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# Effect of extra- and intra-mitochondrial calcium on citrulline synthesis

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**Summary.** A quantitative relationship between rat liver mitochondrial matrix free $Ca^{2+}$ ($[Ca^{2+}]_m$) and citrullinogenesis has been observed. Maximum citrulline synthesis occurred at 100 to 200 nM $[Ca^{2+}]_m$; higher $[Ca^{2+}]_m$ caused inhibition. When $[Ca^{2+}]_m$ was decreased to below 50 nM, by addition of A23187 and EGTA, inhibition also occurred. By incubating mitochondria with ruthenium red ($[Ca^{2+}]_m$ = 200 nM) prior to addition of extra-mitochondrial free $Ca^{2+}$ ($[Ca^{2+}]_o$) it was found that high external $Ca^{2+}$ (800 nM) did not inhibit citrulline synthesis thus demonstrating that $[Ca^{2+}]_m$, not $[Ca^{2+}]_o$ was controlling citrullinogenesis.

**Keywords:** Amino acids – Liver – Mitochondria – Calcium – Citrulline – Ruthenium red

## Introduction

In recent years, considerable evidence has accumulated indicating that the concentrations of free $Ca^{2+}$ in the mitochondrial matrix ($[Ca^{2+}]_m$) plays an important role in various metabolic processes (Moreno-Sánchez and Hansford, 1988; McCormack et al., 1988; Hansford, 1985). It has been suggested by Blackmore et al. (1979) that the changes in the distribution of $Ca^{2+}$ across the inner mitochondrial membrane, such as occur in hepatocytes under the influence of hormones (Corvera and García-Sáinz, 1982; Meijer et al., 1981), can modulate the activity of carbamoyl phosphate synthetase (ammonia). Submicromolar concentrations of extramitochondrial $Ca^{2+}$ stimulate the rate of citrulline in rat liver mitochondria (Johnston and Brand, 1989), as well as the uptake of ornithine (Saavedra-Molina and Piña, 1987) which is carbamoylated to citrulline in rat liver mitochondria. Calcium ions are involved in increasing the synthesis of N-acetylglutamate and therefore the

activity of carbamoyl phosphate synthetase (ammonia) (Johnston and Brand, 1990).

We have reported (Saavedra-Molina et al., 1990), that $[Ca^{2+}]_m$ of isolated rat liver mitochondria can be measured by loading the acetoxymethyl ester of the fluorescent dye fluo-3 (Minta et al., 1989), thus generating the free acid non-permeant form in the matrix, with no significant interference from the mitochondrial auto-fluorescence. The continuous monitoring of $[Ca^{2+}]_m$ gives a quantitative relationship between the ion and the activities of mitochondrial enzymes including those involved in citrulline synthesis. In this paper we demonstrate that in rat liver mitochondria, EGTA stimulated citrulline synthesis and that this synthesis is controlled by the matrix free calcium concentration. Preliminary results have been reported (Saavedra-Molina and Devlin, 1991).

## Materials and methods

All reagents were of analytical grade. Mannitol, sucrose, MOPS (3-[N-morpholino]propanesulfonic acid), EGTA (ethyleneglycol-bis-($\beta$-aminoethylether)N,N,N',N'-tetraacetic acid), L-citrulline, L-ornithine, L-glutamate, malate, succinate, ruthenium red, sodium deoxycholate and rotenone were from Sigma Chemical Co. St. Louis, Mo. Fluo-3-acetoxymethyl (AM) ester (fluo-3/AM) and pluronic acid were from Molecular Probes. Ruthenium red was recrystalyzed as previously described by Luft (1971). All solutions were chelex-treated to deplete calcium (Uribe et al., 1987).

Rat liver mitochondria were prepared as described by Uribe et al., (1987) from male Wistar rats, weighing from 150 to 200 g. All procedures were conducted in ice-cold buffer (isolation medium) containing 220 mM mannitol, 70 mM sucrose, 2 mM MOPS (pH 7.4) and 1 mM EGTA, which was omitted from the last two washes. Protein was determined by the Biuret reaction (Keyser and Vaughn, 1949). Mitochondria (25 mg/ml) were loaded with 10 $\mu$M fluo-3/AM and 0.003% pluronic acid for 20 min at 25°C with shaking at 80 rpm, centrifuged and washed twice with the isolation medium without EGTA in an Eppendorf microfuge for 2 min at room temperature and resuspended in the isolation medium without EGTA (Saavedra-Molina et al., 1990).

Fluorescence of mitochondrial suspensions was monitored with a Hitachi-F2000 spectrofluorometer at 25°C. Excitation wavelength was 506 nm and emmision wavelength 526 nm. Quantitation of mitochondrial matrix $Ca^{2+}$ was determined by measuring the fluorescence minimum (Fmin) at the end of each experiment by the addition of EGTA (600 $\mu$M) and sodium deoxycholate (0.05%), and the fluorescence maximum (Fmax) by subsequent additions of 8 mM $Ca^{2+}$ as described by Saavedra-Molina et al. (1990). Values of Fmin, which represent auto-fluorescence, were always less than 10% of Fmax and did not change under any experimental conditions as measured with non-loaded mitochondria demonstrating that auto-fluorescence did not interfere with the use of fluo-3. $[Ca^{2+}]_m$ was determined from the experimental value of fluorescence. A value of 0.40 $\mu$M was used for the dissociation constant ($K_d$). Calcium-EGTA buffers were used to control extramitochondrial free calcium ($[Ca^{2+}]_o$) as described by Fabiato (1988).

Citrulline was assayed in the medium described in each Figure or Table legends after 10 min incubation at 25°C, and determined by the method of Boyde and Rahmatullah (1980).

## Results and discussion

Preincubation of rat liver mitochondria with EGTA stimulated citrulline synthesis 3 to 4 fold (Table 1) indicating that calcium is involved in the control

of this reaction as has been reported by Meijer et al. (1981). The rate of synthesis was not affected by the presence of ruthenium red which was used in further experiments. The effect of extramitochondrial $Ca^{2+}$ ($[Ca^{2+}]_o$) on $[Ca^{2+}]_m$ was studied in fluo-3 loaded mitochondria. $[Ca^{2+}]_m$ was controlled by adding $Ca^{2+}$ and EGTA to specific concentrations as indicated by the method described by Fabiato (1988). Figure 1 shows the relationship between $[Ca^{2+}]_o$ and $[Ca^{2+}]_m$; between zero and 400 nM $[Ca^{2+}]_o$ there was a 3 fold increase in

**Table 1.** Effect of EGTA on citrulline synthesis of isolated rat liver mitochondria

| EGTA mM | Ruthenium red $\mu$M | Citrulline synthesis nmoles/min/mg |
|---|---|---|
| – | – | 7.0 ± 1.0 (9) |
| | 0.4 | 8.0 ± 1.0 (9) |
| 1.0 | – | 25.0 ± 3.0 (12) |
| 1.0 | 0.4 | 22.0 ± 3.0 (9) |

Incubation medium: 75 mM Tris-HCl (pH 7.4), 5 mM $KH_2PO_4$ buffer, 15 mM KCl, 3 mM $MgCl_2$, 16 mM $KHCO_3$, 10 mM L-ornithine, 10 mM $NH_4Cl$, 10 mM succinate and 5 $\mu$M rotenone. After a 10 min incubation at 25°C, reactions were terminated with perchloric acid (4% final concentration). Results are the mean ± SEM. Numbers in parenthesis indicate separate experiments.

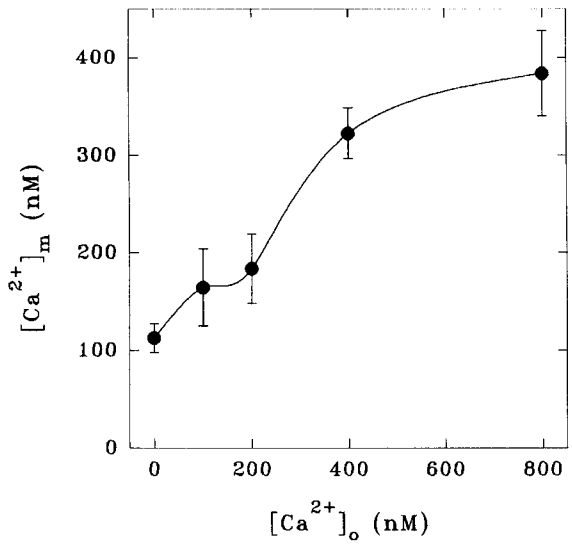


**Fig. 1.** Effect of extramitochondrial $Ca^{2+}$ on the concentration of matrix free $Ca^{2+}$. Fluo-3 loaded mitochondria were incubated in 100 mM KCl, 10 mM MOPS, 16 mM $KHCO_3$, 3 mM $MgCl_2$, 5 mM glutamate, 5 mM malate, pH 7.4 at 25°C. Extramitochondrial calcium was controlled by increasing concentrations of $Ca^{2+}$ and 1 mM EGTA. Results are the mean ± SEM of at least three experiments

$[Ca^{2+}]_m$. Above 400 nM there was only a gradual increase, the $[Ca^{2+}]_m$ leveling off at about 380 nM even though the $[Ca^{2+}]_o$ was increased to 800 nM. The results are similar to that described previously by Saavedra-Molina et al. (1990); the differences may be attributable to the different substrate, glutamate/malate in this study versus succinate in the earlier study.

To determine the effects of $[Ca^{2+}]_m$ on citrullinogenesis, fluo-3 loaded mitochondria were incubated in the presence of L-ornithine and ammonium chloride as substrates for citrullinogenesis. In simultaneous experiments the synthesis of citrulline and $[Ca^{2+}]_m$ were assayed. In order to achieve the minimal value for $[Ca^{2+}]_m$, 6.0 $\mu$M A23187 and 1.0 mM EGTA were added in the absence of added $Ca^{2+}$; for all other values of $[Ca^{2+}]_m$, the $[Ca^{2+}]_o$ was adjusted with a combination of exogenous $Ca^{2+}$ and 1.0 mM EGTA. As presented in Fig. 2, a biphasic effect was observed; at the minimal level of $[Ca^{2+}]_m$ achieved (45 nM), there was a low level of citrulline synthesis. When $[Ca^{2+}]_m$ was adjusted to 100 to 200 nM, maximum synthesis occurred, which was decreased as $[Ca^{2+}]_m$ was increased further. A minimum rate of synthesis was observed above 400 nM.

To rule out the possibility that $[Ca^{2+}]_o$ was involved directly in the citrulline reaction, ruthenium red was employed to inhibit calcium uptake by the mitochondria (Moore, 1970). When mitochondria were incubated with ruthenium red prior to the addition of 800 nM $[Ca^{2+}]_o$, $[Ca^{2+}]_m$ was 208 nM and the rate of citrulline synthesis was 24 nmoles/min/mg (Table 2); but when 800 nM $[Ca^{2+}]_o$ was added before ruthenium red, a value of 322 nM $[Ca^{2+}]_m$

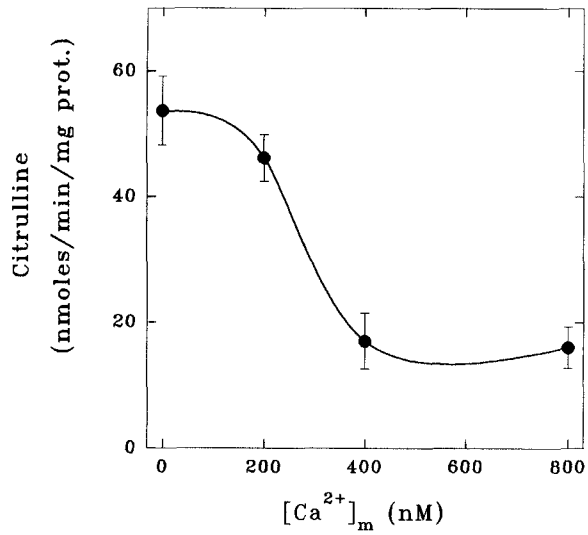


**Fig. 2.** Effect of mitochondrial matrix free $Ca^{2+}$ on the rate of citrulline synthesis. For $[Ca^{2+}]_m$ of 110 nM and higher, fluo-3 loaded mitochondria were incubated in the medium described in Fig. 1, at $[Ca^{2+}]_o$ concentrations to achieve the indicated $[Ca^{2+}]_m$, in the presence of 10 mM L-ornithine plus 10 mM $NH_4Cl$ as substrates. For $[Ca^{2+}]_m$ of 45 nM, 6 $\mu$M A23187 was added to deplete the $[Ca^{2+}]_m$. Simultaneous experiments were carried out to assay citrulline synthesis (2 mg protein/ml) or $[Ca^{2+}]_m$ (0.5 mg protein/ml). Results are the mean ± SEM of 3 to 7 experiments

**Table 2.** Effect of extramitochondrial $Ca^{2+}$ on citrulline synthesis

| Order of addition | | $[Ca^{2+}]_o$ nM | $[Ca^{2+}]_m$ nM | Citrulline synthesis nmoles/min/mg |
|---|---|---|---|---|
| 1st | 2nd | | | |
| RR | $Ca^{2+}$ | 800 | 208 ± 6 (3) | 24 ± 3 (3) |
| $Ca^{2+}$ | RR | 800 | 322 ± 10 (5) | 5 ± 2 (5) |

Incubation medium as described in Fig. 1. Simultaneous experiments were conducted using fluo-3-loaded mitochondria for measuring $[Ca^{2+}]_m$ (0.5 mg protein) or citrulline synthesis (2 mg protein/ml) and treated with 1 $\mu$M ruthenium red (RR) for 2 minutes prior or after the addition of 800 nM $[Ca^{2+}]_o$. Results are the mean ± SEM of at least three experiments in duplicate. Numbers in parenthesis indicate separate experiments.

and the rate of citrulline synthesis was 5 nmoles/min/mg of protein. The results suggest that mitochondrial matrix free calcium and not extramitochondrial calcium is involved in the control of the synthesis of citrulline.

It has been established that low concentrations of calcium ions can be the cause of disorders in the internal mitochondrial membrane (Häusinger, 1990), with which carbamoyl phosphate synthetase I is associated, an enzyme involved in the synthesis of citrulline. This may be the reason for the low level of citrulline synthesis observed when $[Ca^{2+}]_m$ was depleted with A23187. However, there appears to be an inhibitory effect of $Ca^{2+}$ on the synthetic pathway at high $[Ca^{2+}]_m$. Other interactions of mitochondrial matrix $Ca^{2+}$ involve the indirect activation of the pyruvate dehydrogenase complex (PDH) by causing increases in the amount of active non-phosphorylated PDH ($PDH_a$) (Denton et al., 1972). In the same range of concentration (0.1–1.0 $\mu$M), matrix free $Ca^{2+}$ activates 2-oxoglutarate dehydrogenase complex (OGDH) (Denton et al., 1978) and $NAD^+$-isocitrate dehydrogenase (NAD-ICDH) (McCormack and Denton, 1979), by causing a marked decrease in the Km values for their respective substrates. More research is required to obtain the exact nature of the participation of the mitochondrial matrix free calcium ions on this urea cycle reaction.

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