,36,37]. (4) In liver and heart mitochondria (the only tissues in which it has yet been measured), Ca^{2+} uptake can be increased by the presence of spermine in the external medium.

The tissue-specific differences of this rapid uptake between liver mitochondria and heart mitochondria are: (1) The amount of uptake from a single pulse by the RaM in liver mitochondria was significantly larger than that in heart mitochondria. However, because the frequency of cytosolic pulses in heart is much higher than that in liver, the projected amount of uptake per unit time by the RaM in a hepatocyte is not very different from that by the RaM in a myocyte. (2) An important difference relates to the response of the RaM to a second pulse, identical to the first pulse but following it after a period at low $[Ca^{2+}]$. While inhibition of RaM uptake by external Ca^{2+} seems to take place in the same concentration range in both types of mitochondria, it takes a much longer time at low $[Ca^{2+}]$ for RaM uptake of a second pulse to be as large as that in the first pulse in heart mitochondria (over 1 min) as opposed to liver mitochondria (less than 0.3 s) [36,37]. Nevertheless, RaM uptake from the second pulse in heart mitochondria is not zero even at very short (subsecond) times after the first pulse but is 25–30% as large as the uptake from the first pulse [37]. (3) While spermine does activate Ca^{2+} uptake via the RaM in heart mitochondria, its effect is weaker than that of spermine on the RaM in liver mitochondria. The RaM in heart mitochondria is insensitive to activation by ATP or GTP unlike the RaM in liver mitochondria; however, Ca^{2+} uptake via the RaM in heart mitochondria is increased by ADP in a range around 10–20 μM and is strongly inhibited by AMP, unlike the RaM in liver mitochondria [36,37]. (4) The RaM in heart mitochondria seems less sensitive to inhibition by ruthenium red than does the RaM in liver mitochondria [36,37].

Recently, a completely different type of study of RaM characteristics has been initiated in order to determine how quickly RaM uptake of Ca^{2+} occurs. In this work liver mitochondria were suspended in medium containing caged Ca^{2+} (NP-EGTA), a strong Ca^{2+} binder which can be rapidly photodegraded by exposure to UV. The mitochondrial suspension had been incubated beforehand in medium containing fluo-4 AM and after 30 min for conversion to the free acid form washed to remove external fluo-4. The intramitochondrial fluo-4 was then used to assay $[Ca^{2+}]_m$ following exposure of the mitochondria to pulses of UV, which released pulses of Ca^{2+}. The system allowed control of the duration of the UV pulses from 0.5 to 5.5 ms. For the experiments, a drop of mitochondrial suspension containing the caged Ca^{2+} was placed on a coverslip on the stage of an epifluorescence microscope and the response of the system was analyzed using a Till Photonics software package. The mitochondria were then exposed to the desired number of pulses of UV which

caused release of Ca^{2+} pulses to the solution external to the mitochondria. It had been found beforehand that the system had to be set up so that the free calcium concentration in the suspending medium ([Ca^{2+}]) was below about 120 nM in order to see any significant response. While only a fraction of the mitochondria remain bound to the coverslip during the entire experiment, the software allowed "regions of interest" (ROIs) to be drawn around a set of stationary mitochondria and the light emanating from these ROIs to be determined separately throughout the experiment. That is the set of signals shown on the ordinate in the figures below. When the prepulse [Ca^{2+}] was set to around 90 nM the response shown in Fig. 2 was obtained with ten 5.5 ms UV pulses. The interpretation of these data is that while most of the fluo-4 is intramitochondrial, there is a small amount of residual fluo-4 FA outside the mitochondria which was not removed by the wash. The initial pulse gave the largest intramitochondrial $[Ca^{2+}]_m$ response; however, the pulse response decreased with each subsequent pulse and the baseline increased. With each pulse, the external [Ca^{2+}] increased as more Ca^{2+} was released from the caged Ca^{2+}. This was clearly a RaM response and not a uniporter response because a uniporter response should be second order in [Ca^{2+}] (i.e., should increase with increasing external [Ca^{2+}]) while that of the RaM would be inhibited with increasing [Ca^{2+}], as the data show.

Since external [Ca^{2+}] was clearly still a problem, the experimental protocol was modified slightly. The UV pulse was set to 0.5 ms and 3 UV pulses instead of 10 were used. The free calcium concentrations of these pulses in the external medium were determined by calibration with standard solutions to be between 400 and 500 nM in the data shown. An experiment similar to that described above, except as stated, was carried out. While this experiment was in progress, the mitochondrial suspension from which the sample had been drawn was centrifuged and at the end of the experiment a drop of that supernatant representing the external medium without mitochondria was placed on an identical coverslip and treated identically to the treatment of the mitochondrial sample. Also at the end of the mitochondrial experiment, a set of stationary mitochondria were identified in the data and set into ROIs. These ROIs were transferred to data of the supernatant experiment and a pixel by pixel subtraction of light emitted from each ROI during the supernatant experiment subtracted from that emitted during the mitochondrial experiment. This type of treatment takes variation of both UV and fluorescence excitation light into account by using exactly the same geometry. The results are shown in Fig. 3. A blow up of the rise portion of the data in the third pulse is shown in the inset in Fig. 3. Pulses of this type did appear sensitive to inhibition by ruthenium red and CCCP.

These data on Ca^{2+} uptake via the rapid mode into mitochondria suggest several things. First, evidence for a mechanism of very rapid Ca^{2+} uptake into mitochondria can not only be obtained by measuring $^{45}Ca^{2+}$ uptake but also by using sensitive, rapidly responding fluorescent probes such as fluo-4. Total time of uptake seems less than 40 ms. Second, the most striking characteristic of this mode of transport is its speed, the amount of uptake mediated is relatively small when compared with the uniporter at higher [Ca^{2+}]. Third, characteristics of this rapid uptake vary from tissue to tissue probably reflecting the differences in physiological handling of Ca^{2+} pulses in the different tissues. Fourth, this rapid uptake has been seen in rat liver and brain and in chicken heart mitochondria. This suggests that it is conserved over a wide range of vertebrate species.

A highly selective, inwardly rectifying ion channel with most of the features of the mitochondrial Ca^{2+} uniporter has recently been studied through patch clamping of mitoplasts from Cos-7 cells [38]. This channel shows the selectivity series of the uniporter and is inhibited by ruthenium red and ruthenium 360 as expected of the uniporter. The precision of controlling voltage and concentration in experiments of this type far exceeds that of the earlier whole mitochondrial experiments permitting much greater accuracy and allows the

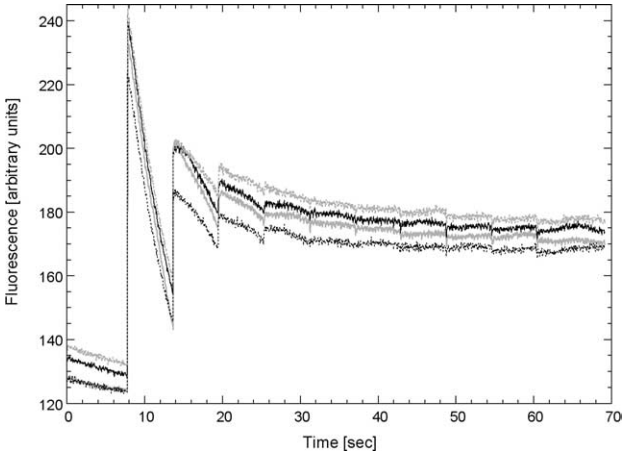

Fig. 2. Fluorescence response (Fluo 4) indicating both intramitochondrial plus external response to ten 5 ms UV pulses which release Ca^{2+} from caged Ca^{2+}. The area around four different mitochondria was followed. In this case, the effects of external Ca^{2+} were not subtracted out. The size of the response to each pulse falls off rapidly, while the fluorescence at the beginning of each pulse increases. The explanation is that the external [Ca^{2+}] increases in a few pulses to a value above 160 nM and RaM response is inhibited by binding of external Ca^{2+} to the inhibition site.

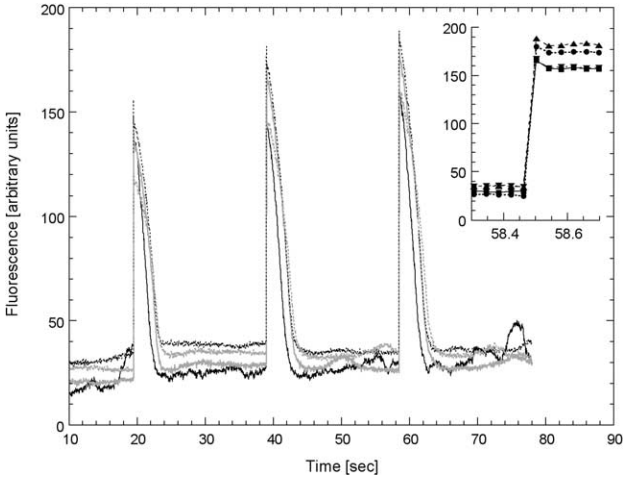

Fig. 3. Fluorescence response (Fluo 4) indicating intramitochondrial free Ca^{2+} concentration in four individual mitochondria during 3 UV pulses of 0.5 ms duration, which release Ca^{2+} from "caged Ca^{2+}". RaM uptake is complete in less than 40 ms; however, $[Ca^{2+}]_m$ remains high in the mitochondrial matrix for seconds. The inset shows a time expanded view of uptake during the third pulse.

firm conclusion that the uniporter is indeed a channel. This channel has multiple subconductance states between 2.6 and 5.2 pS, a $K_{0.5}$ of 19 mM, and a maximum ion flux of approximately 5×10^6 Ca^{2+}/s significantly higher than that estimated earlier [10]. There can be little doubt that this channel is the Ca^{2+} uniporter. However, in two respects these results differ significantly from those which characterize the mitochondrial Ca^{2+} uniporter. First, the Hill coefficient measured for the uniporter is usually a little less than 2 [10], much higher than that measured by the patch clamp work (0.6). Second, the very high affinity (dissociation constant <2 nM) is much higher than that reported for the uniporter which will not transport Ca^{2+} at a concentration below about 200 nM (see Fig. 1). The RaM, on the other hand, transports Ca^{2+} at significantly lower [Ca^{2+}] and because of the inhibition of transport caused by Ca^{2+} binding to the inhibition site, might show a much lower Hill coefficient than the uniporter. Do these differences from classical uniporter behavior, which are more ''RaM-like'', suggest that the RaM and the uniporter are indeed mediated by the same complex in different conformational and conductance states?

7. The mitochondrial Ca^{2+} efflux mechanisms

The role of the efflux mechanisms is to remove the Ca^{2+} sequestered during the pulse during the period between pulses so as to maintain intramitochondrial Ca^{2+} homeostasis for repetitive physiological processes. There is, of course, no such requirement for a pathological process. Two separate mechanisms of Ca^{2+} efflux are known in vertebrate mitochondria, the Na^+-dependent and the Na^+-independent mechanisms (see [10] and references therein). For more detailed reviews of the characteristics of these mechanisms, see reviews, [10,11,14]. Both mechanisms of Ca^{2+} efflux have been found in all the types of vertebrate mitochondria in which they have been sought [10,14]. However, the Na^+-dependent mechanism is dominant in heart, brain, skeletal muscle, parotid gland, adrenal cortex, brown fat, and many other tissues, while the Na^+-independent mechanism is dominant in liver, kidney, lung, and smooth muscle (see [10] and references therein). The kinetics of these two mechanisms are quite different with the Na^+-dependent mechanism being first order in Ca^{2+} and second order in Na^+ [10,14] and the Na^+-independent mechanism being second order in Ca^{2+} [14]. There are many inhibitors of the Na^+-dependent mechanism including tetraphenyl phosphonium, trifluroperazine, diltiazem, verapamil, clonazepam, and amiloride, while there are only a few known inhibitors of the Na^+-independent mechanism such as tetraphenyl phosphonium, cyanide, and low levels of uncouplers [7]. The Na^+ to Ca^{2+} transport stoichiometry of the Na^+-dependent mechanism is probably 3:1 [14] making this mechanism an electrogenic antiporter [39]. The Na^+-independent mechanism is probably a non-electrogenic $2H^+$:Ca^{2+} antiporter which receives some energy from electron transport [14]. While the V_{max} for these efflux mechanisms can vary significantly from tissue to tissue, it is always much smaller than the V_{max} for the uniporter. All of the mechanisms, influx and efflux, play their roles in the hypotheses describing how the system functions to carry out the physiological roles of mitochondrial Ca^{2+} transport.

8. How can these observations be put into perspective with respect to the functions of mitochondrial Ca^{2+} transport? – hypotheses

There are clearly functions of mitochondrial Ca^{2+} transport which require more Ca^{2+} uptake than the minimum necessary for activation of ATP production. The requirements of spatial or temporal modulation of cytosolic Ca^{2+} pulses vary considerably with cell type and the cellular situation and probably often involve the Ca^{2+} uniporter. Activation of ICDH requires an amount of Ca^{2+} uptake which probably also involves the Ca^{2+} uniporter. Induction of the MPT requires more Ca^{2+} and undoubtedly uniporter involvement. Intramitochondrial free Ca^{2+} concentrations of tens to hundreds of micromolar have been measured in cases where uniporter uptake must be strongly involved [2,40]. Under the uptake conditions which seem necessary for some modulations of cytosolic Ca^{2+} transients, the Ca^{2+}-dependence of ATP production must be saturated and the larger amounts of Ca^{2+} uptake could lead to higher levels of ROS production and a faster rate of mitochondrial damage. It is possible that the increased probability of induction of the MPT under these conditions is simply a mechanism leading to faster mitochondrial turnover [19,20].

In all tissues studied, the RaM appears to be a very fast Ca^{2+} transport mechanism which only transports a small amount of Ca^{2+} and transports only briefly at the beginning of a pulse before being inhibited by binding of Ca^{2+} to the external inhibitor site [7,36,37]. This uptake has now been observed by both ^{45}Ca and fluorescence measurements of $[Ca^{2+}]_m$. Furthermore, several groups have reported direct evidence for rapid Ca^{2+} uptake [6,36,37,40] and others have reported evidence for similar appearing mitochondrial uptake at the cellular level (see [42] and references therein). The amount of uptake observed by the RaM of liver mitochondria from a single pulse [36] is enough to satisfy McCormack's uptake criterion of 4 nmol Ca^{2+}/mg protein for stimulation of PDH and α-KGDH [8]. The results of Territo et al. [6] show that rapid uptake of simple 535 nM pulses of Ca^{2+} by isolated heart mitochondria produces the maximum activation of NADH production and oxygen consumption rate. We must conclude that Territo's mitochondrial Ca^{2+} uptake experiments, which were very similar in form and concentration to those used in measuring RaM characteristics, maximally stimulated reduction of intramitochondrial NAD by PDH and α-KGDH and ATP production. So it appears that Ca^{2+} uptake like that mediated via the RaM is capable of activating oxidative phosphorylation.

What can the very fast uptake of small amounts of Ca^{2+} do that slower uptake of the same amounts of Ca^{2+} might not do? The likely answer is that such rapid uptake might produce a larger burst of $[Ca^{2+}]_m$ prior to equilibration. Since increased $[Ca^{2+}]_m$ is obviously an intermediate between Ca^{2+} uptake and activation of intramitochondrial Ca^{2+}-sensitive reactions, this idea suggests that by creating a brief period of high $[Ca^{2+}]_m$ prior to equilibration the function of the RaM is to activate these metabolic reactions with the minimum amount of Ca^{2+}. This strategy would save energy, minimize the probability of inducing the MPT, and greatly decrease the amount of Ca^{2+} which would have to be removed by the relatively slow efflux mechanisms during the periods between pulses in order to maintain intramitochondrial Ca^{2+} homeostasis. While the function of the RaM in this way has been criticized because

over a minute at low [Ca^{2+}] is required with heart mitochondria to completely "reset" the mechanism after inhibition by external Ca^{2+} [1], there is no reason that the RaM needs to be completely "reset". It should be noted that even at very short times between pulses, RaM activity in heart mitochondria never falls below around 25% of maximal activity. Therefore, this long "resetting" time merely represents another means of restricting the amount of Ca^{2+} uptake into heart mitochondria and minimizing the Ca^{2+} that must be removed during the pulse period. We hypothesize that: (1) the RaM is sufficient to transport the minimum Ca^{2+} necessary for activation of ATP production, (2) it does this by producing a burst of $[Ca^{2+}]_m$ prior to equilibration which activates intramitochondrial Ca^{2+}-sensitive metabolic reactions and in this process minimizes both energy expenditure and the amount of Ca^{2+} which must be effluxed within the period of the pulses to maintain intramitochondrial Ca^{2+} homeostasis, and (3) the dichotomy in the amounts of Ca^{2+} transported to activate ATP production or to induce the MPT is the primary way in which the mitochondrion differentiates between signals for these responses.

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