The rapid mode of calcium uptake into heart mitochondria (RaM): comparison to RaM in liver mitochondria

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Abstract

A mechanism of Ca$^{2+}$ uptake, capable of sequestering significant amounts of Ca$^{2+}$ from cytosolic Ca$^{2+}$ pulses, has previously been identified in liver mitochondria. This mechanism, the Rapid Mode of Ca$^{2+}$ uptake (RaM), was shown to sequester Ca$^{2+}$ very rapidly at the beginning of each pulse in a sequence [Sparagna et al. (1995) J. Biol. Chem. 270, 27510-27515]. The existence and properties of RaM in heart mitochondria, however, are unknown and are the basis for this study. We show that RaM functions in heart mitochondria with some of the characteristics of RaM in liver, but its activation and inhibition are quite different. It is feasible that these differences represent different physiological adaptations in these two tissues. In both tissues, RaM is highly conductive at the beginning of a Ca$^{2+}$ pulse, but is inhibited by the rising [Ca$^{2+}$] of the pulse itself. In heart mitochondria, the time required at low [Ca$^{2+}$] to reestablish high Ca$^{2+}$ conductivity via RaM i.e. the ‘resetting time’ of RaM is much longer than in liver. RaM in liver mitochondria is strongly activated by spermine, activated by ATP or GTP and unaffected by ADP and AMP. In heart, RaM is activated much less strongly by spermine and unaffected by ATP or GTP. RaM in heart is strongly inhibited by AMP and has a biphasic response to ADP; it is activated at low concentrations and inhibited at high concentrations. Finally, an hypothesis consistent with the data and characteristics of liver and heart is presented to explain how RaM may function to control the rate of oxidative phosphorylation in each tissue. Under this hypothesis, RaM functions to create a brief, high free Ca$^{2+}$ concentration inside mitochondria which may activate intramitochondrial metabolic reactions with relatively small amounts of Ca$^{2+}$ uptake. This hypothesis is consistent with the view that intramitochondrial [Ca$^{2+}$] may be used to control the rate of ADP phosphorylation in such a way as to minimize the probability of activating the Ca$^{2+}$-induced mitochondrial membrane permeability transition (MPT). © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Mitochondria have an elaborate system for Ca$^{2+}$ transport. Until recently, however, the only specific Ca$^{2+}$ uptake mechanism known to exist in mitochondria was the Ca$^{2+}$ uniporter. Within the last few years, a novel uptake mechanism, the Rapid Mode of Ca$^{2+}$ uptake (RaM) was discovered in liver mito-
chondria which specifically functions to sequester Ca$^{2+}$ from a series of cytosolic calcium pulses [1,2]. This uptake mechanism is significant within mitochondria, since the Ca$^{2+}$: mitochondria are exposed to under physiological conditions is generally in the form of Ca$^{2+}$ oscillations (Ca$^{2+}$ transients or pulses). Several physiological roles have been proposed for the mitochondrial system of Ca$^{2+}$ transport: (1) regulation of Ca$^{2+}$-sensitive metabolic reactions of the mitochondrial matrix to control the cellular metabolic rate [3–6], (2) modulation of cytosolic transients or pulses [7], and (3) a role in initiation of apoptosis through induction of the mitochondrial membrane permeability transition (MPT) [8]. Particularly in heart, where cytosolic Ca$^{2+}$ transients are very short and very frequent, calculations based on the average concentrations of cytosolic Ca$^{2+}$ transients and the kinetics of mitochondrial Ca$^{2+}$ uptake and release predict that the mitochondrial Ca$^{2+}$ uniporter does not have the capacity to sequester sufficient Ca$^{2+}$ from physiological transients or pulses to activate metabolic reactions [9].

Recent evidence from several sources indicates that Ca$^{2+}$ uptake from physiological pulses in heart is greater than that expected via the uniporter alone [10–12]. Schreur et al. [10] used the manganese quench technique pioneered by Miyata et al. [13] to look at mitochondrial calcium uptake in rat heart cells. Trollinger et al. [12] used a technique, which did not rely on manganese being in the proper compartments, which showed rapid increases and decreases in intramitochondrial free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{im}$) within heart myocytes. Griffiths et al. [11] showed increases in [Ca$^{2+}$]$_{im}$ and in NADH fluorescence upon inhibition of mitochondrial Na$^+$/Ca$^{2+}$ exchange. These results suggest that some additional transport mechanism acts to sequester Ca$^{2+}$ from physiological pulses into mitochondria within heart cells. Alternatively, mitochondria may sequester considerable Ca$^{2+}$ within regions of high [Ca$^{2+}$] near the Ca$^{2+}$ release channels of the sarcoplasmic reticulum where the uniporter would be able to sequester Ca$^{2+}$ more rapidly. A third possibility is that a combination of both of these processes may occur.

The RaM mediates very rapid Ca$^{2+}$ uptake at the beginning of each Ca$^{2+}$ pulse in a sequence of pulses. Its conductivity appears to be controlled by binding and release of Ca$^{2+}$ from an external binding site. When no Ca$^{2+}$ is bound to this site, Ca$^{2+}$ conductivity through the RaM is many times higher than that through the uniporter, but when Ca$^{2+}$ is bound to the site, conductivity via the RaM falls. In liver mitochondria, RaM conductivity returns within less than a second after a fall in external [Ca$^{2+}$] to a level near 100 nM and subsequent loss of Ca$^{2+}$ from the external binding–inhibition site. Both spermine and ATP significantly increase the amount of Ca$^{2+}$ uptake via RaM into liver mitochondria. This contrasts with the findings of Litsky and Pfeiffer, where ATP, ADP and AMP inhibited reverse uniporter activity, ATP being the most potent inhibitor [14]. Ca$^{2+}$ uptake of over 7 nmol/mg protein has been observed from a single 600 nM pulse via RaM into liver mitochondria. This contrasts with concentrations of spermine and ATP are in the physiological range [2,9]. This amount of uptake would be sufficient to permit [Ca$^{2+}$]$_{im}$ to activate the cellular metabolic rate or to permit mitochondrial modulation of cytosolic Ca$^{2+}$ transients or waves. Clearly, this amount of uptake per pulse would be excessive in heart mitochondria where the pulse frequency and numbers of pulses are so much greater than in liver.

There has been a recent report of rapid Ca$^{2+}$ uptake in heart mitochondria in a study using electron probe microanalysis in conjunction with the very rapid flash freezing technique. In this study the peak of intramitochondrial total [Ca$^{2+}$] was found to occur only 20 ms after the peak in cytosolic [Ca$^{2+}$] causing intramitochondrial [Ca$^{2+}$] to reflect cytosolic [Ca$^{2+}$] on a beat-to-beat basis [24,25]. Behavior of this sort would require a very rapid mitochondrial Ca$^{2+}$ influx mechanism in heart similar to that which we report here.

Here we describe the results of experiments designed to determine whether heart mitochondria contain a Ca$^{2+}$ transport mechanism similar to the RaM found in liver mitochondria and to characterize this mechanism in heart.

2. Experimental procedures

Heart mitochondria were prepared from ~4 week old White Leghorn chickens using a modification of the technique of Palmer, et al. [15]. 0.5 mg/ml of isolated mitochondria were placed in a medium con-
containing 150 mM KCl, 24 mM K-HEPES, 0.1 mM KPi, 5 mM K-succinate, 0.1 mM sucrose and 3 μM fura-2 FA at pH 7.2. [3H]Sucrose was added to the solution as follows: 1 μCi/ml of [3H]sucrose in 70% ethanol was diluted with distilled deionized water to a concentration of 0.25 μCi/ml. Then ~3 μl of [3H]sucrose per ml of sample were added to the sample. Unless otherwise noted, 400 μM cyclosporin A (CsA) and 7.5 μM tetraphenyl phosphonium (TPP) were present in the experimental media to inhibit induction of the MPT and to inhibit the Na\(^+\)-dependent efflux mechanism. Pulses were created by adding a buffered calcium solution of 3.05 mM \(^{45}\)Ca labeled CaCl\(_2\) and 20 mM HEDTA at pH 7.2 (free calcium ~1 μM) with a total activity of 50 μCi/ml and then either 10 or 100 mM BAPTA at pH 7.2. These solutions were added via a dual syringe automatic pipettor (Hamilton, Reno, NV) to a cuvette placed in a Deltascan fluorimeter (PTI, New Brunswick, NJ, USA) at 23°C. The sample was excited with 340 and 380 nm light and emission was measured at 510 nm. Fluorescence data were analyzed using Felix software. Further details of this experimental setup are described in Sparagna et al. [1]. Upon centrifugation at 4°C in a Hermle centrifuge at 12 800 rpm for 8 min (g\(_{\text{max}}\) = 13 666) the pellet and aliquots of the supernatant were added to separate vials and put in scintillation fluid. Samples were counted via a dual label technique and calcium uptake was calculated using the procedure found in Gunter et al. [16].

Interfibrillar (polytron) and subsarcolemmal (nagarse-treated) mitochondria were prepared using the technique of Palmer et al. [15]. Ruthenium red stainable mitochondria and mitochondria resistant to staining by ruthenium red were separated by use of a 10%/16% Percoll Gradient step centrifuged at 16 krpm for 30 min using a Beckman fixed angle centrifuge rotor JA-20.

Adenylate kinase activity was determined by a fluorescence technique similar to that used by Brdiczka and colleagues [17]. Samples of isolated mitochondria were placed in a cuvette containing 150 mM KCl, 100 mM triethanolamine, 16 mM MgSO\(_4\) 10 mM EDTA, 5 μM rotenone and 3 nmol/mg oligomycin at pH 7.4 along with sufficient enzyme and substrate to drive the set of reactions:

\[
\text{ATP} + \text{AMP} \xrightarrow{\text{adenylate kinase}} 2 \text{ADP}
\]

\[
\text{ADP} + \text{Phosphoenolpyruvate} \xrightarrow{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate}
\]

\[
\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{lactate dehydrogenase}} \text{lactate} + \text{NAD}^+
\]

The rate-limiting step in oxidation of NADH after mixing is the activity of adenylate kinase. Excitation was at 340 nm and emission was measured at 450 nm. After comparison of the activity of known standards, adenylate kinase activity was determined (Table 1).

### 3. Results

#### 3.1. Evidence of the RaM in heart mitochondria

When Ca\(^{2+}\) uptake into heart mitochondria from ‘square-wave’-shaped Ca\(^{2+}\) pulses is plotted as a function of pulse duration and pulse intensity ([Ca\(^{2+}\)]) as shown in Fig. 1, the results are similar

<table>
<thead>
<tr>
<th>Type of mitochondria</th>
<th>Type of substrate</th>
<th>Adenylate kinase activity (mUnits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>5 mM succinate</td>
<td>3.31 ± 0.20</td>
</tr>
<tr>
<td>Heart</td>
<td>5 mM glutamate/5 mM malate</td>
<td>3.88 ± 0.97</td>
</tr>
<tr>
<td>Liver</td>
<td>5 mM succinate</td>
<td>12.88 ± 1.39</td>
</tr>
</tbody>
</table>

Adenylate kinase activity in isolated mitochondria. Isolated mitochondria were suspended in a mannitol sucrose media containing either 5 mM succinate or 5 mM glutamate and 5 mM malate. Adenylate kinase activity was then determined by measuring the maximum rate of NADH production as described in Section 2 and compared to standards of rabbit muscle adenylate kinase of known activity.
to what was observed in liver mitochondria [1,2]. The slope of a line fit to each set of data at a given [Ca\(^{2+}\)] corresponds to the rate of Ca\(^{2+}\) uptake via the uniporter, while the intercept of the fit line with the y-axis is always significantly above zero (see Fig. 1). Since mitochondria exposed to a Ca\(^{2+}\) pulse for zero time cannot sequester any Ca\(^{2+}\), the uptake represented by this intercept must indicate either binding of Ca\(^{2+}\) to the exterior of the mitochondria, rapid exchange of unlabeled internal Ca\(^{2+}\) for labeled external Ca\(^{2+}\), or rapid net Ca\(^{2+}\) uptake. Since the pulses are terminated by addition of either EGTA or BAPTA, Ca\(^{2+}\) binding to the mitochondrial exterior is an unlikely explanation for these data. Moreover, pulses of 45Ca\(^{2+}\) that were followed by addition of AMP and ruthenium red and then exposed to 100 times as much unlabeled calcium as the labeled Ca\(^{2+}\) used in the pulse showed no significant difference in uptake rates and only a small difference in total uptake (see Fig. 2). AMP was shown to inhibit calcium uptake from the rapid mechanism (shown below in Fig. 8B) and ruthenium red is known to inhibit uniporter uptake. These results indicate that the effect of binding was minimal. Furthermore, this type of rapid uptake into non-Ca\(^{2+}\)-depleted mitochondria (total Ca\(^{2+}\) approximately 10 nmol/mg protein) is equivalent to uptake observed with Ca\(^{2+}\)-depleted heart mitochondria (total Ca\(^{2+}\) approximately 2 nmol/mg protein). This uptake is also equivalent to net uptake into heart mitochondria which have been incubated for over 20 min with labeled Ca\(^{2+}\) and then depleted of Ca\(^{2+}\) to bring their total internal Ca\(^{2+}\) down to around 2 nmol/mg protein so that initial labeled Ca\(^{2+}\) can be subtracted from the final amount of label. The results are shown in Fig. 3. These data show that there is no significant difference between the results in these three cases. These data also show that ruthenium red does not completely inhibit calcium uptake in heart mitochondria. The parity in uptake between the depleted and nondepleted cases suggests that rapid exchange of unlabeled internal Ca\(^{2+}\) for labeled external Ca\(^{2+}\) is not the explanation for these data. The observation that the rapid uptake
into both Ca\(^{2+}\)-depleted and nondepleted mitochondria is the same as net uptake of 45Ca\(^{2+}\) identifies this process as rapid net uptake, similar to that observed in liver mitochondria and termed RaM. This rapid net uptake is completely inhibited by 10 nmol/mg carbonyl cyanide 3-chlorophenyl hydrazone (CCCP) but is not inhibited by 3.33 µg/ml oligomycin or 1 µM thapsigargin, indicating that this mitochondrial uptake, like uniporter uptake, requires the mitochondrial electrochemical proton gradient (data not shown).

### 3.2. Activation and resetting requirements of the RaM

RaM in liver mitochondria showed evidence of an external calcium binding site which led to inactivation of RaM in less than 0.75 s [2]. This is true for heart mitochondria as well. Calcium uptake by RaM in heart mitochondria is complete by 0.75 s and remains inactive for the remainder of the calcium pulse. The evidence for this is seen in Fig. 1. A straight line fits these data well and there is no curvature towards zero uptake at zero pulse width as would be expected for slower uptake. If the RaM is activated at the beginning of a pulse of calcium and then quickly inactivated, under what conditions can it be reactivated or ‘reset’ so that uptake during

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**Fig. 3.** Calcium loading and depletion. Heart mitochondria were prepared and resuspended in mannitol sucrose media (uptake shown by closed circles) as described in Section 2 (without TPP and CsA) and then exposed to a pre-pulse calcium concentration of 94.3 ± 2.7 nM, a pulse calcium concentration of 263.0 ± 14.6 nM and a post-pulse calcium concentration of 78.6 ± 2.0 nM. Mitochondria treated with 10 nmol/mg ruthenium red prior to pulse generation are represented by the closed diamonds. A second portion of mitochondria were then depleted of their calcium to concentration of 1–2 nmol/mg protein by resuspension in a solution containing 169 mM mannitol, 56 mM sucrose, 2 mM HEPES, 0.5 mg/ml BSA, 1 mM EGTA, 0.1 mM MgCl\(_2\), 50 µM Pi and 20 mM NaCl at 22°C for 20 min. After being placed in KCl media as described in Section 2, mitochondria were exposed to pulses of pre-pulse calcium concentration 92.9 ± 3.3 nM, pulse calcium concentration 262.7 ± 17.7 nM and post-pulse calcium concentration 80.4 ± 3.0 nM. Uptake from these mitochondria is represented by the closed squares. A third portion of mitochondria were preloaded with 45Ca (by adding 30 nmol/mg protein radiolabeled 45Ca to the suspension) then resuspended in the same mannitol sucrose depletion media as described above at 22°C for 20 min resulting in depletion down to 1–2 nmol/mg of total Ca\(^{2+}\). After placement in KCl media, these mitochondria were exposed to a pulse of pre-pulse calcium concentration 91.3 ± 3.1 nM, pulse calcium concentration 258.7 ± 7.1 nM and post-pulse pulse calcium concentration of 80.2 ± 4.8 nM. The net uptake of labeled calcium by these samples after exposure to calcium pulses is shown by the closed triangles.

**Fig. 4.** Variation of inter-pulse time. Heart mitochondria were exposed to a sequence of two 5 s pulses of calcium (pre-pulse calcium concentration 95.7 ± 5.8 nM, inter-pulse calcium concentration 98.2 ± 6.9 nM, and post-pulses calcium concentration 88.9 ± 6.4 nM) the first pulse contained no radiolabeled 45Ca (pulse concentration 198.5 ± 15.8 nM) and the second contained 50 Ci/ml, as described in Section 2 (pulse concentration 208.7 ± 15.6 nM). Calcium uptake was then measured while varying the interpulse period (represented by the closed squares). This uptake was then compared to calcium uptake from a single pulse of radiolabeled calcium (pre-pulse calcium concentration 198.5 ± 15.8 nM) and the second contained 50 µCi/ml, as described in Section 2 (pulse concentration 208.7 ± 15.6 nM). Calcium uptake was then measured while varying the interpulse period (represented by the closed squares).
a subsequent pulse is as large as the uptake during the first pulse? We answered this question with heart mitochondria through experiments using two pulses of calcium similarly to the way it was answered with liver mitochondria [2]. First, we exposed the heart mitochondria to two similar pulses, dropping the [Ca$^{2+}$] during the interpulse period to the initial [Ca$^{2+}$] of 90 nM, and making only the second pulse radioactive in order to determine uptake from the second pulse only. The time between pulses was varied. This experiment indicated that 60–90 s (Fig. 4) is the required duration of the interpulse period for uptake during the second pulse to be as large as uptake during a single, similar pulse of calcium. In other words, it takes 60–90 s to totally reset all the RaM transporters. It is significant that calcium uptake from the second pulse of a two pulse sequence does not drop to zero even at very small interpulse periods, suggesting that not all the RaM transporters are inactive at very short interpulse periods. In fact, at the shortest possible interpulse period that could be created with this pulse generation system (0.75 s), ~0.09 nmol/mg Ca$^{2+}$ was taken up per pulse.

Next we determined how low the [Ca$^{2+}$] must fall during the interpulse period before uptake during the second pulse is equivalent to uptake during the first pulse. We exposed the heart mitochondria to two similar pulses, dropping the [Ca$^{2+}$] during the interpulse period to the initial [Ca$^{2+}$] of 90 nM, and making only the second pulse radioactive in order to determine uptake from the second pulse only. The time between pulses was varied. This experiment indicated that 60–90 s (Fig. 4) is the required duration of the interpulse period for uptake during the second pulse to be as large as uptake during a single, similar pulse of calcium. In other words, it takes 60–90 s to totally reset all the RaM transporters. It is significant that calcium uptake from the second pulse of a two pulse sequence does not drop to zero even at very small interpulse periods, suggesting that not all the RaM transporters are inactive at very short interpulse periods. In fact, at the shortest possible interpulse period that could be created with this pulse generation system (0.75 s), ~0.09 nmol/mg Ca$^{2+}$ was taken up per pulse.

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pulse. In the second type of double pulse experiment, 
$\text{Ca}^{2+}$ uptake was measured from a series of two indentical pulses, the interpulse calcium concentration was varied, and uptake from the second pulse was compared to that from a single calcium pulse of identical size and shape. The results in Fig. 5 show that this interpulse $[\text{Ca}^{2+}]$ must fall to a value near or below 175 nM before uptake during the second pulse is equivalent to uptake of the first pulse. In this regard, RaM in heart is similar to RaM in liver mitochondria. The results shown in Figs. 4 and 5 together indicate that all that is necessary to reset the mechanism mediating rapid $\text{Ca}^{2+}$ uptake into heart mitochondria is a fall in $[\text{Ca}^{2+}]$ below 175 nM for the order of a minute. In heart mitochondria the system must be exposed to this lower $[\text{Ca}^{2+}]$ for a much longer period of time than was found for liver mitochondria which required less than 1 s. These results predict that if the pulses are separated by approximately 60 s of $[\text{Ca}^{2+}]$ near 100 nM, the mitochondria will sequester significant amounts of $\text{Ca}^{2+}$ during each pulse. This is verified by the data shown in Fig. 6, where calcium uptake was observed from sev-

Fig. 7. Effects of spermine on RaM uptake. Heart mitochondria were placed in KCl media as specified in Section 2 in the presence of varying amounts of spermine and then exposed to pulses of calcium (pre-pulse calcium concentration 19.5 ± 6.7 nM, pulse calcium concentration 225.2 ± 52.4 nM and post-pulse calcium concentration 22.0 ± 8.4 nM) of different duration. Calcium uptake from the RaM was estimated by taking the $y$-intercept of a line fitted to a calcium uptake vs. pulse width curve, similar to Fig. 1. The rate of uniporter uptake was measured by taking the slope of the line. Error bars are one standard deviation for slope and intercept of linear least squares fitted lines.

Fig. 8. Effects of AMP on RaM uptake in liver and heart. (A) Liver mitochondria were prepared and calcium uptake was measured as described in Sparagna et al. (Sparagna, 1995). Calcium pulses (pre-pulse calcium concentration 112.6 ± 8.3 nM, pulse magnitude 311.1 ± 21.7 nM and post-pulse calcium concentration 108.1 ± 7.7 nM) were produced in the presence and absence of 2 mM AMP and 2 mM cAMP. The closed circles represent calcium uptake with 0 AMP and 0 cAMP added. The closed squares represent calcium uptake with 2 mM AMP added. The closed diamonds represent calcium uptake with 2 mM cAMP added. Error bars represent one standard deviation of two repeats. (B) Heart mitochondrial calcium uptake was measured as described in Section 2 (without TPP and CsA). Calcium pulses (pre-pulse calcium concentration 59.3 ± 6.1 nM, pulse calcium concentration 500.2 ± 50.2 nM and post-pulse calcium concentration 11.5 ± 1.4 nM) were made in the presence and absence of 2 mM AMP and 2 mM cAMP. The closed circles represent calcium uptake with 0 AMP and 0 cAMP added. The closed squares represent calcium uptake in the presence of 2 mM added AMP. The closed diamonds represent calcium uptake in the presence of 2 mM added cAMP. Error bars represent one standard deviation of two repeats.
eral pulses of calcium and compared to uptake from a single high-Ca$^{2+}$ pulse of identical duration.

These results, like RaM behavior in liver mitochondria, are consistent with a model in which the high conductivity of the RaM, manifest at the initiation of the Ca$^{2+}$ pulse, is inhibited by binding of Ca$^{2+}$ from the pulse to a modulation site on the external or cytosolic side of the RaM complex. A difference in the behavior of the RaM in heart mitochondria from that of liver mitochondria is that it takes 60–90 s below 175 nM [Ca$^{2+}$] to reset sufficient RaM transporters in heart mitochondria to get the same uptake during the second pulse as that obtained during the first, while it takes less than a second at this calcium concentration in liver mitochondria.

### 3.3. Modulators and effectors of the RaM

The polyamine spermine is known to increase or decrease Ca$^{2+}$ uptake via the uniporter depending on the [Ca$^{2+}$] [18]. The physiological level of spermine in heart cells is around 300 μM [19]. The effect of this amount of spermine on Ca$^{2+}$ uptake via RaM in heart mitochondria is shown in Fig. 7. Physiological amounts of spermine slightly enhance uptake of Ca$^{2+}$ via RaM from physiological-type pulses in heart mitochondria, whereas in liver mitochondria physiological amounts of spermine increase Ca$^{2+}$ uptake via RaM from physiological-type Ca$^{2+}$ pulses by a factor of six or more [2]. The general effect of spermine on both types of mitochondria seems to be the same, but spermine levels in liver cells are higher (about 1 mM), and the effects of spermine on liver mitochondria are greater than those on heart mitochondria even at similar spermine concentrations.

Fig. 8A shows that 2 mM AMP has no effect on uptake via the RaM in liver mitochondria whereas that amount of AMP severely inhibits uptake via RaM in heart mitochondria (shown in Fig. 8B). Fig. 9A shows that the effect of 1 mM ATP is to almost double the Ca$^{2+}$ uptake via RaM in liver mitochondria. GTP causes a similar enhancement of uptake via the RaM in liver mitochondria (data not shown). Fig. 9B shows that even a considerably greater amount of ATP does not effect uptake via the RaM in heart mitochondria. Fig. 9B also shows that a high concentration of ADP inhibits the RaM in heart mitochondria while having little effect on uniporter uptake. One way in which ADP might inhibit RaM is through its conversion to AMP by adenylate kinase. Fig. 10 shows that ADP inhibits Ca$^{2+}$ uptake...
via RaM in heart mitochondria even when adenylate kinase has been completely inhibited by 1 mM of the potent noncompetitive inhibitor P1,P5-di(adenosine-5’)pentaphosphate (AP5A).

Fig. 10. Effects of ADP upon adenylate kinase inhibition. Isolated heart mitochondria were exposed to pulses of calcium (pre-pulse calcium concentration 51.3 ± 7.7 nM, pulse calcium concentration 332.1 ± 24.0 nM and post-pulse calcium concentration 79.1 ± 19.0 nM) without the addition of ADP (uptake shown by the closed circles), in the presence of 1 mM added ADP (uptake represented by closed squares) or in the presence of 1 mM ADP and 1 μM AP5A (uptake represented by closed diamonds).

Fig. 11. ADP dose response. Heart mitochondria were exposed to pulses of calcium as described in Section 2 with or without 3 nmol/mg protein of oligomycin present in the pulse generation media. Pulses were made in the absence or presence of different amounts of ADP. Pre-pulse calcium concentration was 58.4 ± 10.6 nM, pulse calcium was 274.1 ± 35.2 nM and post-pulse calcium concentration was 74.8 ± 11.1 nM. RaM uptake was determined by the intercept of calcium uptake vs. pulse width curves. The closed circles show RaM uptake in the absence of oligomycin and the closed squares show uptake in the presence of oligomycin. Error bars were one standard deviation for the intercept of least squares fitted lines.

via RaM in heart mitochondria even when adenylate kinase has been completely inhibited by 1 μM of the potent noncompetitive inhibitor P1,P5-di(adenosine-5’)pentaphosphate (AP5A).

Fig. 11 shows that the response of RaM to ADP is biphasic. ADP activates Ca2+ uptake via the RaM throughout the range of accepted physiological concentration. However, at higher concentrations it inhibits Ca2+ uptake via the RaM. Data are shown in both the presence and absence of oligomycin, an inhibitor of phosphorylation. Although the data in the absence of oligomycin are more physiological, the ADP concentration in the presence of oligomycin should deviate less. This stability of ADP concentration is most important at the lowest ADP concentration levels. Note that the data in the presence of oligomycin seem to be shifted slightly toward lower ADP concentrations, consistent with the expected effect of oligomycin. These effects of ADP are particularly interesting in view of the measurements of Balaban showing that the concentration of ADP in metabolically active cells is around 20 μM [20], studies indicating the Km for ADP association with the F1ATPase is 20–27 μM in Escherichia coli [21,22] and studies showing the Km for ADP in heart mitochon-
dria is 3–150 μM depending on the proton motive force [23]. The possible effects of the biphasic response of RaM to ADP are discussed below. AMP inhibition of RaM Ca$^{2+}$ uptake is shown in Fig. 12. RaM and uniporter are both inhibited by AMP in hyperphysiological concentrations. Inhibition by ruthenium red varied with different preparations of heart mitochondria. Sometimes ruthenium red inhibited RaM and other times it had little effect. A qualitative correlation between the amount of red staining of mitochondrial pellets and the amount of inhibition by ruthenium red was observed. Heart mitochondria were separated into interfibrillar and subsarcolemmal populations [15] in order to determine if this variability was related to different admixtures of these two known types of heart mitochondria. Although pellets formed from mitochondria prepared using the nagarse technique showed more staining by ruthenium red than those prepared using the polytron technique, both populations of mitochondria showed staining by ruthenium red and inhibition of the RaM and uniporter (data not shown). Running either preparation of mitochondria through a Percoll density gradient allows separation into two populations, one of which could be stained by ruthenium red while the other could not. Mitochondria insensitive to staining by ruthenium red showed no significant RaM inhibition by ruthenium red as well as a greater resistance to uniporter inhibition when compared with mitochondria sensitive to staining by ruthenium red. Fig. 13 shows a typical heart mitochondrial preparation in which ruthenium red inhibits the uniporter, but does not significantly inhibit RaM. A second preparation of mitochondria (data not shown) contained mostly mitochondria sensitive to ruthenium red staining. These mitochondria showed similar behavior to liver mitochondria, in which the RaM was inhibited by ruthenium red. However, the RaM in ruthenium red-stainable heart mitochondria required a 100-fold higher concentration of ruthenium red for inhibition than the uniporter, compared to a 10-fold higher concentration for liver mitochondria. The respiratory control ratios of mitochondria sensitive to ruthenium red staining are less than those of mitochondria resistant to ruthenium red staining, suggesting that mitochondria sensitive to ruthenium red staining include a greater portion of damaged mitochondria (data not shown). A mixed population of the same mitochondria has respiratory control ratios that are between the two values. No population of mitochondria resistant to ruthenium red staining was observed in liver mitochondria.

4. Discussion

4.1. Similarities and differences in the RaM in heart and liver mitochondria

RaM in heart mitochondria has many of the same characteristics as RaM in liver mitochondria:

1. The rapid uptake at the beginning of each pulse leads to frequency-modulated signaling in both tissues.
2. When the mitochondria are suspended in simple HEPES-buffered KCl medium containing substrate, the amount of Ca$^{2+}$ uptake via RaM into heart mitochondria is about the same as that into liver mitochondria.
3. In both types of tissue, RaM provides a much faster pathway of mitochondrial Ca\(^{2+}\) uptake than the uniporter.

4. RaM in both types of tissue is inhibited by [Ca\(^{2+}\)] in the suspending medium greater than 150–200 nM. This inhibition is mediated by Ca\(^{2+}\) binding to an external binding site, occupancy of which reduces RaM conductivity at least to the level of that of the uniporter. RaM characteristics fit a proposed model (described below) in which the RaM could serve to control the rate of oxidative phosphorylation and also to minimize the probability of induction of the mitochondrial MPT.

There are significant differences between the characteristics of the RaM in heart and liver mitochondria as well:

1. A population of heart mitochondria resistant to staining by ruthenium red was found, where no such population was observed in liver mitochondria. These healthier mitochondria had no significant inhibition of RaM Ca\(^{2+}\) uptake by ruthenium red. (shown in Section 3)

2. The RaM in liver is capable of sequestering as much Ca\(^{2+}\) from a second identical pulse as from the first pulse within a fraction of a second following a fall in extramitochondrial [Ca\(^{2+}\)] to around 100 nM, whereas in heart it requires 1 min or longer for complete ‘resetting’ of all the RaM transporters. These differences between the RaM in liver and heart in the time required for Ca\(^{2+}\) to come off the inhibition site probably relate to the different characteristics of Ca\(^{2+}\) pulses seen in these two tissues. In heart, Ca\(^{2+}\) pulses are very short (perhaps half a second or less in duration) and occur every second or less. In contrast, in liver the pulses are 5–15 s long and may occur as often as every 20 s or as infrequently as every several minutes following activation by hormone [6]. If the heart RaM sequestered Ca\(^{2+}\) from each pulse as efficiently as the liver RaM, far more Ca\(^{2+}\) would be sequestered than necessary for activation of ATP production. Because increasing intramitochondrial [Ca\(^{2+}\)] increases the possibility of the MPT, the altered RaM characteristics in heart mitochondria could provide protection against inducing the MPT, while supplying sufficient Ca\(^{2+}\) to control the metabolic rate, as described below.

3. While spermine, ATP, and GTP greatly increase the Ca\(^{2+}\) sequestered under physiological conditions by liver mitochondria, RaM Ca\(^{2+}\) uptake by heart mitochondria is unaffected by ATP and GTP and is only slightly enhanced by a physiological level of spermine: the maximum Ca\(^{2+}\) uptake per pulse in the presence of physiological amounts of these agents was above 7 nmol/mg protein with liver mitochondria but maximum uptake under physiological amounts of these agents was only around 1 nmol/mg protein in heart mitochondria (data not shown).

4. The heart RaM has a biphasic response to ADP, while the liver RaM is unaffected by variation in ADP. ADP, of course, also directly stimulates the rate of ATP production. The data shown in Fig. 12 suggest that direct stimulation through ADP and indirect stimulation through ADP-enhanced RaM uptake act synergistically within the physiological range of ADP concentration. The maximum observed ADP stimulation of RaM is in the region of measured ADP levels in metabolically active cells (20 μM) [20] and of the \(K_m\) for ADP interaction with the F\(_1\)ATPase (20–27 μM in E. coli and 3–150 μM in heart mitochondria, depending on the proton motive force) [21–23]. Above this concentration range, RaM Ca\(^{2+}\) uptake falls off dramatically with increasing ADP concentration. Direct ADP stimulation of the phosphorylation rate is very high in this range. Additional stimulation via the RaM is probably unnecessary and eliminating it decreases intramitochondrial Ca\(^{2+}\) providing further protection against induction of the MPT.

5. AMP is a strong inhibitor of RaM in heart mitochondria, while it has no effect on RaM in liver. Although the concentration of AMP that inhibits RaM is hyperphysiological, this inhibition can be almost complete at high AMP concentrations. ADP also inhibits RaM at concentrations which might occur pathologically. Under pathological conditions of high [AMP] or [ADP], the heart cell would require a great amount of energy. AMP potently activates glycolysis by activation of phosphofructokinase [26] and ADP can also activate glycolysis. Glycolysis can produce a faster
rate of ATP production than oxidative phosphorylation for a short period after which acidification of the cytosol inactivates glycolysis [27–29]. High [AMP] could be a signal to the cell to inactivate oxidative phosphorylation (by inhibiting RaM) and upregulate glycolysis which not only temporarily increases ATP production but also acidifies the cytosol and provides further protection against the MPT.

Under conditions where [ATP] is low and [ADP] and [AMP] are high, several factors act to protect the cell against induction of the Ca\(^{2+}\)-induced MPT which could otherwise be fatal: (1) Direct inhibition of the MPT by ADP [30], (2) acidification of the cytosol following activation of glycolysis which is also a potent MPT inhibitor [31], (3) inhibition of Ca\(^{2+}\) uptake via RaM by high ADP or AMP concentration may be another factor.

### 4.2. An hypothesis as to how the RaM may function to activate ATP production in liver and heart

Clearly, the regulation and control of the RaM in heart and in liver are quite different. Consideration of the differences between the RaM in heart and liver mitochondria and between the uniporter and RaM in both tissues suggest a novel strategy for Ca\(^{2+}\) signaling via RaM. The heart data in particular suggest that minimizing the amount of Ca\(^{2+}\) necessary for activation of the Ca\(^{2+}\)-sensitive metabolic reactions is an important RaM characteristic. Essentially every difference between RaM in liver and in heart caused uptake via heart RaM to be lower relative to that in liver. It is doubtful that evolution would have caused

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**Fig. 14.** Possible RaM function: how it may differ in liver and heart mitochondria. The cytosolic calcium pulses to which liver and heart mitochondria are exposed in vivo are different. While the amplitude (calcium concentration) of the pulses may be in the same range (typically 0.6–1.2 \(\mu\)M), the grouping of pulses, their frequency and their duration differ markedly. In the cytosol of liver cells there may be 6–10 pulses in a sequence following exposure of the cell to a hormone, such as vasopressin or catecholamine. These pulses may be spaced 20 s to over a minute apart and are between 5 and 15 s in duration. In contrast, a heart cell has continual pulses which may be less than half a second in duration and temporally separated by a fraction of a second to longer than a second. Because the RaM can function only during the first fraction of a second of each pulse, without differences in control, it could sequester much more calcium in heart mitochondria than in liver mitochondria. Consistent with the data, two models that illustrate how RaM may function to provide a sufficient calcium signal in liver while avoiding calcium overload and induction of the MPT in heart are depicted in A and B, respectively. (A) With an approximate rate of 1 calcium pulse per minute in liver, all of the RaM transporters function in phase to sequester an amount of calcium from each transporter sufficient to activate intramitochondrial calcium-sensitive reactions. (B) With an approximate rate of one calcium pulse per second in heart, only a few RaM transporters function at each pulse, producing regions of high local free calcium concentration which differ from pulse to pulse. Over the course of a minute or two, however, the free calcium concentrations present in the micro-region of a calcium sensitive enzyme on the inner surface of the inner mitochondrial membrane may not be very different in the two cases.
the RaM in these tissues to diverge in this way unless Ca^{2+} poses a real threat to heart mitochondria. This also suggests that a significant function of the RaM is to minimize the need for total Ca^{2+} in metabolic Ca^{2+} signaling.

How might this be done? The most striking difference between the Ca^{2+} sequestered via RaM and that sequestered via uniporter is the speed of the uptake. In liver mitochondria, maximum RaM conductivity is 300 times (or more) faster than that of the uniporter. RaM conductivity is also much faster than that of the uniporter in heart mitochondria, although the lower limit of demonstratable maximum permeability is less than that of the liver RaM. Electron paramagnetic resonance studies have shown that intramitochondrial Mn^{2+} and Ca^{2+} compete with protons and must displace protons before binding [32–35]. This slows the process of Ca^{2+} or Mn^{2+} binding inside mitochondria. Because intramitochondrial Ca^{2+} binding is slow and RaM uptake is so fast, during the very rapid Ca^{2+} uptake via RaM, uptake could outpace binding and cause the intramitochondrial free Ca^{2+} concentration ([Ca^{2+}]_{m}) to rise much higher during RaM uptake than during the much slower Ca^{2+} uptake via the uniporter. Ca^{2+} binding to all intramitochondrial metabolic activation sites must be controlled by [Ca^{2+}]_{m}. If Ca^{2+} binding to an activation site is fast, then the process could be sensitive to the difference between RaM uptake of Ca^{2+} and uniporter uptake of Ca^{2+}. This hypothesis, exploiting differences in [Ca^{2+}]_{m} and binding for Ca^{2+}-mediated events within the matrix, provides a possible novel strategy for control of these processes of activation. Because the ratio of [Ca^{2+}]_{m} to total matrix Ca^{2+} would also be significantly increased during this brief period of elevated [Ca^{2+}]_{m}, the total amount of mitochondrial Ca^{2+} uptake necessary for metabolic signaling could be minimized.

A model of possible RaM function in liver and heart mitochondria is shown in Fig. 14A,B. It is assumed that the high rate of Ca^{2+} uptake via RaM causes regions of elevated [Ca^{2+}]_{m} in a local region around each RaM mechanism. In liver mitochondria (Fig. 14A), all of the RaM transporters function in phase at the beginning of each Ca^{2+} transient causing a large increase in [Ca^{2+}]_{m} within the crista for a brief period at the beginning of the pulse. In heart, on the other hand (Fig. 14B), with many more Ca^{2+} pulses per minute, only a small fraction of the RaM mechanisms are conductive at the beginning of each pulse. However, if the loss of Ca^{2+} inhibition of RaM conductivity is stochastic, then a small and different set of RaM transporters is conductive at the beginning of each pulse. Over a minute and around 60 pulses, the regions of high local Ca^{2+} concentration vary stochastically over the crista, such that exposure to high [Ca^{2+}] over the course of a minute in any local region may not be very different from the exposure in liver mitochondria over the same time frame.

The observed activation of Ca^{2+} uptake via RaM by ADP at physiological ADP concentrations and inhibition at very high ADP concentrations support the concept that the physiological role of RaM is rapid sequestration of Ca^{2+}, potentially to enhance the rate of ATP production under physiological conditions. From this perspective, it is probably not coincidence that the maximum ADP activation of RaM Ca^{2+} uptake in heart mitochondria occurs (to within precision of measurement) at the level of ADP found in metabolically active heart cells and at the K_{m} for ADP association with the F_{1}ATPase.

These considerations use the RaM’s observed characteristics in heart and liver mitochondria and the observed Ca^{2+} transients in these tissues to provide a model of how the RaM in both these tissues may function as a part of the mechanism controlling the rate of oxidative phosphorylation while minimizing the probability of induction of the MPT.

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References