Journal of Bioenergetics and Biomembranes, Vol. 26, No. 5, 1994

Mitochondrial Free Ca^{2+} Concentration in Living Cells

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Received June 15, 1994

Evidence has accrued during the past two decades that mitochondrial Ca^{2+} plays an important role in the regulation of numerous cell functions such as energy metabolism. This implies that mitochondrial Ca^{2+} transport systems might be able to relay the changes of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) into mitochondrial matrix for regulating biochemical activities. To substantiate this idea, measurements of intramitochondrial free Ca^{2+} concentration ($[Ca^{2+}]_m$) become essential. In this article, we review the results from recent studies attempting to measure $[Ca^{2+}]_m$ in living cells. In addition, the significance of each study is discussed.

KEY WORDS: Mitochondria, free Ca^{2+} concentration, living cells.

INTRODUCTION

The quantitation of $[Ca^{2+}]_m$ is a crucial step in our understanding of what physiological role the mitochondrial Ca^{2+} transport system plays. Consequently, research questions dealing with $[Ca^{2+}]_m$ measurements have been increasing significantly during the last decade. Prior to the development of fluorescent Ca^{2+} indicators by Tsien and his associates (Tsien, 1981; Grynkiewicz *et al.*, 1985), the only method available for measuring $[Ca^{2+}]_m$ was the "null-point" technique (Coll *et al.*, 1982; Hansford and Castro, 1982). In this approach, net flux of Ca^{2+} in a mitochondrial suspension was measured using a metallochromic indicator arsenazo III. The Ca^{2+} concentration in the suspending medium was varied while the mitochondrial membrane was made permeable to Ca^{2+} with the $Ca^{2+}/2H^+$ exchange ionophore A23187. It was defined that the $[Ca^{2+}]_m$ was equivalent to the free Ca^{2+} concentration of the suspending medium when there was no net flux into or out of mitochondria. In this way, the first measurement of $[Ca^{2+}]_m$ was made. However, three major limitations exist for this method: (1) the sensitivity of metallochromic indicators to Ca^{2+} ($K_d > 10\,\mu M$) is in a range much higher than the physiological level of Ca^{2+}; (2) the use of an ionophore in conjunction with mitochondrial uncouplers perturbs mitochondrial constituents such as ATP content, and (3) isolated mitochondria may not transport Ca^{2+} in the same manner as those in living cells.

The introduction of fluorescent indicators with Ca^{2+} dissociation constants in the submicromolar range has opened a new era in the research of cell Ca^{2+} homeostasis. Not only have these indicators been applied to measurement of $[Ca^{2+}]_c$, but they have also been applied to the study of $[Ca^{2+}]_m$ in suspensions of isolated mitochondria (Davis *et al.*, 1987; Lukács and Kapus, 1987; Gunter *et al.*, 1988; Moreno-Sanchez and Hansford, 1988; Reers *et al.*, 1989; McCormack *et al.*, 1989). In addition, artificial Ca^{2+} pulses, simulating physiological Ca^{2+} oscillations, have been generated to study uptake of Ca^{2+} during pulses by isolated mitochondria (Leisey *et al.*, 1993; Sparagna *et al.*, 1994). The most obvious advantage of using isolated mitochondria to investigate mitochondrial Ca^{2+} transport is that the compositions of the incubating solutions are well defined. Therefore, it is rather simple to study the effects of different factors in the bathing media such as pH, Mg^{2+}, and spermine on mitochondrial Ca^{2+} transport system. However, as indicated previously, isolated mitochondria may not have properties

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0145-479X/94/1000-0487$07.00/0

identical to those in intact cells. Furthermore, the artificial Ca^{2+} pulses may not have similar spatial and temporal characteristics as the cytosolic Ca^{2+} pulses induced by hormones and other physiological stimulations. For example, the existence of microdomains with high Ca^{2+} close to IP_3-sensitive-gated channels that are sensed by neighboring mitochondria has been demonstrated recently in living cells (Rizzuto *et al.*, 1993).

The present review focuses specifically on studies in which $[Ca^{2+}]_m$ has been measured in living cells.

MEASUREMENTS OF $[Ca^{2+}]_m$ WITH FLUORESCENCE DIGITAL IMAGING MICROSCOPY

The development of fluorophores with high affinity for Ca^{2+} plus the availability of fluorescence digital imaging microscopy (FDIM) have made it possible to monitor the spatial and temporal distribution of intracellular Ca^{2+} concentration in single living cells (Tsien and Poenie, 1986). Using these techniques, several studies have shown a striking heterogeneity of intracellular Ca^{2+} distribution in different types of cells. One reason why FDIM can reveal spatial heterogeneity of intracellular Ca^{2+} concentration is the ability of intracellular organelles to trap the fluorescent indicators by converting the acetyoxymethyl ester form of Ca^{2+} fluorophores to their Ca^{2+}-sensitive form. For example, spatial heterogeneity of intracellular Ca^{2+} concentration, originating from differences in Ca^{2+} distribution in the cytoplasm, nucleus, and sarcoplasmic reticulum, has been detected in fura-2-loaded toad stomach smooth muscle cells (William *et al.*, 1985).

Evidence that fura-2 is trapped by mitochondria in living cells was first reported in 1987 (Steinberg *et al.*, 1987). In this study, microscopic examination of bovine aortic endothelial cells loaded with fura-2 by exposure to its permeant ester revealed a distinct filamentous pattern of fluorescence distribution that correlated with mitochondrial location. The authors also found that most of the mitochondria-associated fura-2 was in its Ca^{2+} sensitive form. These observations demonstrated mitochondrial localizations of fura-2 and suggested a possible method of monitoring $[Ca^{2+}]_m$ in living cells.

In one of the first attempts to measure $[Ca^{2+}]_m$ in living cells, it was observed that the fura-2 flurorescence ratio image of single myocytes isolated from guinea pig ventricle showed discrete and clustered "hot spots," which occupied approximately 20–50% of an individual cell's area (Williford *et al.*, 1990). The fluorescence intensity and the area of the hot spots were increased by agents that deplete Ca^{2+} in the sarcoplasmic reticulum, namely, ryanodine and caffeine. However, when cells were exposed to agents that diminish the driving force for Ca^{2+} uptake by the mitochondrial Ca^{2+} uniporter, such as the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), or the inhibitor of electron transport antimycin A, the fluorescent intensity and the area of the hot spots were reduced. These results indicate that Ca^{2+} compartmentation in the mitochondria is largely responsible for the intracellular Ca^{2+} heterogeneity seen in the ventricular myocytes of guinea pig. The observations that hot spots were enhanced by caffeine and ryanodine suggest that mitochondria can sequester Ca^{2+} released from sarcoplasmic reticulum. Based on *in vitro* calibration, the apparent $[Ca^{2+}]_c$ was approximately 75 nM and the $[Ca^{2+}]_m$ in the hot spots was in the range of 150–350 nM. The most significant problem limiting accuracy in quantifying $[Ca^{2+}]_m$ in this study arose from the fluorescence from out-of-focus planes. The thickness of an adult guinea pig ventricular myocyte is around 10 μm. Therefore, the apparent view of the focused plane is distorted by out-of-focus fluorescence. This problem can be resolved either by using microscopes with capabilities to reduce out-of-focus fluorescence in the z-axis such as a confocal microscope (White *et al.*, 1987) or by using thinner cells such as cultured heart cells.

To achieve a better spatial resolution of $[Ca^{2+}]_m$, a cultured cell line of porcine kidney (LLC-PK1) that has thinner morphology was used (Vamvakas *et al.*, 1990). Loading of LLC-PK1 cells with fura-2AM at 37°C in the absence of the anion transport inhibitor probenecid resulted in a marked accumulation of the dye in discrete spots in the mitochondria-rich perinuclear areas, as shown by rhodamine 123 staining. This identical distribution of fura-2 and rhodamine reinforces the idea that $[Ca^{2+}]_m$ can be monitored in living cells. The authors also studied the role of $[Ca^{2+}]_m$ in cell injury. They found that the distribution of $[Ca^{2+}]_m$ was perturbed by the nephrotoxic cysteine *S*-conjugate *S*-(1,2-dichlorovinyl)-L-cysteine, before the formation of blebs at cell surface and subsequent cell death. These observations confirm the idea that mitochondria are primary cellular targets

for cytotoxic *S*-conjugates (Lash and Anders, 1987). In addition, these experiments demonstrate that the combination of fura-2 and FDIM provides an opportunity for studying the role of cellular and subcellular Ca^{2+} distribution in cell injury. For example, the role of $[Ca^{2+}]_m$ in doxorubicin-induced cardiac injury was reported recently by Chacon *et al.*, (1992) using fura-2 and FDIM. The results showed that little difference existed between $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ under control condition. However, in the presence of a highly effective cancer chemotherapeutic agent doxorubicin, a greater than 2-fold increase in $[Ca^{2+}]_m$ occurred before changes in $[Ca^{2+}]_c$ could be detected. This increase in $[Ca^{2+}]_m$ accompanied the observed dissipation in mitochondrial membrane potential and cellular ATP levels. Therefore, mitochondrial Ca^{2+} overload, not cytosolic Ca^{2+} overload, is an early event in the onset of doxorubicin-induced cell injury. These studies suggest that several agents cause cell injury by disturbing mitochondrial Ca^{2+} homeostatsis without much effect on $[Ca^{2+}]_c$ initially. In addition, the FDIM provides a useful tool to study this kind of subcellular Ca^{2+} alterations during cell injury.

It has recently been reported that $[Ca^{2+}]_m$ was approximately twofold higher than $[Ca^{2+}]_c$ in cultured neonatal rat ventricular myocytes (Fig. 1A). In these studies, mitochondrial areas were also identified by rhodamine 123 staining (Fig. 1B). When $[Ca^{2+}]_c$ was raised by KCl depolarization, elevated external Ca^{2+}, exposure to Na^+-free medium, or addition of caffeine and ryanodine, $[Ca^{2+}]_m$ also increased, suggesting that the mitochondrial Ca^{2+} uniporter sequesters Ca^{2+} during physiological fluctuations of $[Ca^{2+}]_c$ (Jou and Sheu, 1990, 1992).

The ability of mitochondria to sequester cytosolic Ca^{2+} under physiological stimulation has also been shown in AR4-2J pancreatoma cells (Glennon *et al.*, 1992). In unstimulated cells, $[Ca^{2+}]_m$ is in the range of $K_d(\approx 200\,nM)$ for fura-2. When the cells were treated with methacholine, an agonist that activates phospholipase C, $[Ca^{2+}]_c$ increased rapidly to a peak value followed by a much smaller sustained elevation. $[Ca^{2+}]_m$ also rose abruptly on agonist activation; however, it decayed more slowly than $[Ca^{2+}]_c$. These findings indicate that Ca^{2+} can translocate from IP_3-sensitive endoplasmic reticulum to mitochondria. This cycling of Ca^{2+} between IP_3-sensitive pool and mitochondria was also observed in NIH-3T3 fibroblasts responding to IP_3 injections (Connor, 1993). Similarly, the cycling of Ca^{2+} between caffeine-sensitive pool (sarcoplasmic reticulum) and mitochondria was found in rabbit cardiac myocytes (Bassani *et al.*, 1993) and guinea pig ventricular myocytes (Isenberg *et al.*, 1993).

All the studies described above have an intrinsic technical limitation regarding the use of FDIM and fura-2 for measuring $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ simultaneously: the contamination with out-of-focus fluorescence. To reduce this problem, three methods have been adopted: (1) quenching of cytosolic fluorescence by Mn^{2+} (Miyata *et al.*, 1991); (2) using confocal microscopy (Chacon *et al.*, 1994); and (3) using confocal microscopy and a mitochondrially specific Ca^{2+} indicator, rhod-2 (Minta *et al.*, 1989; Jou and Sheu, 1994).

To measure $[Ca^{2+}]_m$ *in situ* in single cardiac myocytes, Miyata *et al.* (1991) took advantage of the fact that exposure of cells to indo-1 AM leads to the localization of indo-1 in the mitochondrial compartment as well as in the cytosol. Application of $100\,\mu M$ $MnCl_2$ to the buffer caused a progressive quenching of the fluorescence to approximately 50% by 30 min. Figure 2 is an example showing the effectiveness of Mn^{2+} in quenching the cytosolic fura-2 in a cultured rat ventricular myocyte after exposing it to $100\,\mu M$ Mn^{2+} for 20 min (Jou and Sheu, unpublished result). This pattern of fura-2 distribution was similar to the rhodamine 123 pattern, suggesting that it was due to dye compartmentation in mitochondria. Similarly, the finding that the remaining fluorescence after Mn^{2+} quenching in the study by Miyata *et al.*, (1991) was due to intramitochondrial indo-1 has been proved by its resistance to treatment of the cell with digitonin at concentrations that release cytosolic but not mitochondrial enzymes and by the finding that ruthenium red and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone prevent its response to elevated $[Ca^{2+}]_c$. this preferential quenching of the cytosolic fraction of indo-1 with Mn^{2+} then allowed the authors to measure $[Ca^{2+}]_m$ continuously based on the residual fluorescence from the cell. In resting cells, $[Ca^{2+}]_m$ is found to be $83 \pm 14\,nM$. This value was below the value of 150 nM for resing $[Ca^{2+}]_c$. In low $[Na^+]_0$ or high $[K^+]_0$ solution $[Ca^{2+}]_m$ rose significantly. The relationship between $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ was nonlinear. When $[Ca^{2+}]_c$ exceeded 500 nM, $[Ca^{2+}]_m$ rose sharply and became higher than $[Ca^{2+}]_c$. While significant Ca^{2+} uptake was not observed from a single electrical stimulation, and increase of the beat frequency to 4 Hz resulted in an increase of $[Ca^{2+}]_m$ up to 600 nM. This technique of measuring $[Ca^{2+}]_m$

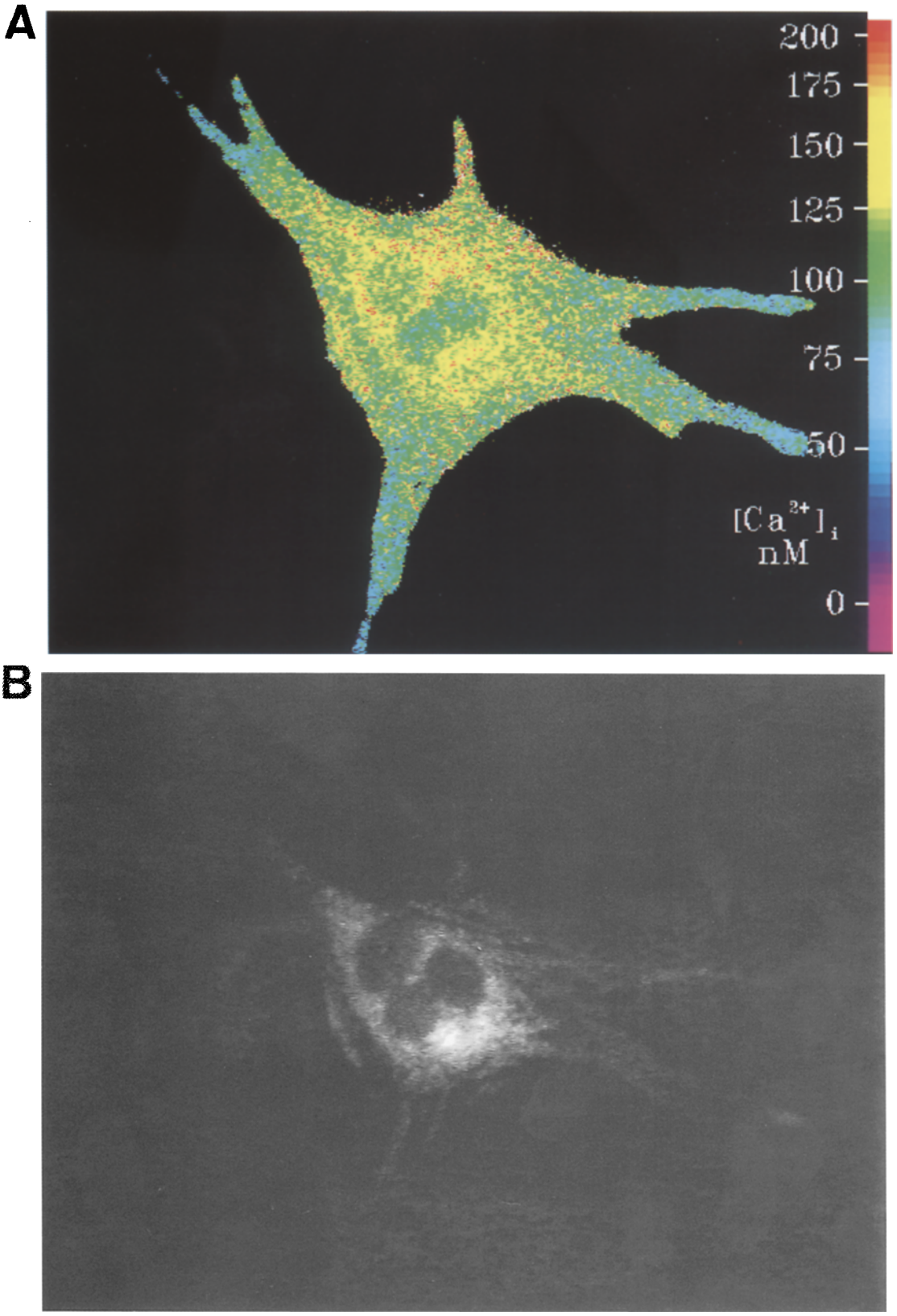

Fig. 1. Mitochondrial contribution to the heterogeneous distribution of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in a 2-week-old cultured neonatal rat ventricular myocyte. (A) Pseudocolor fluorescence ratio image (340 nm/380 nm) of a fura-2-loaded ventricular myocyte. (B) Rhodamine 123 fluorescence image of the same cell taken by a camera to reveal the distribution of mitochondria. The cell length is approximately 100 μm.

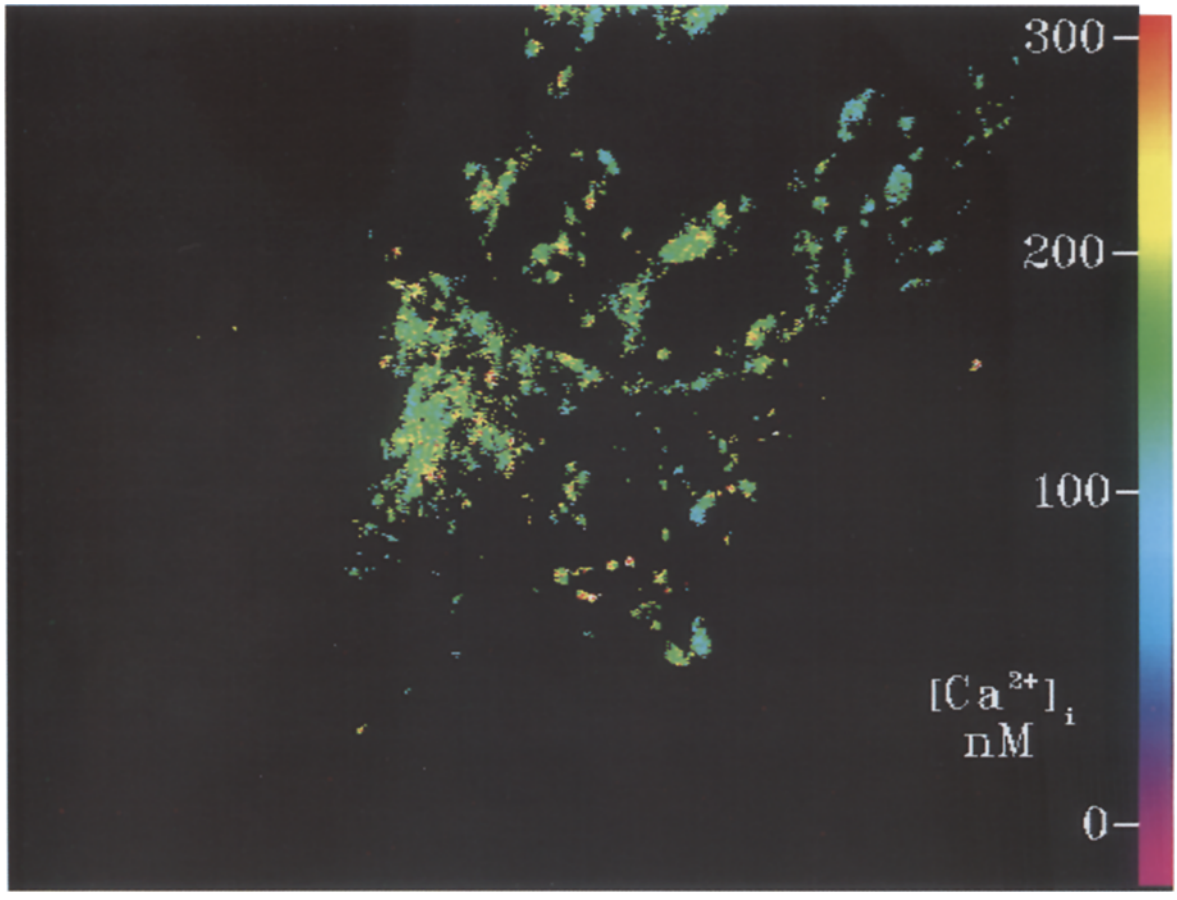

Fig. 2. Pseudocolor image of a 2-week-old cultured neonatal rat ventricular myocyte after quenching cytosolic fura-2 with Mn^{2+} (100 μM) for 20 min. The size of each mitochondrion is approximately 1 μm in width and 2–5 μm in length.

without $[Ca^{2+}]_c$ contamination could be of great value in quantitating $[Ca^{2+}]_m$. However, Mn^{2+} is also sequestered by mitochondria (Gunter and Puskin, 1972) and inhibits mitochondrial Ca^{2+} efflux mechanisms (Gavin *et al.*, 1990). Therefore, this technique should be tested by comparing the results to experiments performed without the use of Mn^{2+}.

Application of the optical sectioning capabilities of the laser scanning confocal microscope to measure $[Ca^{2+}]_m$ in cultured adult rabbit cardiac myocytes was reported recently (Chacon *et al.*, 1994). In the control solution, the fluorescence images showed that $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ had identical values. After inhibition of cellular ATP synthesis with cyanide and 2-deoxyglucose, mitochondrial ΔpH gradually collapsed. Subsequently, cytosolic and mitochondrial Ca^{2+} began to increase. Mitochondrial Ca^{2+} loading became especially prominent just at the onset of hypercontraction. At this time, mitochondria depolarized and finally released their Ca^{2+} through the cyclosporin A-sensitive Ca^{2+} channels (Gunter and Pleiffer, 1990). These events were followed ultimately by breakdown of the plasma membrane permeability barrier and cell death.

A novel approach has been reported recently in an abstract in which cells were loaded with a cationic Ca^{2+} dye, rhod-2, that localized primarily inside the mitochondria due to their very high internally negative membrane potential. The fluorescent signal of mitochondria was then detected using a laser confocal microscope that significantly reduced the undesired signal from out-of-focus planes. It was demonstrated that in cultured neonatal rat ventricular myocytes, $[Ca^{2+}]_m$ increased as $[Ca^{2+}]_c$ was elevated by high extracellular KCl solution, removal of extracellular Na^+, and heartbeat (Jou and Sheu, 1994).

MEASUREMENTS OF $[Ca^{2+}]_m$ WITH RECOMBINANT AEQUORIN TARGETED TO THE MITOCHONDRIA

A novel procedure to monitor $[Ca^{2+}]_m$ in living cells has been reported recently (Rizzuto *et al.*, 1992). The complementary DNA for the Ca^{2+}-sensitive photoprotein aequorin was fused in frame with that of a mitochondrial protein-encoding subunit VIII of human cytochrome C oxidase. The hybrid cDNA was transfected into bovine endothelial cells to obtain stable clones expressing variable amounts of aequorin targeted to the mitochondria. This allowed the parallel measurements of $[Ca^{2+}]_c$ with fura-2 and $[Ca^{2+}]_m$ with targeted aequorin. Activation of phospholipase C-coupled P_{2y} receptor by exogenous ATP caused a rapid rise in $[Ca^{2+}]_c$, higher than 500 nM. Concurrently, ATP also induced a rapid and transient increase in $[Ca^{2+}]_m$. The upstroke of the $[Ca^{2+}]_m$ increase was almost identical in time course to that of $[Ca^{2+}]_c$. However, the decline of the $[Ca^{2+}]_m$ was much faster, with return to basal level within 10 s, whereas $[Ca^{2+}]_c$ remained elevated at a slowly declining plateau for several minutes. The resting $[Ca^{2+}]_m$ was estimated to be approximately 200 nM. Upon stimulation with ATP, $[Ca^{2+}]_m$ increased to above 5 μM in less than 5 s. Therefore, the kinetics and amplitude of the $[Ca^{2+}]_m$ changes appeared to be fast and large.

The fast and large increase in $[Ca^{2+}]_m$ appeared to be most pronounced when $[Ca^{2+}]_c$ was increased through IP_3-induced Ca^{2+} release from intracellular stores (Rizzuto *et al.*, 1993). For instance, in the absence of extracellular Ca^{2+}, histamine-induced a somewhat smaller and faster increase in $[Ca^{2+}]_c$ (400 nM; 2–3 s to peak) by releasing Ca^{2+} from intracellular stores than the subsequent increase in $[Ca^{2+}]_c$ when extracellular Ca^{2+} was added back to the medium and resulted in an increase in $[Ca^{2+}]_c$ through Ca^{2+} influx ($\approx$ 800 nM, 10–12 s to peak). The changes in $[Ca^{2+}]_m$ induced by histamine under the same conditions were different. The increase in $[Ca^{2+}]_m$ due to release from intracellular stores (histamine in Ca^{2+}-free medium) was larger and faster, whereas that induced by Ca^{2+} influx from extracellular Ca^{2+} was smaller and slower. These results indicate that the same average increase in $[Ca^{2+}]_c$ caused by intracellular Ca^{2+} mobilization or influx across the plasma membrane have different effects on the uptake of Ca^{2+} into mitochondria. Mobilization of Ca^{2+} caused an increase in $[Ca^{2+}]_m$ more than one order of magnitude faster than that predicted on the basis of the amplitude of the mean rise in $[Ca^{2+}]_c$. To explain these observations, the authors hypothesized that microdomains exist where high $[Ca^{2+}]_c$ close to the IP_3-sensitive channels is sensed by neighboring mitochondria.

To investigate further the relationship between the influx of Ca^{2+} across the plasma membrane and mitochondrial Ca^{2+} uptake, pancreatic β-cells were used (Rutter *et al.*, 1993). Unlike endothelial cells and HeLa cell clone, physiological stimuli such as ATP and glucose raised $[Ca^{2+}]_c$ largely by stimulating Ca^{2+} influx and not by mobilizing internal Ca^{2+}. Exposure of β-cells to ATP elicited increases in $[Ca^{2+}]_c$ largely by stimulating Ca^{2+} influx. ATP also caused an increase in $[Ca^{2+}]_m$ that parallel the increase in $[Ca^{2+}]_c$, but the increase was one order of magnitude higher than the change in $[Ca^{2+}]_c$. In addition, a similar or greater increase in $[Ca^{2+}]_m$ was induced by plasma membrane depolarization with KCl. An unexpected observation is the 10 times larger change in $[Ca^{2+}]_m$ than in $[Ca^{2+}]_c$. Assuming that mitochondria occupy 5% of total cell volume and have a ratio of free:bound Ca^{2+} similar to that of cytosol, one can calculate that the influx of Ca^{2+} into the mitochondria during stimulation may represent as much as 50% of the total Ca^{2+} flux into the cell. This implies that the mitochondrial Ca^{2+} transport system has a significant Ca^{2+} buffering effect during physiological Ca^{2+} pulses.

Finally, although measurement of $[Ca^{2+}]_m$ with the specific targeting recombinant aequorin has proved successful as described above, several questions need to be addressed. For example, the intensity of signal was considerably small for detection at the single-cell level. Therefore, 1,000–10,000 cells were used for measurements. This use of cell suspensions for experiments could induce errors due to the heterogeneous responses of individual cells.

CONCLUSION

In order for Ca^{2+} to act as a key mediator in mitochondrial functions such as energy metabolism (Denton and McCormack, 1990; Hansford, 1985), the mitochondria Ca^{2+} transport system must respond to changes in $[Ca^{2+}]_c$ under physiological conditions. With the advances in fluorescence microscopy and targeting recombinant aequorin to specific

intracellular organelles, quantitative measurements of $[Ca^{2+}]_m$ in living cells have become possible. We expect that in the next few years the physiological and pathological role of intramitochondrial Ca^{2+} will be more precisely elucidated (Gunter *et al.*, 1994).

ACKNOWLEDGMENTS

We thank Robert Raphael and Henry Colecraft for reading the manuscript. This work was supported by NIH grants HL-33333 and GM-35550, and American Heart Association/NY State Affiliate Grant-in-Aid 94-421 (to S-S.S.) and NIH grant HL-33333 and American Heart Association/NY State Affiliate post-doctoral fellowship (to M-J.J.).

REFERENCES

Bassani, J. W. M., Bassani, R. A., and Bers, D. M. (1993). *J. Physiol.* **460,** 603–621.

Chacon, E., Ulrich, R., and Acosta, D. (1992). *Biochem. J.* **281,** 871–878.

Chacon, E., Reece, J. M., Nieminen, A-L., Zahrebelski, G., Herman, B., and Lemasters, J. J.(1994). *Biophys. J.* **66,** 942–952.

Coll, K. E., Joseph, S. K. Corkey, B. E., and Williamson, J. R., (1982). *J. Biol. Chem.* **257,** 8696–8704.

Connor, J. A. (1993). *Cell Calcium* **14,** 185–200.

Davis, M. H., Altschuld, R. A., Jung, D. W., and Brierley, G. P. (1987). *Biochem. Biophys. Res. Commun.* **149,** 40–45.

Denton, R. M., and McCormack, J. G. (1990). *Annu. Rev. Physiol.* **52,** 451–466.

Gavin, C. E., Gunter, K. K., and Gunter, T. E. (1990). *Biochem. J.* **266,** 329–334.

Glennon, M. C., Bird, G. St. J., Takemura, J., Thastrup, O., Leslie, B. A., and Putney, J. W., Jr. (1992). *J. Biol. Chem.* **267,** 25568–25575.

Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985). *J. Biol. Chem.* **260,** 3440–3450.

Gunter, T. E., and Pfeiffer, D. R. (1990). *Am. J. Physiol.* **258,** C755–C786.

Gunter, T. E., and Puskin, J. S. (1972). *Biophys. J.* **12,** 625–635.

Gunter, T. E., Restrepo, D., and Gunter, K. K. (1988). *Am. J. Physiol.* **225,** C304–C310.

Gunter, T. E., Gunter, K. K., Sheu S-S., and Gavin, C. E. (1994). *Am. J. Physiol.*, **267,** C313–C339.

Hansford, R. G. (1985). *Rev. Physiol. Biochem. Pharmacol.* **102,** 1–72.

Hansford, R. G., and Castro, F. (1982). *J. Bioenerg. Biomembr.* **14,** 361–376.

Isenberg, G., Han, S., Schiefer, A., and Wendt-Gallitelli, M. F. (1993). *Cardiovasc. Res.* **27,** 1800–1809.

Jou, M-J., and Sheu, S-S. (1990). *Biophys. J.* **57,** 343a.

Jou, M-J., and Sheu, S-S. (1992). *Biophys. J.* **61,** A164.

Jou, M-J., and Sheu, S-S. (1994). *Biophys. J.* **66,** A94.

Lash, L. H., and Anders, M. W. (1987). *Mol. Pharmacol.* **32,** 549–556.

Leisey, J. R., Grotyohann, L. W., Scott, D. A., and Scaduto, R. C., Jr. (1993). *Am. J. Physiol.* **265,** H1203–H1208.

Lukács, G. L., and Kapus, A. (1987). *Biochem. J.* **248,** 609–613.

McCormack, J. G., Browne, H. M., and Dawes, N. J. (1989). *Biochim. Biophys. Acta* **973,** 420–427.

Minta, A., Kao, J. P., and Tsien, R. Y. (1989). *J. Biol. Chem.* **264,** 8171–8178.

Miyata, H., Silverman, H. S., Sollott, S. J., Lakatta, E. G., Stern, M. D., and Hansford, R. G. (1991). *Am. J. Physiol.* **261,** H1123–H1134.

Moreno-Sánchez, R., and Hansford, R. G. (1988). *Biochem. J.* **256,** 403–412.

Reers, M., Kelly, R. A., and Smith, T. W. (1989). *Biochem. J.* **257,** 131–142.

Rizzuto, R., Brini, M., Murgia, M., and Pozzan, T. (1993). *Science* **262,** 744–747.

Rizzuto, R., Simpson, A. W. M., Brini, M., and Pozzan, T. (1992). *Nature (London)* **358,** 325–327.

Rutter, G. A., Theler, J-M., Murgia, M., Wollheim, C. B., Pozzan, T., and Rizzuto, R. (1993). *J. Biol. Chem.* **268,** 22385–22390.

Sparagna, G. C., Gunter, K. K. and Gunter, T. E. (1994). *Anal. Biochem.* **219,** 96–103.

Steinberg, S. F., Bilezikian, J. P., and Al-Awqati, Q. (1987). *Am. J. Physiol.* **253,** C744–C747.

Tsien, R. Y. (1981). *Nature (London)* **290,** 527–528.

Tsien, R. Y., and Poenie, M. (1986). *Trends Biochem. Sci.* **11,** 450–455.

Vamvakas, S., Sharma, V. K., Sheu, S-S., and Anders, M. W. (1990). *Mol. Pharmacol.* **38,** 455–461.

White, J. G., Amos, W. B., and Fordham, M. (1987). *J. Cell Biol.* **105,** 41–48.

Williams, D. A., Forgarty, K. E., Tsien, R. Y., and Fay, F. S. (1985). *Nature (London)* **318,** 558–561.

Williford, D. J., Sharma, V. K., Korth, M., and Sheu, S-S. (1990). *Circ. Res.* **66,** 241–248.