Mitochondrial Calcium Uptake from Physiological-type Pulses of Calcium

A DESCRIPTION OF THE RAPID UPTAKE MODE*

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A controversy in the field of bioenergetics has been whether mitochondria are capable of sequestering enough Ca$^{2+}$ from cytosolic Ca$^{2+}$ pulses to raise their intramitochondrial free Ca$^{2+}$ level ([Ca$^{2+}$]$_{mi}$). This is significant because an increase in [Ca$^{2+}$]$_{mi}$ has been linked to an increase in cellular metabolic rate through various mechanisms. To resolve this question, we exposed isolated liver mitochondria to physiological-type pulses of Ca$^{2+}$ produced using a pulse-generating system (Sparagna, G. C., Gunter, K. K., and Gunter, T. E. (1994) Anal. Biochem. 219, 96–103). We then measured the resulting mitochondrial Ca$^{2+}$ uptake. The unipporter was previously thought to be the only specific Ca$^{2+}$ uptake mechanism in mitochondria. Our studies have uncovered an additional uptake mechanism, the rapid mode of uptake or RaM, which functions at the beginning of each pulse and allows mitochondria to sequester a considerable amount of Ca$^{2+}$ from short pulses. We have shown that the RaM is reset by decreasing the [Ca$^{2+}$] between pulses for a very short time, making this uptake mode ideally suited for Ca$^{2+}$ sequestration from Ca$^{2+}$ pulse sequences. With rapid Ca$^{2+}$ uptake occurring at the beginning of each pulse, liver mitochondria may be able to sequester sufficient Ca$^{2+}$ from a short sequence of pulses to activate the cellular metabolic rate.

It has been suggested that the intramitochondrial free calcium concentration ([Ca$^{2+}$]$_{mi}$) can function as a metabolic mediator, acting at several loci to control the cellular metabolic rate (1–4). Key steps within several important metabolic processes are activated by [Ca$^{2+}$]$_{mi}$. At least two dehydrogenases important in the control of the Krebs cycle, pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, can be controlled by [Ca$^{2+}$]$_{mi}$ of changes of a few nanomoles per milligram of protein (1, 5) (equivalent to [Ca$^{2+}$]$_{mi}$, values of a micromolar or less). A third dehydrogenase, isocitrate dehydrogenase, while also activated by [Ca$^{2+}$]$_{mi}$, requires a matrix [Ca$^{2+}$]$_{mi}$ above 3 μM for significant activation and therefore may or may not be involved in physiological responses (6).

The activities of other important metabolic steps have also been suggested to be controlled by matrix [Ca$^{2+}$]$_{mi}$. Halestrap and Griffiths (7) have suggested that the rate of electron transport in liver mitochondria is enhanced through a Ca$^{2+}$-induced swelling process mediated by accumulation of pyrophosphate. Both the F$_1$ ATPase (8, 9) and the adenine nucleotide translocase (10–13) have also been identified as sites for Ca$^{2+}$ modulation of metabolic rate.

The primary controversy has been whether or not mitochondria are capable of sequestering a sufficient amount of Ca$^{2+}$ from physiological pulses to mediate these Ca$^{2+}$-activated metabolic steps. Most of the results of electron probe microanalysis studies used in conjunction with rapid freezing of the tissue have suggested that little if any Ca$^{2+}$ is sequestered by mitochondria from physiological pulses (14–18). Extrapolation of existing data (19) on Ca$^{2+}$ uptake by mitochondria to the short times relevant to rapid pulses of Ca$^{2+}$ has also suggested that mitochondrial uptake of Ca$^{2+}$ from these pulses would probably be insufficient for [Ca$^{2+}$]$_{mi}$, to function as an intramitochondrial metabolic mediator.

Conversely, other experiments have suggested that significant Ca$^{2+}$ may in fact be sequestered by mitochondria from physiological pulses. One of these experiments involves electron probe microanalysis (20), and the two described below utilize fluorescence techniques. In the first set of experiments, increases in intramitochondrial [Ca$^{2+}$]$_{mi}$ were measured following an increase in the frequency of Ca$^{2+}$ pulses in cardiac myocytes using the fura-2 fluorescence technique by using Mn$^{2+}$ to selectively quench the fluorescence of cytosolic fura-2 (21). In the second set of experiments, Rizzuto et al. (22–24) fused the Ca$^{2+}$-sensitive photoprotein aequorin in frame with a mitochondrially directed leader sequence from subunit VIII of human cytochrome oxidase. This hybrid DNA was transfected into bovine endothelial cells, obtaining stable lines that express variable amounts of intramitochondrial aequorin. Activation of these cells with external ATP caused an increase in the cytosolic free calcium concentration ([Ca$^{2+}$]$_o$) (measured using fura-2 techniques) from 100 to 500 nM and in [Ca$^{2+}$]$_{mi}$ (measured using aequorin luminescence) from around 200 nM to over 5 μM. This observed mitochondrial response was very rapid and returned to baseline in about 10 s. It was also drastically reduced by exposure to the uncoupler FCCP, which, by dissipating the electrochemical proton gradient, Δψ, would greatly reduce the driving force for mitochondrial Ca$^{2+}$ accumulation via the unipporter.

With few exceptions (25, 26), studies of Ca$^{2+}$ uptake via the mitochondrial unipporter have at best utilized steady buffered levels of free calcium ([Ca$^{2+}$]$_o$) (see Refs. 19 and 27), while in many cell types the exposure of the mitochondria within a cell to significant [Ca$^{2+}$]$_o$ occurs because of cytosolic Ca$^{2+}$ pulses. In view of the controversy as to whether mitochondria can actually sequester significant Ca$^{2+}$ from physiological pulses, it would seem appropriate to reinvestigate Ca$^{2+}$ sequestration by isolated mitochondria, focusing on uptake of Ca$^{2+}$ from pulses. The use of isolated mitochondria would greatly increase the.

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accuracy with which this uptake data could be obtained. Furthermore, on the basis of extrapolations of data in the existing literature, it is difficult to understand how the [Ca\textsuperscript{2+}]\textsubscript{m} levels could increase so rapidly upon exposure to a pulse of cytosolic [Ca\textsuperscript{2+}], as was observed by Rizzuto et al. (22–24).

We have set up an apparatus that allows us to create and measure Ca\textsuperscript{2+} pulses in a cylindrical cuvette similar to those observed in vivo in many types of tissue. At the same time, this apparatus permits us to measure mitochondrial Ca\textsuperscript{2+} uptake accurately using a dual label isotope technique. A detailed description of this apparatus and of the results of preliminary experiments studying Ca\textsuperscript{2+} sequestration by mitochondria has been published (26). We report here the results of a more extensive study of sequestration of Ca\textsuperscript{2+} from pulses by isolated liver mitochondria. Our studies indicate that mitochondria can sequester Ca\textsuperscript{2+} very rapidly for a short period of time via a process unsuspected prior to the initiation of these studies (26), the rapid mode of Ca\textsuperscript{2+} uptake (RaM).

**MATERIALS AND METHODS**

Rat liver mitochondria were prepared as described in Sparagna et al. (26) and Wingrove and Gunter (28) using Sprague-Dawley specific pathogen-free rats weighing 180–200 g. Experiments were carried out in either medium A or B. Medium A contained (in mM) 150 KCl, 24 potassium-HEPES, 5 potassium succinate, 0.1 sucrose, and 0.1 potassium P, at pH 7.2. Medium B was medium A plus 3 \(\mu\)M fura-2 FA (Texas Fluorescence Labs). Where appropriate, components of these solutions at high concentration were passed through a Chelex-100 column to remove divalent cations. Ca\textsuperscript{45+}-HEDTA and EGTA solutions were made using separate standard solutions (in mM) of 100 CaCl\textsubscript{2}, 500 EGTA, and 100 HEDTA diluted with medium A. The standard solutions had been titrated as described (29) to check the accuracy of their concentrations. The Ca\textsuperscript{2+}-HEDTA solution contained 3.05 mM \(^{45}\text{CaCl}_2\) (20 nCi/ml) and 20 mM HEDTA at pH 7.2. The EGTA solution contained either 2.5 or 100 mM EGTA (see figure captions) at pH 7.2. The \(K_w\) of fura-2 FA was determined to be 275 nM (29). A dual label technique was used to determine mitochondrial Ca\textsuperscript{2+} uptake as described (30). In addition to the \(^{45}\text{Ca}\) in the Ca\textsuperscript{45+}-HEDTA solution, a \[^{3}H\text{Isoucrose\ solution was used for uptake studies containing 1 \(\mu\)Ci/ml [\[^{3}H\text{Isoucrose (DuPont NEN) in 70} \%\text{ ethanol diluted with double distilled water to a specific activity of 0.25 \(\mu\)Ci/ml. All solutions used were Na\text{+-free}. For each experiment, 3 ml of medium B, 0.5 mg/ml isolated mitochondria, and 8 \(\mu\)l of [\[^{3}H\text{Isoucrose were used. The pulse-generating and monitoring system used contains a dual syringe automatic pipettor (Microlab 940, Hamilton) and an MS-III fluorimeter (Photon Technology International) controlled by IBM-compatible 286 and 486 computers, respectively. The details of the experimental set up and characteristics of the pulse apparatus are as described previously (26).**

**RESULTS**

Amount of Calcium Uptake from Rapid Pulses—In an earlier paper (26), we showed that mitochondria were capable of sequestering Ca\textsuperscript{2+} from artificially generated, rapid pulses designed to be similar to those observed in the cytosol of many types of cells in vivo following hormonal activation. Control experiments (see “Discussion”) indicated that what was being observed was actual net uptake and not either external binding or rapid exchange. The Ca\textsuperscript{2+} appeared to be sequestered in two distinct “modes,” a rapid uptake (or high conductivity) mode (RaM) of very short duration followed by a slower uptake (or lower conductivity) mode, which has the characteristics of the Ca\textsuperscript{2+} uniporter. The terminology “rapid mode of uptake” and “slower mode of uptake” was chosen so as not to bias the issue of whether or not both uptake modes were mediated by the mitochondrial Ca\textsuperscript{2+} uniporter. Uptake via both uptake modes increased with the height ([Ca\textsuperscript{2+}]) of the pulses; however, below around 200 nM all uptake appeared to be via the RaM.

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1 The abbreviations used are: RaM, rapid mode of Ca\textsuperscript{2+} uptake; HEDTA, N-hydroxyethylendiamine triacetate acid.

**Fig. 1.** A schematic diagram of our Ca\textsuperscript{2+} pulses. The letters in the diagram represent the following: \(h_1\), pulse height; \(i\), interpulse period; \(h_0\), initial [Ca\textsuperscript{2+}] level of the medium before the pulse; \(h_2\), [Ca\textsuperscript{2+}] height of the pulse; \(h_3\), [Ca\textsuperscript{2+}] level during the interpulse period.

**Fig. 2.** Mitochondrial rapid and slower Ca\textsuperscript{2+} uptake. Mitochondria were exposed to single Ca\textsuperscript{2+} pulses with widths between 1 and 10 s, and the resulting amount of Ca\textsuperscript{2+} uptake was determined for pulses of various heights. All pulses were made by adding 2.5 mM EGTA and the Ca\textsuperscript{45+}-HEDTA solution as defined under “Materials and Methods.” Mitochondria were suspended at 0.5 mg of protein/ml in 3 ml of medium B, to which 33 \(\mu\)l EGTA (final concentration) had been added before pulses were made. The (closed circles) data represent pulses with an average height of 484 ± 38 nM [Ca\textsuperscript{2+}], which were made using 17 \(\mu\)l of Ca\textsuperscript{45+}-HEDTA and 100 \(\mu\)l EGTA. The (closed triangles) data represent pulses with an average height of 281 ± 15 nM [Ca\textsuperscript{2+}], which were made using 10 \(\mu\)l of Ca\textsuperscript{45+}-HEDTA and 60 \(\mu\)l of EGTA. The (closed squares) data represent pulses with an average height of 171 ± 5 nM [Ca\textsuperscript{2+}], which were made using 5 \(\mu\)l of Ca\textsuperscript{45+}-HEDTA and 40 \(\mu\)l of EGTA. In the control pulses (×), ruthenium red (4 nmol/mg of mitochondrial protein) was added before the pulses were made. The control pulses had an average height of 329 ± 64 nM [Ca\textsuperscript{2+}] and were made using 10 \(\mu\)l of Ca\textsuperscript{45+}-HEDTA and 60 \(\mu\)l of EGTA. Error bars represent one standard deviation of three repetitions.

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**Fig. 1** is a diagram of a sequence of two Ca\textsuperscript{2+} pulses showing the parameters that were varied in our experiments. The pulses we generated are trapezoidal-shaped, and the width (w) is therefore taken as the width at halfway between the initial Ca\textsuperscript{2+} level before the pulse (\(h_0\) for the first pulse, \(h_2\) for the second) and the height of the pulse (\(h_2\)). The interpulse period (i) is also measured at halfway between these two Ca\textsuperscript{2+} levels.

**Fig. 2** is a graph showing Ca\textsuperscript{2+} uptake from a single calcium pulse as the width (w) is varied. Each curve represents a series of single pulses of a single height (\(h_2\)). For clarity, curves having only three different heights and one to which ruthenium red (a potent inhibitor of the uniporter) was added are shown, but we have carried out this experiment with pulses of many heights (data not shown). All pulses have an initial [Ca\textsuperscript{2+}] (\(h_0\)) of between 30 and 60 nM and an ending [Ca\textsuperscript{2+}] of less than 10 nM.

Clearly, a mitochondrial suspension that is exposed to a Ca\textsuperscript{2+} pulse for zero time can sequester no Ca\textsuperscript{2+} from that pulse. Therefore, the observation that an extrapolation of all uptake curves of the type shown in Fig. 2 invariably intercepts the zero time line at a value of sequestered Ca\textsuperscript{2+} significantly above zero must be interpreted as an indication that a brief period of
with those reported earlier as Ca\(^{2+}\)). The slope of the lines fit to each set of data in Fig. 2 is referred to as the lower conductivity or unipporter mode. The rates of uptake observed for the lower conductivity mode are consistent with those reported earlier as Ca\(^{2+}\) sequestration via the unipporter (19, 31). The amount of rapid uptake in mitochondria seems to be relatively independent of pulse height (h\(_1\)) in pulses with heights above 500 nM Ca\(^{2+}\) (data not shown), whereas the amount of slower uptake increases rapidly as pulse heights increase at pulse heights above 400 nM Ca\(^{2+}\).

Is the RaM Uptake Mediated by the Mitochondrial Uniporter Complex?—As shown previously (26) and in Fig. 2, when a sufficient amount of ruthenium red is added to the suspension prior to the Ca\(^{2+}\) uptake, the Ca\(^{2+}\) unipporter is mixed into the suspension prior to the Ca\(^{2+}\) uptake, and the Ca\(^{2+}\) uptake is virtually zero. In other words, in certain quantities, ruthenium red inhibits both the high and the slow conductivity phases. However, as we will discuss below, it inhibits these two phases differently.

To determine precisely how ruthenium red effects both the rapid (RaM) and slower modes of mitochondrial Ca\(^{2+}\) uptake, we performed titration studies using this inhibitor. Fig. 3 shows the percent inhibition for the RaM (closed circles) and slower uptake (open circles) with increasing ruthenium red concentration. To determine the ruthenium red inhibition of the RaM and slower uptake, we calculated the y intercept (RaM uptake) and slope (slower uptake) from curves like those shown in Fig. 2 with pulses having an average height (h\(_2\)) of 534 nM for a series of ruthenium red concentrations. The percent inhibition was based on Ca\(^{2+}\) uptake with no ruthenium red added. From Fig. 3, we can clearly see that at a concentration of 0.1 nmol of ruthenium red/mg of mitochondrial protein, the slower uptake is inhibited, whereas the RaM is not. At concentrations below 0.1 nmol/mg of mitochondrial protein, we consistently find that ruthenium red activates Ca\(^{2+}\) uptake via the RaM. At concentrations below 0.003 nmol/mg of protein, it may also activate the slower mode of Ca\(^{2+}\) uptake, but that is less certain. What is clear from this data is that at higher concentrations, ruthenium red inhibits both modes of mitochondrial Ca\(^{2+}\) uptake and that the amount of ruthenium red necessary to inhibit the slower uptake is over an order of magnitude less than that required to inhibit the RaM.

Cyclosporin A has been shown to inhibit the mitochondrial permeability transition (32, 33). To rule out the possibility that calcium uptake during the RaM is related to the mitochondrial permeability transition, we added 1 \(\mu\)g cyclosporin A to our medium before making the pulses. The Ca\(^{2+}\) uptake resulting from these pulses was identical to uptake from pulses made without cyclosporin A (data not shown).

Fig. 3. Ruthenium red titration curve. Ruthenium red of various concentrations was added to 3 ml of medium B plus 33 \(\mu\)g EGTA (final concentration) in the cuvette. Mitochondria at a concentration of 0.5 mg of protein/ml were then added, and pulses were made. Curves similar to those shown in Fig. 2 having an average height of 534 ± 31 nM [Ca\(^{2+}\)] were made for each ruthenium red concentration. The slope and y intercept, corresponding to slower and rapid mitochondrial uptake, respectively, were determined using linear regression, and the percent inhibition for rapid uptake (closed circles) and slower uptake (open circles) was calculated using the curve with 0 nmol of ruthenium red/mg of mitochondrial protein as a standard for 0% inhibition. All pulses were made by adding 100 nm EGTA and the Ca\(^{2+}\)-HEDTA solution as defined under "Materials and Methods." The pulses were made using 10 \(\mu\)l of Ca\(^{2+}\)-HEDTA and 12 \(\mu\)l of EGTA. Error bars represent 95% confidence limits on the slope and y intercept converted to percentages.

Can the RaM be Reset by a Decrease in Medium [Ca\(^{2+}\)]?—The RaM could not serve as a mechanism for significantly increasing the total amount of mitochondrial Ca\(^{2+}\) uptake unless it could be reset by the low level of Ca\(^{2+}\) encountered between pulses in vivo, thereby causing a significant amount of Ca\(^{2+}\) uptake at the beginning of each pulse. To see if the RaM could be reset in this way, we carried out experiments using more than one pulse.

The first experiment we performed was to expose the mitochondria to two 5-s-wide Ca\(^{2+}\) pulses under conditions in which the [Ca\(^{2+}\)] between pulses (h\(_0\)) was approximately 100 nM and the time between the pulses (i) was varied from 0 to 10 s. The zero time point was actually a 10-s-wide pulse where both the Ca\(^{2+}\) and the EGTA used to create other pulses in this set were added simultaneously after 5 s so as to produce a pulse whose total Ca\(^{2+}\) and EGTA concentrations were identical to that experienced by the mitochondria in other experiments in this set. We also made single 5-s-wide pulses, which were the same height as the double pulses. The Ca\(^{2+}\) uptake during the second pulse was then determined by subtracting the Ca\(^{2+}\) uptake due to a single pulse only (the first pulse) from the uptake resulting from both pulses. The results can be seen in Fig. 4 where the closed circles represent the uptake from the second pulse only. We can see from Fig. 4 that the uptake from the second 5-s-wide pulse when the two pulses are separated by as little as 0.75 s is twice as great as the uptake from the last 5 s
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FIG. 5. Variation of the [Ca\(^{2+}\)] level between pulses. Two pulses of average heights 397 ± 18 and 408 ± 24 nM [Ca\(^{2+}\)], respectively, and widths of 10 s were made, and the interpulse period was fixed at 1 s. The interpulse [Ca\(^{2+}\)] was varied between 118 and 407 nM. Mitochondria were suspended at 0.5 mg of protein/ml in 3 ml of medium B. All pulses were made by adding 2.5 mM EGTA and the Ca\(^{2+}\)-HEDTA solution as defined under "Materials and Methods." The (open triangles) data represent uptake from both pulses made with 15 μl of EGTA, 9 μl of Ca\(^{2+}\)-HEDTA, 0.5–25 μl of EGTA, 1–18 μl of Ca\(^{2+}\)-HEDTA, and 25–60 μl of EGTA. The (open squares) data represent uptake from either one 10-s-wide or one 11-s-wide pulse made with 15 μl of EGTA, 9 μl of Ca\(^{2+}\)-HEDTA, and 30 μl of EGTA. The (closed circles) data represent uptake from the second pulse only by subtracting the uptake of the pulse from the second pulse. Error bars represent one standard deviation of five repetitions.

FIG. 6. Uptake from steady state versus pulsed [Ca\(^{2+}\)]. Uptake from pulses with widths of 5–25 s and heights of 412 ± 22 nM [Ca\(^{2+}\)] (open circle) are compared with uptake from one to five 5-s-wide pulses with an interpulse period of 2 s and average heights of 437 ± 35 nM [Ca\(^{2+}\)] (closed triangles). Mitochondria were suspended at 0.5 mg of protein/ml in 3 ml of medium B, to which 33 μM EGTA (final concentration) had been added before pulses were made. All pulses were made by adding 2.5 mM EGTA and the Ca\(^{2+}\)-HEDTA solution as defined under "Materials and Methods." The (steady state) pulses were made with 2.5 μl of Ca\(^{2+}\)-HEDTA and 7 μl of EGTA. The multiple pulses were made with all or part of 2.5 μl of Ca\(^{2+}\)-HEDTA, 7 μl of EGTA, 2.5 μl of Ca\(^{2+}\)-HEDTA, 9 μl of EGTA, 3 μl of Ca\(^{2+}\)-HEDTA, 14 μl of EGTA, 4 μl of Ca\(^{2+}\)-HEDTA, 20 μl of EGTA, 8 μl of Ca\(^{2+}\)-HEDTA, and 28 μl of EGTA. Error bars represent one standard deviation of three repetitions.

of a single 10-s-wide pulse. This indicates that the RaM is occurring at the beginning of the second pulse as well as the first.

We next set out to determine the minimum level that the [Ca\(^{2+}\)] between pulses must be dropped to reset the RaM. To do this, the [Ca\(^{2+}\)] level \(h_2\) between two 10-s-wide pulses separated by an interpulse period \(i\) of 1 s was varied over the range from 118 nM up to the 402 nM height of the pulses. This is shown in Fig. 5 where the x axis indicates the amount that the Ca\(^{2+}\) level between pulses was lowered from the pulse height of 402 nM.

Fig. 5, again, shows the amount of calcium uptake from the second pulse only (closed circles) by subtracting the uptake of one pulse from the uptake from both pulses. If the [Ca\(^{2+}\)] between pulses is not lowered and the pulse is, in effect, a 21-s-wide pulse instead of two 10-s-wide pulses separated by 1 s, the uptake from the second pulse is due to uptake during the lower conductivity mode only and is therefore less than uptake from the second pulse when \(h_2\) is lowered by more than 200 nM. Therefore, decreasing the interpulse [Ca\(^{2+}\)] level \(h_2\) by more than 200 nM appears to completely reset the high conductivity phase of Ca\(^{2+}\) uptake (RaM) for a 400 nM high Ca\(^{2+}\) pulse.

These results clearly show that the RaM is reset very rapidly (within a time of less than 0.75 s) when the [Ca\(^{2+}\)] falls to a level of less than 200 nM between pulses for pulses with heights of 400 nM. It appears then that the high conductivity phase can easily be reset under conditions similar to those encountered physiologically during the period between Ca\(^{2+}\) pulses.

The Effect of Pulse Sequences on Total Calcium Uptake—If a rapid mode (RaM) of Ca\(^{2+}\) uptake into mitochondria exists having the characteristics described above, namely that it can be reset if the interpulse [Ca\(^{2+}\)] \(h_2\) falls for a very short period of time \(i\), then it would be predicted that the Ca\(^{2+}\) uptake obtained with mitochondria from a series of separate pulses would be significantly greater than that obtained from exposure of the mitochondria to the same constant [Ca\(^{2+}\)] for the same overall time. This is indeed the case as can be seen quite dramatically in Fig. 6.

The uptake observed during the continual exposure (open circles) corresponds to high conductivity plus low conductivity contributions for one pulse period followed by an addition of one low conductivity contribution for each succeeding 5-s pulse period. The uptake observed from the multiple pulse exposure (closed triangles), on the other hand, corresponds to both a high conductivity plus a low conductivity contribution from each 5-s pulse encountered, resulting in more total calcium uptake from separate pulses than from a prolonged exposure to an elevated Ca\(^{2+}\) level.

The uptake of spermine on the RaM—The levels of Ca\(^{2+}\) uptake mediated by the rapid mode (RaM) of mitochondria are small but not negligible with respect to the levels of uptake necessary to activate several metabolically linked Ca\(^{2+}\)-mediated reactions (see "Discussion"). In considering whether or not the high conductivity phase of Ca\(^{2+}\) uptake by mitochondria could have physiological significance, it would be useful to evaluate its possible activation by common cellular components such as spermine.

Spermine is known to be activated by spermine, which is present in many types of cells at a concentration of around 1 mM. Fig. 7 shows the effect of spermine concentrations ranging from 100 nM to 1 mM on both the RaM and slower uptake mode. At a concentration of 0.1 mM and higher, spermine appears to increase the RaM by approximately a factor of 2 and the lower conductivity uptake by about a factor of 2. The great increase in Ca\(^{2+}\) uptake during even a single pulse, mediated by this endogenous activating agent, brings the levels of intramitochondrial [Ca\(^{2+}\)] into the range in which it could significantly increase the metabolic rate as considered in the Introduction.

**DISCUSSION**

Uptake during RaM was identified as net uptake as opposed to either external binding or rapid exchange of labeled external Ca\(^{2+}\) for unlabeled intramitochondrial Ca\(^{2+}\) in a series of experiments described earlier (26). External binding was ruled out by 1) showing that the addition of small amounts of the inhibitor ruthenium red added prior to the Ca\(^{2+}\) pulse elimi-
Curves of Ca\textsuperscript{2+} concentration of 0.5 mg of protein/ml were then added, and pulses were made. Curves of Ca\textsuperscript{2+} uptake versus pulse width (not shown) were made for each spermine concentration, and the slope and y intercept for each curve was calculated using linear regression. The y intercept (left axis) corresponding to rapid mitochondrial uptake is shown with the closed circles, and the slope (right axis) corresponding to slower mitochondrial uptake is shown with open circles. All pulses were made by adding 2.5 mg EGTA and the Ca\textsuperscript{2+}-HEDTA solution as defined under “Materials and Methods.” Pulses had heights of 925 ± 14 nm [Ca\textsuperscript{2+}] and were made using 5 μl of Ca\textsuperscript{2+}-HEDTA and 25 μl of EGTA. Error bars represent 95% confidence limits.

The observation that the RaM is reset by simply dropping the Ca\textsuperscript{2+} into the range usually observed after or between pulses short enough to allow us to measure the duration of the RaM was unsuccessful, data such as that shown in Fig. 6 are reassuring. This is because these results demonstrate that even if our techniques are not fast enough to permit us to directly measure conductivity during the high conductivity mode, this type of uptake is real and reproducible, and it occurs at the initiation of each pulse as the data of Figs. 2, 4, and 5 suggest that it should. The data of Fig. 7 demonstrate that the amount of uptake through this mechanism can be sufficient for very significant activation of the Ca\textsuperscript{2+}-activated metabolic reactions in the mitochondrial matrix and is consistent with the high levels of [Ca\textsuperscript{2+}]\textsubscript{m} observed following physiological activation of Ca\textsuperscript{2+} pulses by Rizzuto et al. (22–24). The current data along with considerable data already in the literature (see Ref. 4 for a review) provide very strong support for the hypothesis that [Ca\textsuperscript{2+}]\textsubscript{m} can function as a metabolic mediator and probably is a primary mechanism controlling the metabolism of some cells.

As shown in Fig. 7, the maximum amount of transport observed via the high conductivity mode during only a single pulse can be between 1 and 2 nmol of Ca\textsuperscript{2+}/mg of mitochondrial protein and in the range where Ca\textsuperscript{2+} modulation of the Krebs cycle Ca\textsuperscript{2+}-sensitive dehydrogenases, pyruvate dehydrogenase and \(\alpha\)-ketoglutarate dehydrogenase, and other Ca\textsuperscript{2+}-sensitive metabolic processes occurs (4). The observations described above clearly possess the power to reconcile recent observations describing relatively high levels of [Ca\textsuperscript{2+}]\textsubscript{m}, following Ca\textsuperscript{2+} pulses in cells (21–24) and older measurements of Ca\textsuperscript{2+} sequestration by isolated mitochondria using buffered Ca\textsuperscript{2+} (4, 19, 27).

We have found that most of the Ca\textsuperscript{2+} sequestered into liver mitochondria from pulses with heights below 400 nM or from narrow pulses like those seen in the cytosol of heart cells is taken up via the RaM. In many cell types, pulse intensities fall within a limited range (4). It is possible that, under some conditions, the total amount of Ca\textsuperscript{2+} sequestered within a given period and consequently the level of metabolic stimulation could depend more on the pulse frequency than on pulse intensity. In other words, metabolic signaling to the mitochondria could show some of the properties of frequency modulation. Mitochondria would be expected to sequester Ca\textsuperscript{2+} more effectively from a pulse that reaches its peak very rapidly than one that may reach the same level more slowly, since the duration of the RaM is clearly short. This may explain the very rapid mitochondrial [Ca\textsuperscript{2+}] uptake observed by Rizzuto et al. (23). In some respects the possible frequency-modulated behavior of mitochondrial Ca\textsuperscript{2+} uptake seems akin to the relationship between the strength of a stimulus and the frequency of action potential spikes, i.e. the stronger the signal, the higher the frequency of the action potential and the stronger the response.

The reason why the system might function in this way could be simple. The buffering power of the cytosol for Ca\textsuperscript{2+} is not constant as a function of [Ca\textsuperscript{2+}], but increases significantly as [Ca\textsuperscript{2+}] increases above 1 μM. This is because there are more low affinity than high affinity Ca\textsuperscript{2+} binding sites present in the cytosol; consequently, it requires much more than twice the Ca\textsuperscript{2+} to produce a 1 μM Ca\textsuperscript{2+} pulse than one of 500 nm. The energetic costs of ion pumping are not negligible. It surely requires less energy to produce two 500 nm Ca\textsuperscript{2+} pulses than one 1 μM pulse; it may require less to produce even three or four 500 nm pulses. With a significant fraction of the uptake during a 500 nm pulse being from the RaM, the uptake from two narrow 500 nm pulses could be as large as the uptake from one 1 μM pulse. The uptake from three or four 500 nm pulses would probably be significantly greater. Furthermore, increasing the strength of the stimulus by increasing the pulse frequency rather than pulse height has the additional advantage of caus-
ing a smaller perturbation on the mechanisms maintaining
Ca\textsuperscript{2+} homeostasis within the cell by allowing them to function
over a smaller range of [Ca\textsuperscript{2+}].

Interestingly, if one considers the types of Ca\textsuperscript{2+} pulses observed
in various types of cells, the primary variation is in
pulse duration, number of pulses in a sequence, and pulse frequency but not in pulse intensity (4). For example, in considering
pulses in cells that have a high relative metabolic rate,
such as cardiac myocytes, cells that have an intermediate metabolism rate, such as hepatocytes, and cells that have a low metabolic rate, such as chondrocytes (4), the Ca\textsuperscript{2+} pulse intensity varies by only a factor of two from around 600 nM to above
1 \mu M. On the other hand, the pulse duration varies by a factor of
200, from less than 0.5 s to around 2 min, the number of
pulses in a sequence varies from an indeterminately large number
with cardiac myocytes to one with chondrocytes, and the
pulse frequency varies from around 1 Hz with cardiac myocytes
to near zero with chondrocytes. The pulse intensities observed
physiologically in the cytosol of various types of cells (approximately 1 \mu M) are large enough to be unambiguously interpreted as “pulse on” against a background (approximately 100
nM), which is “pulse off” while not large enough to pose a
significant danger to the cell due to activation of proteases etc.
Ca\textsuperscript{2+} signaling at the cellular level could well have evolved
toward a “frequency modulated” mode of action both for reasons of economy and safety. With the inclusion of the rapid
uptake mode, described here, the mitochondrial Ca\textsuperscript{2+}
transport system seems well adapted to utilize this Ca\textsuperscript{2+} signaling
system.

It is clearly important to determine the duration of the RaM
and to determine whether activation such as that by spermine
represents an increase in the conductivity of the mechanism or an increase in the duration of the high conductivity mode or both. We have been unable to resolve this problem with the
shortest Ca\textsuperscript{2+} pulses, which we have produced to date with our
pulse-generating system. Further experiments have been
planned to address this question.

Particularly, if it should turn out that the RaM is a separate functional mode of the uniporter, this would represent a very
unusual transporter. The slower conductivity mode of the
uniporter has been recognized as a fast mechanism in its own
right (31). The high conductivity mode is considerably faster,
particularly at low [Ca\textsuperscript{2+}]. One possibility is that the gate of a
gated pore moves out of the transport path as the high conduc-
tivity mode is reset, leaving a higher conductivity channel open
during RaM uptake. While this view is currently very specula-
tive, this is clearly both an interesting and functionally impor-
tant mechanism.

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