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STEADY STATE REGULATION OF EXTRAMITOCHONDRIAL Ca^{2+} BY RAT LIVER MITOCHONDRIA

EFFECTS OF Mg^{2+} AND ATP

GERALD L. BECKER

Department of Biochemistry, University of Alabama Medical Center, Birmingham, AL 35294 (U.S.A.)

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Summary

An electrode-based system capable of monitoring ionized Ca^{2+} concentrations ($[Ca^{2+}] < 1$ μM was used to examine the regulation of extramitochondrial $[Ca^{2+}]$ by rat liver mitochondria. At the point of steady state balance between Ca^{2+} uptake and release, $[Ca^{2+}]$ ranged between 0.5 and 1.0 μM in a KCl/Hepes/succinate medium. When 1 mM Mg^{2+} was included in this basal medium, the range of steady state $[Ca^{2+}]$ values was 1—2 μM. Further additions (3 mM MgATP and 2 mM P_i) lowered extramitochondrial $[Ca^{2+}]$ to 0.4—0.8 μM. Thus under experimental conditions simulating the control of cytosolic $[Ca^{2+}]$, liver mitochondria buffered extramitochondrial $[Ca^{2+}]$ at constant values within the range of $[Ca^{2+}]$ estimated for liver cytosol; and cytosolic levels of Mg^{2+} and ATP significantly affected those steady state $[Ca^{2+}]$ values in directions consistent with previously reported effects of those modulators on mitochondrial Ca^{2+} uptake and release.

Introduction

A central role for mitochondria in the regulation of cytosolic $[Ca^{2+}]$ was first proposed by Drahota et al. [1], based on their finding that energized mitochondria lowered total Ca^{2+} of a suspending medium to apparently constant levels in the micromolar range. These workers suggested that a steady state balance between simultaneous influx and efflux of mitochondrial Ca^{2+} could have accounted for their findings. Operation of such a steady state under con-

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediamine tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ditions simulating those in vivo has recently been demonstrated directly in several laboratories [2—5]. Emphasizing physiologically realistic rather than kinetically rigorous conditions, Becker [4] and Nicholls [5] independently used Ca^{2+}-selective electrodes to show that the net uptake of added Ca^{2+} by rat liver mitochondria and the net release of Ca^{2+} following the addition of chelating agents each stopped at identical values of extramitochondrial $[Ca^{2+}]$ in the micromolar range, reflecting attainment of a steady state equilibrium beween mitochondrial Ca^{2+} influx and efflux.

This finding not only confirmed the close relationship between mitochondrial Ca^{2+} transport and regulation of cytosolic $[Ca^{2+}]$ but also showed the importance of steady state conditions in the maintenance of intracellular Ca^{2+} homeostasis. This paper describes further studies of steady state Ca^{2+} transport by rat liver mitochondria, using a medium mimicking cytosol in ionic composition and in content of modulators of Ca^{2+} transport. Under such conditions extramitochondrial $[Ca^{2+}]$ values ranged below 1 μM and were significantly affected by Mg^{2+} and ATP at their typical cytosolic concentrations.

Methods

Mitochondrial fractions were isolated from the livers of fasted 200—300 g male Sprague-Dawley rats by the method of Johnson and Lardy [6], except that the isolation medium contained 5 mM Tris-Hepes (pH 7.2) and 0.5 mM Na_2H_2EGTA in addition to 0.25 M sucrose. Respiratory control ratios ranged between 4 and 6 with succinate as substrate.

For studies of Ca^{2+} movements, mitochondria (10 mg protein) were added to 5 ml basal medium (120 mM KCl, 2 mM potassium succinate, 25 mM Tris-Hepes, pH 7.0) and the suspension magnetically stirred at 25 ± 1°C in an open-topped chamber. Extramitochondrial $[Ca^{2+}]$ was monitored through a Radiometer F2112 Ca^{2+}-selective electrode connected to a Radiometer PHM64 pH meter, as described by Madeira [7]. The logarithmic relation between electrode potential and $[Ca^{2+}]$ was linearized by feeding the pH meter output into a logarithmic amplifier (Model 4127KG, Burr-Brown Corporation) wired for antilog operation according to the manufacturer's instructions. The following values were chosen for the external resistors required by the antilog circuit: $R_0 = 31$ kΩ, $R_1 = 10$ MΩ, $R_2 = 750$ Ω. The output from the antilog transducer was recorded continuously (Omniscribe, Houston Instruments). Electrode readings were calibrated against absolute values of $[Ca^{2+}]$ by adding to fresh medium; all additions (except mitochondria) present when experimental measurements were made plus 0.2 mM $Ca(NO_3)_2$ plus the concentration of EGTA (disodium salt) calculated to achieve the desired value of $[Ca^{2+}]$ [8]. The value $4.7 \cdot 10^6$ M^{-1} was used for the association constant of the CaEGTA complex at pH 7.0. The response of the measuring system was found to be a linear function of $[Ca^{2+}]$ for $[Ca^{2+}] \geqslant 0.25$ μM.

Results

Fig. 1 is a recording of $[Ca^{2+}]$ in a mitochondrial suspension which had already reached steady state equilibrium ($[Ca^{2+}]_o$) through the uptake of

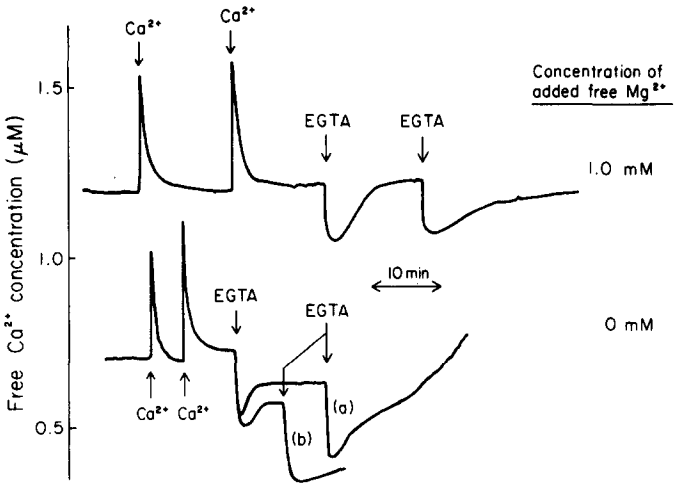

Fig. 1. Changes in $[Ca^{2+}]$ produced by Ca^{2+} and EGTA additions to mitochondrial suspensions. Mitochondria (10 mg protein) were suspended in 5.0 ml of medium containing 120 mM KCl, 25 mM Tris-Hepes (pH 7.0) ± 1 mM $MgCl_2$, as indicated. After $[Ca^{2+}]$ had become constant, pulsed additions of 10 μM $Ca(NO_3)_2$ or 10 μM Na_2H_2EGTA were made at the times marked by arrows.

endogenous Ca^{2+} (⩽25 nmol = 2.5 nmol/mg mitochondrial protein) from the medium. As reported previously [4], pulsed additions of either Ca^{2+} or EGTA were followed by mitochondrial Ca^{2+} uptake or release, respectively, tending to restore the original value of $[Ca^{2+}]_0$. Comparison of the two traces of Fig. 1 illustrates the effects of physiologic levels of Mg^{2+} in the basal medium. Added Mg^{2+} at $[Mg^{2+}] = 1$ mM [9] significantly raised the values of $[Ca^{2+}]_0$ and facilitated return of $[Ca^{2+}]$ to that same value of $[Ca^{2+}]_0$ after EGTA addition. $[Ca^{2+}]_0$ values were consistently 0.5 to 1.0 μM higher in the presence of Mg^{2+} for each of 5 different preparations incubated both with and without added Mg^{2+} (Table I).

Extramitochondrial $[Ca^{2+}]_0$ values were lower when the medium was supplemented to extend its resemblance to cytosol: to the basal (KCl/Hepes/succinate) medium were added ATP 3 mM and ADP 0.5 mM [10], along with P_i 2 mM and $MgCl_2$ 4 mM (to maintain $[Mg^{2+}] = 1$ mM, assuming that all ATP complexed with Mg^{2+}). In this so-called 'complete' medium, $[Ca^{2+}]_0$ values

TABLE I

EFFECT OF MODIFIERS OF MITOCHONDRIAL Ca^{2+} TRANSPORT ON EXTRAMITOCHONDRIAL STEADY STATE $[Ca^{2+}]$ VALUES

Rat liver mitochondria were incubated in basal medium (KCl 125 mM, Hepes 25 mM, succinate 2 mM) with modifiers added as shown. Numbers in parenthesis represent number of different mitochondrial preparations on which measurements were made.

Modifiers present	Extramitochondrial $[Ca^{2+}]$ (μM)
None (basal medium)	0.5—1.0 (5)
Mg^{2+} 1 mM	1—2 (5)
Mg^{2+} 1 mM, MgATP 3 mM, potassium phosphate 2 mM	0.4—0.8 (8)

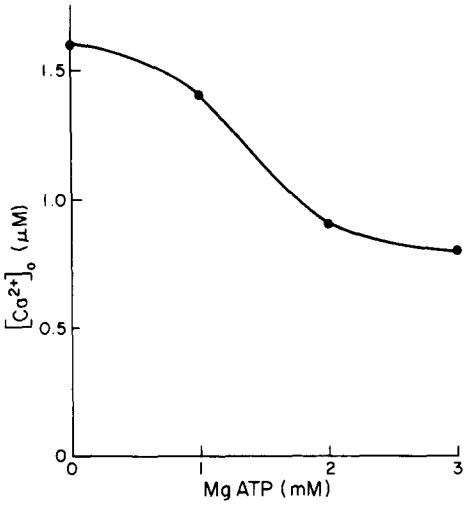

Fig. 2. Effect of MgATP concentration on $[Ca^{2+}]_0$ values attained in mitochondrial suspensions. Incubation conditions were the same as for Fig. 1, except for the addition of the indicated amounts of MgATP to the medium.

varied between 0.4 and 0.8 μM (Table I). Fig. 2 shows that the difference in $[Ca^{2+}]_0$ in the 'complete' versus Mg^{2+}-only media, since both contained $[Mg^{2+}]$ = 1 mM, was accounted for by differences in the amount of MgATP: increasing amounts of added MgATP produced a corresponding decline in $[Ca^{2+}]_0$, from a value in the Mg^{2+}-only range at MgATP = 0 to a value typical of the 'complete' medium at MgATP = 3 mM. The omission of ADP, P_i or succinate from the complete medium or the inclusion of rotenone or oligomycin produced no detectable effect on observed $[Ca^{2+}]$ dynamics.

Discussion

The experiments described above have attempted to study mitochondrial Ca^{2+} transport in terms of its extramitochondrial consequences rather than as a strictly mitochondrial process. Since the principal cellular function of mitochondrial Ca^{2+} transport is the regulation of ionized Ca^{2+} levels in the cytosol, extramitochondrial $[Ca^{2+}]$ is the transport-related variable of interest. The physiological significance of these measurements of $[Ca^{2+}]$ is enhanced through inclusion in the medium of typical cytosolic levels of those species known to influence mitochondrial Ca^{2+} transport (including the amount of Ca^{2+} available to the mitochondria) along with direct monitoring of extramitochondrial $[Ca^{2+}]$. Net uptake and net release of mitochondrial Ca^{2+} could be visualized within the same incubation, another feature important to the physiological relevance of these studies, since both morphological and biochemical evidence [11] suggests that cytosolic $[Ca^{2+}]$ is regulated within a range of $[Ca^{2+}]$ values for which mitochondrial Ca^{2+} uptake and release are in balance or nearly so. The effectiveness of mitochondrial Ca^{2+} transport in maintaining $[Ca^{2+}]$ homeostasis within this range is indicated by the similarity of $[Ca^{2+}]_0$ values to generally accepted values for cytosolic $[Ca^{2+}]$ [11].

While Nicholls [5] has analyzed the relative contributions of mitochondrial Ca^{2+} influx and efflux processes at various $[Ca^{2+}]$ values and characterized the dependence of $[Ca^{2+}]_0$ on the charge and concentration components of chemiosmotic energy stores, this work has focussed more on detailing the overall pattern of ambient $[Ca^{2+}]$ regulation by mitochondria and the effects of potential regulatory agents. The effects of Mg^{2+} and ATP on steady state $[Ca^{2+}]_0$ were consistent with those seen using more traditional measures of Ca^{2+} transport (see Ref. 12 for review). The higher values of $[Ca^{2+}]_0$ obtained with Mg^{2+} as the only addition to the basal medium (a finding previously reported [5] but based on transmembrane $^{45}Ca^{2+}$ distribution rather than on direct measurement of $[Ca^{2+}]$) are consistent with the selective inhibition of Ca^{2+} influx described by others [13,14]. Since Ca^{2+} efflux remains relatively unaffected by external Mg^{2+} (Fig. 1), the equal Ca^{2+} influx necessary to attain the steady state was reached only through a higher extramitochondrial $[Ca^{2+}]$ in compensation for the lowering by Mg^{2+} of the rate constant for Ca^{2+} influx. The reverse situation prevailed when the additional components of the complete medium were present — i.e. Ca^{2+} release was preferentially inhibited (data not shown), as would have been expected from the lower $[Ca^{2+}]_0$ values observed compared to incubations in which Mg^{2+} was the only addition. As shown in Fig. 2, this effect was attributable to the presence of MgATP in the complete medium. Adenine nucleotides have long been known to enhance the uptake and/or retention of Ca^{2+} by mitochondria [15—17], and selective inhibition of Ca^{2+} efflux from heart mitochondria by either ATP or ADP has recently been shown [18]. The experiments reported here provide no information on the mechanism(s) through which Mg^{2+} prevented the incomplete buffering of $[Ca^{2+}]$ which followed EGTA addition (lower trace of Fig. 1, curves (a) and (b)), although other studies have also shown salutary effects of Mg^{2+} on the transport of Ca^{2+} as well as on other energy-linked mitochondrial activities dependent on inner membrane integrity [19,20].

The actual contribution of mitochondria to the control of cytosolic $[Ca^{2+}]$ in vivo cannot of course be determined from these studies, since the system excluded non-mitochondrial Ca^{2+} pumps able to compete with mitochondria for available Ca^{2+} [21], as well as those cytosolic species not clearly established as having effects on mitochondrial Ca^{2+} transport. However, the approach described here is being extended to include microsomal fractions in an attempt to improve the in vitro simulation of cytosolic $[Ca^{2+}]$ regulation (Becker, G.L. and Fiskum, G., unpublished data).

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