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Parallel measurement of oxoglutarate dehydrogenase activity and matrix free Ca^{2+} in fura-2-loaded heart mitochondria

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The entrapment of the Ca^{2+}-sensitive fluorescence indicators fura-2 or quin2 in the matrix space of isolated heart mitochondria renders possible the direct monitoring of the matrix free Ca^{2+} ($[Ca^{2+}]_m$) [(1987) Biochem. J. 248, 609–613]. In this paper the correlation between the $[Ca^{2+}]_m$ and the in situ activity of oxoglutarate dehydrogenase (OGDH) in fura-2-loaded mitochondria is shown. At the initial value of $[Ca^{2+}]_m$, 64 nM, which corresponded to 0.36 nmol/mg mitochondrial Ca content, the OGDH activity was 12% of the maximal. Half-maximal and maximal activation were attained at 0.8 and 1.6 μM $[Ca^{2+}]_m$, respectively. The results indicate that an increase of the mitochondrial Ca content in the physiological range enhances the OGDH activity by means of elevation of $[Ca^{2+}]_m$.

Ca^{2+}; Oxoglutarate dehydrogenase; Fura-2; (Mitochondria)

1. INTRODUCTION

In the last few years the regulatory role of the mitochondrion as the main cytoplasmic Ca^{2+} buffer became unlikely. Instead it was suggested that the mitochondrial Ca^{2+} transport systems could modulate the free Ca^{2+} concentration in the range 0.05–5 μM of the matrix space. By this mechanism three intramitochondrial dehydrogenases (pyruvate dehydrogenase, oxoglutarate dehydrogenase, isocitrate dehydrogenase) could be regulated (review [1–5]). These dehydrogenases are Ca^{2+} sensitive in mitochondrial extracts [6–8] and were also shown to respond to changes in Ca content of mitochondria in situ [9–11]. Furthermore, hormonal signals which elevate cytoplasmic Ca^{2+} were shown to increase the activity of these intramitochondrial dehydrogenases and the rate of oxidative metabolism [9–12].

In the above experiments the $[Ca^{2+}]_m$ was estimated only indirectly using either equilibration of Ca^{2+} gradient by A23187 in uncoupled mitochondria [13] or applying the null-point titration method ([14,15] but see also [3]).

Recently we have developed a method to monitor continuously the $[Ca^{2+}]_m$ after loading isolated heart mitochondria with the Ca^{2+}-sensitive fluorescent indicators fura-2 or quin2 [16]. This offers a possibility to compare directly the change of the $[Ca^{2+}]_m$ with the OGDH activities in the same mitochondrial preparation.

2. MATERIALS AND METHODS

Rat heart mitochondria were prepared as in [16].

All types of measurements were carried out in a basic medium containing 240 mM sucrose, 20 mM Tris-HCl, 2 mM KCl, 0.5 mM malate, 0.05 mM ADP, 0.1 mM NH_4Cl, 200 μM Ca^{2+}, 0.1% (w/v) bovine serum albumine pH 7.4 at 22–24°C.

Mitochondrial Ca^{2+} uptake and Ca content were determined with Ca^{2+}-sensitive electrode and atomic absorption spectrophotometry as in [16].

OGDH activity was assayed by measuring fluorimetrically the 2-oxoglutarate-dependent steady-state extent of mitochondrial $NAD(P)^+$ reduction at non-saturating concentration of

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Abbreviations: OGDH, oxoglutarate dehydrogenase; CCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $[Ca^{2+}]_m$, intramitochondrial (matrix) free $[Ca^{2+}]$; fura-2/AM, acetoxymethylester of fura-2

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2-oxoglutarate (0.17–0.68 mM) [14,18]. This was shown to be proportional with the initial rate of OGDH activity [19] and also specific, proved by $^{14}CO_2$ production from labelled oxoglutarate [19]. Fluorescence was monitored at 365 nm (excitation) and 460 nm (emission wavelength). $NAD(P)H^+$ production was calculated on the basis of the fluorescence signal of a known amount of exogenous $NADH^+$ added to the mitochondrial suspension or expressed as a percentage of the maximal Ca^{2+}-dependent activation [18,19]. In the basic medium the Ca content of mitochondria did not change during the measurements of OGDH activity (controlled by Ca^{2+} electrode and see [10]).

Loading of mitochondria with fura-2 was carried out principally as described in [16] with the modification that mitochondria (approx. 50 mg protein/ml) were incubated in the presence of 12 μM fura-2/AM at 35°C for 8 min (see section 3.1). Fluorescence was detected and the $[Ca^{2+}]_m$ was calculated as described in [16].

3. RESULTS AND DISCUSSION

3.1. *Loading mitochondria with fura-2*

Fura-2 was chosen as an indicator of the $[Ca^{2+}]_m$ because fura-2/AM was hydrolysed faster and chelated less intramitochondrial Ca^{2+} than quin2 [16]. The previously used loading period (30 min) had to be reduced (8 min) to prevent the loss of OGDH activity. Under these conditions 80% of the non-loaded mitochondrial OGDH activity was preserved.

To verify the hydrolysis of fura-2/AM to fura-2 the spectral properties of the loaded mitochondria were studied in a basic medium containing Triton X-100. The shift of the excitation peak of the dye (fig.1A,a) towards 340 nm which is characteristic of the fura-2-Ca complex [20], was observed in 5 min and continuously increased for 20 min (fig.1A,b–d). The fluorescence peak of fura-2-Ca was retained in the mitochondria separated from the loading medium (fig.1B,a). This fluorescence maximum was gradually decreased or totally quenched by EGTA (fig.1B,b–d) or Mn^{2+} (fig.1B,g) added to the solubilized suspension of mitochondria [20,21]. The appearance of the isosbestic point after decreasing the medium $[Ca^{2+}]$ by EGTA shows that the generation of other fluorescent intermediates [22] during the loading process is unlikely [23]. In agreement with this, addition of exogenous fura-2 free acid to the

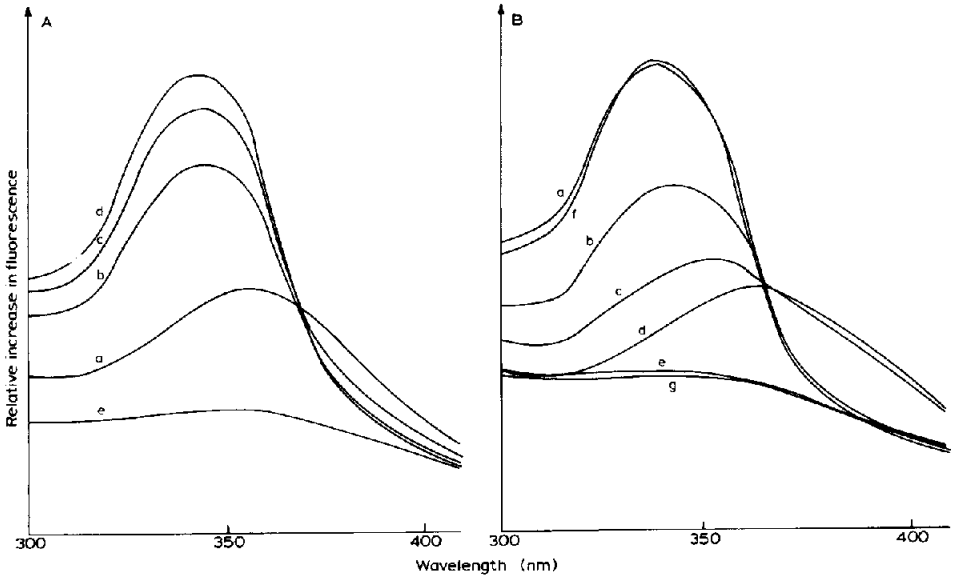

Fig.1. Spectral characteristics of the fura-2/AM-loaded mitochondria. (A) Heart mitochondria were incubated with 12 μM fura-2/AM. After incubation for (a) 0, (b) 5, (c) 10, (d) 20 min, aliquots of 0.5 mg mitochondrial protein were transferred in 2 ml of the basic medium containing 0.063% Triton X-100, (e) unloaded mitochondria. Excitation spectrum was recorded at an emission wavelength of 500 nm. (B) Retention of the fura-2-Ca peak after separation of the mitochondria from the loading medium. Mitochondria after being loaded for 8 min with fura-2/AM were washed and recentrifuged. Thereafter 0.5 mg mitochondrial protein was resuspended (a) as in A. Fluorescence was recorded in the same sample after reducing the $[Ca^{2+}]$ by the addition of (b) 0.1, (c) 0.5, (d) 5 mM EGTA, (e) unloaded mitochondria, (f) fura-2 free acid (27 nM) was added to sample e, (g) addition of 2 mM Mn^{2+} to sample a.

non-loaded mitochondria mimicked the spectral properties of the fura-2-loaded mitochondria (fig.1B,f).

3.2. *Localization of fura-2*

The fact that fura-2 was localized within the mitochondria and did not leak out was proved by using Mn^{2+} as a quencher of the fura-2-Ca fluorescence [21] and also by compounds which influenced the Ca^{2+} transport of the mitochondria. (A similar procedure was applied for quin2-loaded mitochondria, see fig.2 in [16].) The fluorescence of the dye-loaded mitochondria was only increased if mitochondria accumulated Ca^{2+} from the medium (fig.2a). If either Ruthenium red, a specific inhibitor of the Ca^{2+} uptake pathway [24], CCCP, an uncoupler (not shown), or EGTA was present in the medium the fluorescence signal remained at a low level (fig.2b,c). Ca^{2+} movements were also verified by Ca^{2+} electrode (not shown). The high fluorescence attained after mitochondrial Ca^{2+} accumulation was rapidly quenched by Mn^{2+} (fig.2a) which is transported via the Ca^{2+} uptake pathway [25]. Therefore the Mn^{2+} quench was substantially reduced by previously added Ruthenium red (fig.2d). This observation together with the fact that EGTA did not decrease the high level of fluorescence (fig.2e) pointed to the absence of free fura-2 outside the mitochondria. In the presence of Ruthenium red ionomycin, which permeabilized mitochondria for both Ca^{2+} and Mn^{2+}, restored the quenching effects of EGTA and Mn^{2+} (fig.2d,e) or elevated the low mitochondrial fluorescence (fig.2c).

3.3. *Correlation between $[Ca^{2+}]_m$ and OGDH activity*

By limiting the time of Ca^{2+} accumulation either by Ruthenium red or EGTA we could change the mitochondrial Ca content and simultaneously the OGDH activity. Fig.3 shows the OGDH activity and the Ca content of non-loaded (fig.3A) and

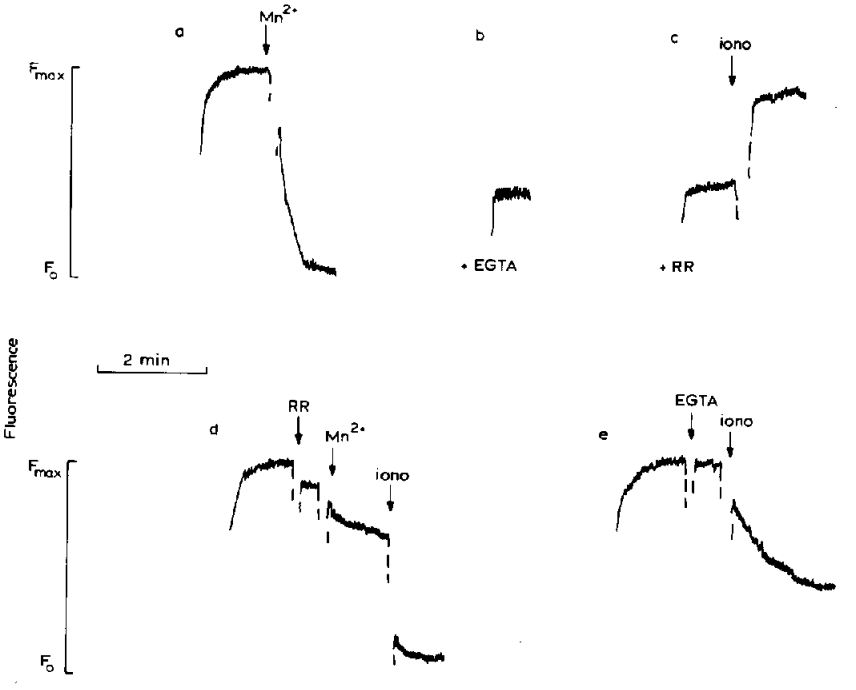

Fig.2. Localisation of fura-2 in the matrix space. Dye-loaded mitochondria were incubated in the basic medium and fura-2 fluorescence was recorded as described in section 2. Further additions: 1.5 mM $MnCl_2$ (Mn^{2+}), 3 mM EGTA with 20 mM Tris-OH (EGTA), 4 μM Ruthenium red (RR), 2 μM ionomycin (iono). In b and c EGTA and RR were present before mitochondria were added. The autofluorescence of mitochondria incubated only with dimethyl sulfoxide instead of fura-2/AM was influenced only by Ruthenium red (not shown).

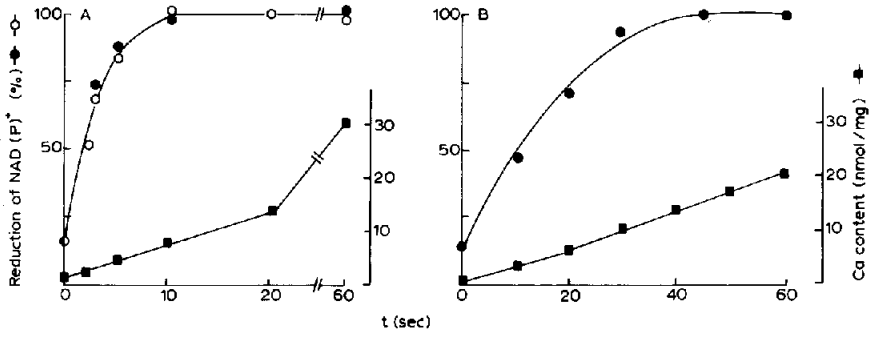

Fig.3. Correlation between mitochondrial Ca content and OGDH activity in non-loaded (A) and fura-2-loaded (B) mitochondria. Before determination of OGDH activity as described in section 2, Ca^{2+} uptake was inhibited at the indicated times (A) either by 7 μM Ruthenium red (O) or 3.8 mM EGTA (●) or (B) 3.8 mM EGTA addition. Ca content was calculated as the sum of the initial and accumulated Ca^{2+}.

dye-loaded (fig.3B) mitochondria as a function of Ca^{2+} accumulation time. The Ruthenium red and the EGTA stop method gave identical results (fig.3A). An eventual alkalization of the matrix space was not the cause of the increased $NAD(P)H^+$ level as the presence of permeating proton-donor anions (0.1 mM K^+-propionate or 0.2 mM trisphosphate) did not alter the response (not shown). In the absence of Ca^{2+} accumulation the OGDH activity of dye-loaded (0.51 ± 0.21 nmol $NAD(P)H^+$/mg, SD, $n = 8$) and non-loaded mitochondria (0.55 ± 0.31 nmol/mg, $n = 5$) was almost identical.

On fig.4 the activation of intramitochondrial OGDH is demonstrated as a function of $[Ca^{2+}]_m$, measured by fura-2. At the initial Ca content (0.36 ± 0.16 nmol/mg, $n = 5$) the $[Ca^{2+}]_m$ (determined as in fig.2b) was 64 nM (± 21, $n = 4$) and the OGDH activity was 12.2% of its maximum. Half-maximal activation was attained at a Ca content of 4.35 nmol/mg (± 1.54, $n = 5$) (fig.3B), corresponding to a $[Ca^{2+}]_m$ of 800 nM resulting in a 4-fold increase of OGDH activity over the starting value (fig.4). An increase in Ca content from the initial 0.95 to 4.35 nmol/mg of non-loaded mitochondria caused 85% activation of OGDH (fig.3A). The discrepancy is probably due to the enhanced Ca^{2+} chelation of the incubated and fura-2-loaded mitochondria. The maximal enzyme activity was achieved at 1600 nM $[Ca^{2+}]_m$ (fig.4). The correlation between the $[Ca^{2+}]_m$ and the OGDH activity observed by us was very similar to that obtained on mitochondrial extracts [1].

Earlier publications proved unambiguously that hormonal signals which elevated the cytoplasmic $[Ca^{2+}]$ increased both the mitochondrial Ca content and the OGDH activity [2,3]. The present results revealed direct correlation between the $[Ca^{2+}]_m$ and the intramitochondrially located OGDH activity and indicate that fluctuation of the mitochondrial Ca content occurring in vivo (1–6 nmol/mg) elicit appropriate changes in the $[Ca^{2+}]_m$ to regulate the matrix dehydrogenases.

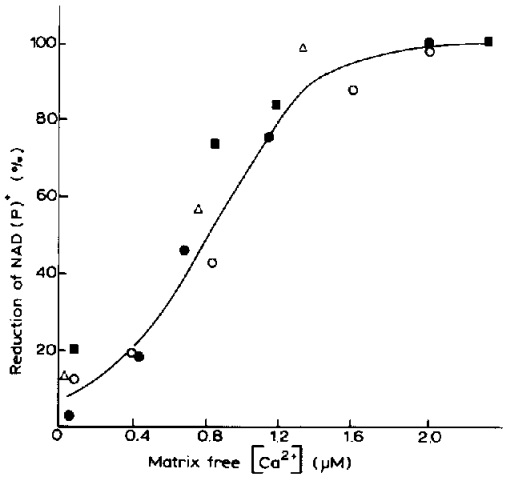

Fig.4. The OGDH activity as a function of the matrix free $[Ca^{2+}]$. After resuspending the fura-2-loaded mitochondria OGDH activity was measured after blocking the Ca^{2+} uptake with 3.8 mM EGTA and in parallel sample $[Ca^{2+}]_m$ was determined from the fluorescence record similar to 1A (as described in section 2). The 100% represents 4.2 (± 0.5, $n = 4$) nmol $NAD(P)H^+$ production/mg. The different symbols indicate four separate experiments.

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