Vol. 167, No. 1, 1990
February 28, 1990

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 148-153

CONTROL OF MITOCHONDRIAL MATRIX CALCIUM: STUDIES USING FLUO-3 AS A FLUORESCENT CALCIUM INDICATOR

Alfredo Saavedra-Molina,* Salvador Uribe,** and Thomas M. Devlin*

*Department of Biological Chemistry, Hahnemann University School of Medicine, Philadelphia, PA 19102 and ** Instituto de Fisiologia Celular, Universidad Nacional Autonoma de Mexico, D.F., Mexico

Received December 21, 1989

Fluo-3, a fluorescent Ca^{2+} indicator, is sequestered by isolated rat liver mitochondria and is an effective probe for evaluating the concentration and kinetics of change of mitochondrial matrix ionized calcium ($[Ca^{2+}]m$) under a variety of conditions. At the wavelengths employed, there is no significant interference by auto-fluorescence. There is an insignificant release of the indicator over four hours and the loading and presence of fluo-3 has no effect on respiratory rate or oxidative phosphorylation. The $[Ca^{2+}]m$ steady state can be altered by the assay conditions, i.e. the presence of extra-mitochondrial Ca^{2+}, Mg^{2+}, phosphate and respiratory inhibitors. The total matrix ionized calcium represents a small percent (<0.01%) of the total mitochondrial calcium.

The concentration of free Ca^{2+} of the mitochondrial matrix ($[Ca^{2+}]m$) influences the activity of various mitochondrial metabolic processes (1,2). Total mitochondrial calcium is controlled by an electrophoretic influx mechanism and a separate efflux pathway (3). Until recently, $[Ca^{2+}]m$ could be determined only by indirect measurements (4,5) and it has been assumed that most of the calcium is sequestered or present as unionized complexes. It has been demonstrated recently (6-9) that isolated rat heart and liver mitochondria can sequester the fluorescent Ca^{2+} indicators fura-2 and indo-1 which have been used to measure changes in cytosolic Ca^{2+} of intact cells. These Ca^{2+} indicators, however, have excitation and emission wavelengths in the same range as mitochondrial components, thus, requiring correction for changes in the auto-fluorescence of mitochondria. Loading with larger quantities of indicator can partially alleviate this problem but increases the possibility of a greater influence of buffering by the indicator (6).

We report here on the use of fluo-3 (10), a long wavelength fluorescent Ca^{2+} indicator, which can be loaded into isolated rat liver mitochondria to measure $[Ca^{2+}]m$. Mitochondrial auto-fluorescence does not interfere with $[Ca^{2+}]m$ measurements because

0006-291X/90 $1.50

the excitation and emission wavelengths are above 500 nm. The Kd is 0.40 uM which is in the range of rat liver mitochondrial matrix free calcium; fura-2 and indo-1 have lower Kd values making measurements more difficult (11). Another advantage is that the use of fluo-3 does not require ratio recording of fluorescence because there is over a 40 fold increase in fluorescence on binding to Ca^{2+}.

Our measurements with fluo-3 indicate that the mitochondrial matrix free calcium is controlled by a variety of factors including the extramitochondrial Ca^{2+} concentration, the presence of Mg^{2+} and phosphate, and the redox state of the respiratory chain.

Materials and Methods

All chemicals were reagent grade or of high purity; fluo-3 free acid and fluo-3-acetoxymethyl (AM) ester (fluo-3/AM) were purchased from Molecular Probes. Rat liver mitochondria were prepared from male Wistar rats weighing from 150 to 200 g as described previously (12) in ice cold medium containing 220 mM mannitol, 70 mM sucrose, 2 mM MOPS (pH 7.4) and 1 mM EGTA. EGTA was omitted in the final two washes. Protein was determined by the Biuret method (13). Mitochondria (25 mg/ml) were incubated with 10 uM fluo-3/AM and 0.003% pluronic acid for 20 minutes at 25°C with shaking at 80 rpm. Mitochondria were then centrifuged and washed twice in an Eppendorf microfuge for 2 minutes and resuspended in the isolation medium without EGTA.

Fluorescence of mitochondrial suspensions were monitored with a Perkin Elmer MPF-44 spectrofluorometer or a Spex Industries spectrofluorometer at 25°C; excitation wavelength was 506 nm (slit 10 nm) and emission was 526 nm (slit 10 nm). Quantitation of mitochondrial matrix Ca^{2+} was determined by measuring the fluorescence minimum (Fmin) at the end of each experiment by addition of EGTA (600 uM), ionomycin (10 uM) and deoxycholate (0.05%), and the fluorescence maximum (Fmax) by subsequent addition of 6 mM Ca^{2+}. Values of Fmin, which represent auto-fluorescence, were always less than 10% of Fmax and did not change under any of the experimental conditions as measured with non-loaded mitochondria demonstrating that auto-fluorescence was not a complicating problem with the use of fluo-3. The $[Ca^{2+}]m$ was determined from the equilibrium equation, $[Ca^{2+}]m = Kd\ (F - Fmin)/(Fmax - F)$, where F was the experimental value of fluorescence. A value of 0.40 uM was used for the Kd (10); variations of pH between 7.0 and 7.4 do not alter the Kd significantly. Calcium EGTA buffers were used to maintain extramitochondrial free calcium ($[Ca^{2+}]o$) as described by Fabiato (14). Specific experimental conditions are presented in the legends to the figures.

Results and Discussion

The conditions for loading of mitochondria with fluo-3/AM including dye and mitochondrial concentrations, pluronic acid concentration and temperature were determined empirically by measuring loading efficiency to maximize the amount of dye uptake. After incorporation, less than 4% of the incorporated dye was found in the external mitochondrial medium after four hours of incubation at 4°C. With glutamate-malate as substrates, there were no differences in the P/O ratios, respiratory control

ratios, and state 3 and state 4 respiratory rates between control and fluo-3 loaded mitochondria. Thus, the loading procedure and the presence of fluo-3 did not affect critical mitochondrial functions.

The results presented in Figure 1 demonstrate the method for determining $[Ca^{2+}]m$; in 1-A, mitochondria were added to a medium where the extramitochondrial Ca^{2+} concentration was maintained at 400 nM using EGTA as a Ca^{2+} buffer (14). After

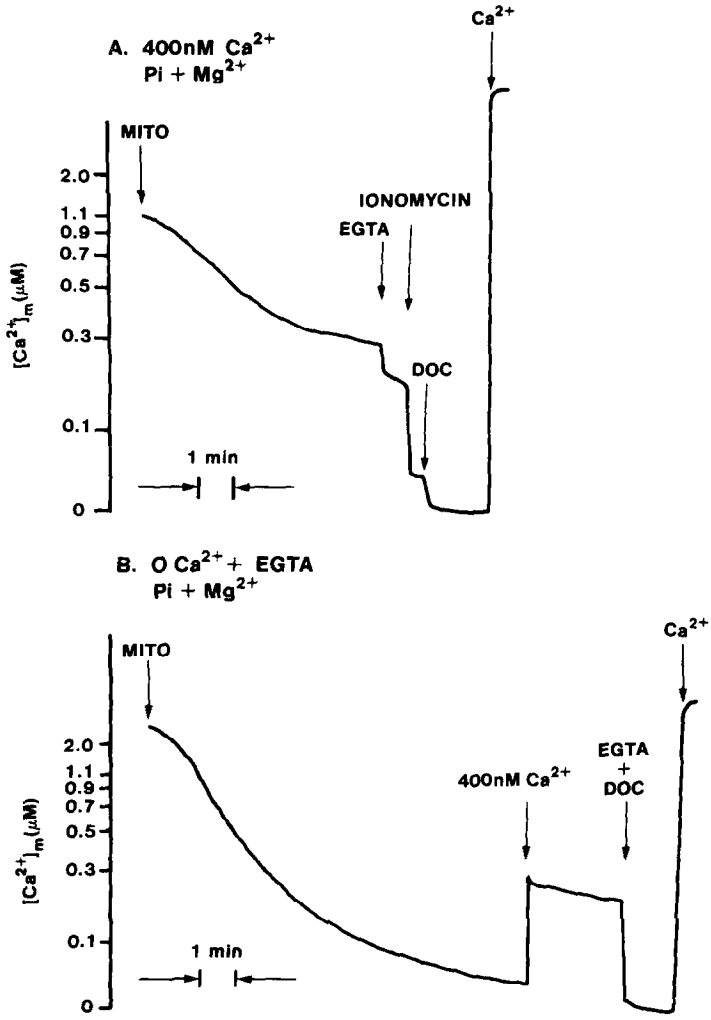

FIGURE 1. DETERMINATION OF MATRIX FREE Ca^{2+} OF RAT LIVER MITOCHONDRIA. Fluo-3 loaded mitochondria (0.5 mg/ml) were subjected to 400 nM (A) or zero nM (B) extramitochondrial Ca^{2+} in the presence of 2 mM phosphate-tris and 2 mM $MgCl_2$ plus 5 mM succinate-Tris at zero time. Additions made where indicated. Minimum fluorescence was determined by the addition of EGTA (600 uM), ionomycin (10 uM) and deoxycholate (0.01%), and maximum fluorescence was obtained by addition of 8 mM $CaCl_2$.

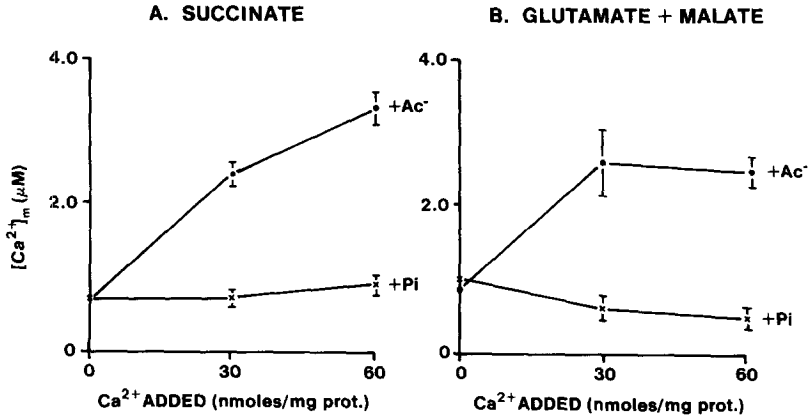

FIGURE 2. EFFECT OF PERMEANT ANIONS ON MATRIX FREE Ca^{2+} OF MITOCHONDRIA LOADED WITH CALCIUM. Fluo-3 loaded mitochondria (0.5 mg/ml) were incubated in 220 mM mannitol, 70 mM sucrose, 2 mM MOPS, pH 7.4 chelex-treated solution, plus 5 mM succinate-Tris or 5 mM glutamate-Tris plus 5 mM malate-Tris as substrates with either 5 mM sodium acetate or 2 mM phosphate-Tris. Mitochondria were preloaded with either 30 or 60 nmoles Ca^{2+}/mg protein. The final steady state matrix ionized calcium was determined. Results are the mean ± SEM of at least 3 experiments.

approximately 6 to 8 minutes the $[Ca^{2+}]m$ reached an equilibrium at approximately 280 nM. This figure also demonstrates the determination of the Fmin and Fmax as described in Methods. As presented in Figure 1B, when mitochondria were incubated in the absence of external Ca^{2+}, the $[Ca^{2+}]m$ equilibrated at less than 100 nM; upon addition of 400 nM $[Ca^{2+}]o$, $[Ca^{2+}]m$ reached approximately the same value as when incubated initially in Ca^{2+} containing medium. Mitochondria in the isolation medium at 0°C had high and variable $[Ca^{2+}]m$ which decreased during incubation at 25°C, reaching a constant level depending on the assay system. Thus, the results in Figure 1 demonstrate that the steady state concentrations of $[Ca^{2+}]m$ and kinetics of change can be determined easily. In subsequent figures, only the steady state values of $[Ca^{2+}]m$ are reported.

The effect of preloading mitochondria with exogenous Ca^{2+} on the $[Ca^{2+}]m$ is presented in Figure 2. With phosphate as the permeant anion, preloading with either 30 or 60 nmoles of Ca^{2+}/mg protein did not significantly alter the $[Ca^{2+}]m$; presumably the Ca^{2+} was being sequestered as calcium phosphate salts. A similar observation has been made by Gunter, et. al. (8). In contrast, $[Ca^{2+}]m$ increased with increasing exogenous Ca^{2+} in the presence of acetate as the permeant anion. Regardless of the permeant anion employed, however, the total amount of $[Ca^{2+}]m$ represented less than 0.01 % of the total calcium present in the mitochondria. The mitochondrial matrix volume is approximately 1 ul/mg protein and if $[Ca^{2+}]m$ were 1 uM, the total matrix free calcium would be 0.001 nmoles/mg protein. In the experiments presented in Figure

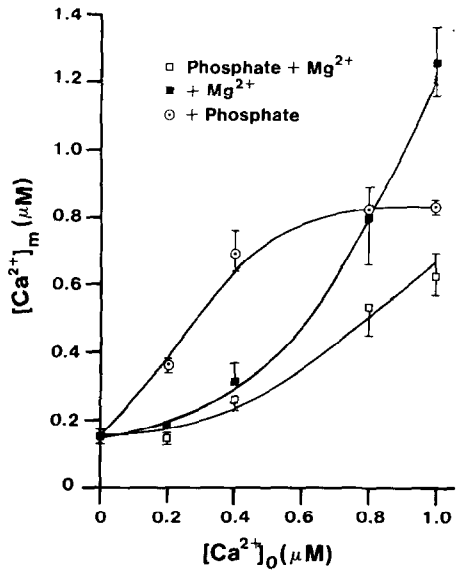

FIGURE 3. RELATIONSHIP BETWEEN EXTRA-MITOCHONDRIAL FREE Ca^{2+} AND MITOCHONDRIAL MATRIX FREE Ca^{2+}. Fluo-3 loaded mitochondria (0.5 mg/ml) were incubated in 150 mM KCl, 20 mM MOPS, pH 7.4, 5 mM succinate as substrate, and 2 mM phosphate-Tris and/or 2 mM $MgCl_2$ where indicated. Extra-mitochondrial Ca^{2+} ($[Ca^{2+}]o$) was controlled with added Ca^{2+} and 1 mM EGTA (14). Values were determined after the steady state was established. Results are the mean ± SEM of at least three experiments.

2, the total Ca^{2+} taken up by the mitochondria was 30 or 60 nmoles/mg protein. Thus, only a very small percent of the total mitochondrial calcium is present as $[Ca^{2+}]m$. This would also be the case even in the absence of exogenous calcium, because isolated mitochondria usually contain between 1 and 5 nmoles of calcium/mg protein.

The effect of phosphate and/or Mg^{2+} in the incubation medium with varying concentrations of $[Ca^{2+}]o$ is presented in Figure 3. The values for $[Ca^{2+}]m$ are steady-state values after a preincubation period during which the $[Ca^{2+}]o$ was maintained by the EGTA buffer. The presence of both phosphate and Mg^{2+} led to the lowest concentrations of $[Ca^{2+}]m$. At the lower concentrations of $[Ca^{2+}]o$ tested, the low levels of $[Ca^{2+}]m$ in the presence of Mg^{2+} may be attributable to an inhibition by Mg^{2+} of the Ca^{2+} uniporter. As indicated, the values of $[Ca^{2+}]m$ were either lower or higher than the $[Ca^{2+}]o$ depending on the incubation system. Addition of either rotenone or antimycin A with succinate as substrate decreased the $[Ca^{2+}]m$ to a level equal to that in the absence of external $[Ca^{2+}]o$ regardless of the external Ca^{2+} concentration, demonstrating that the $[Ca^{2+}]m$ is dependent upon the redox state of the respiratory chain.

The results demonstrate that fluo-3 can be used to quantitate $[Ca^{2+}]m$ under a variety of conditions and to study the kinetics of change of $[Ca^{2+}]m$. $[Ca^{2+}]m$ will vary depending on the assay conditions including the external Ca^{2+} concentration.

Acknowledgments

Our thanks to Mr. A. Kalenak for measurements of mitochondrial function and to Mrs. Colleen Healy for typing the manuscript. These studies were supported by the Office of Naval Research, grants N00014-84-C-0215 and N00014-89-J-1738, the U.S. - Mexico Cooperative Science Program-NSF and the CONACYT-Mexico.

References

1. Hansford, R.G. (1985) Rev. Physiol. Biochem. Pharm. 102, 1-72.
2. Denton, R.M., and McCormick, J.G. (1985) Am. J. Physiol. 249, E543-E554.
3. Crompton, M. (1985) Curr. Top. Membr. Transp. 25, 231-276.
4. Coll, K.E., Joseph, S.K., Corkey, B.E., and Williamson, J.R. (1982) J. Biol. Chem. 257, 8696-8704.
5. Hansford, R.G., and Castro, R. (1982) J. Bioenerg. Biomembr. 14, 361-375.
6. Lukacs, G.L., and Kapus, A. (1987) Biochem. J. 248, 609-613.
7. Davis, M.H., Altschuld, R.A., Jung, D.W., and Brierley, G.P. (1987) Biochem. Biophys. Res. Commun. 149, 40-45.
8. Gunter, T.E., Restrepo, D., and Gunter, K.K. (1988) Am. J. Physiol. C304-C310.
9. McCormack, J.G., Browne, H.M., and Dawes, N.J. (1989) Biochim. Biophys. Acta. 973, 420-427.
10. Minta, A., Kao, J.P.Y., and Tsien, R.Y. (1989) J. Biol. Chem. 264, 8171-8178.
11. Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440-3450.
12. Uribe, S., Ohnishi, S.T., Israelite, C., and Devlin, T.M. (1987) Biochim. Biophys. Acta. 924, 87-98.
13. Gornal, A.G., Bardavill, C.J., and David, M.M. (1949) J. Biol. Chem. 177, 751-760.
14. Fabiato, A. (1988) Methods in Enzymology (Fleischer, S., and Fleischer, B., eds) Vol. 157, pp. 378-416. Academic Press, San Diego, CA.