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Novel oxaloacetate effect on mitochondrial Ca^{2+} movement

Yuri N. Leikin*, Tatjana V. Zharova, Olga V. Tjulina

Department of Biochemistry, School of Biology, Moscow State University, Moscow 119899, Russian Federation

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Mitochondrial Ca^{2+} movement was investigated in the presence of oxaloacetate, which is widely known as a 'Ca^{2+}-releasing' agent [1978, Proc. Natl Acad. Sci. USA 75, 1690-1694]. It is demonstrated that rat liver mitochondria are capable of net Ca^{2+} accumulation from the oxaloacetate supplemented assay mixture. Both the membrane energization and the cation uniport at the expense of oxaloacetate are shown to be specifically blocked by either arsenite or ammonium chloride. With respiratory inhibitors present, ADP is shown to be a prerequisite for a high Ca^{2+} capacity, which can be alternatively enlarged with a concomitant loss of the arsenite effect by an addition of an $NADP^+$-specific reductant (isocitrate). Arsenite-sensitive production of NADPH is observed, thus suggesting coupling between pyridine nucleotide transhydrogenation and the cation uniport in mitochondria. The role of such a coupling mechanism in the uniporter-mediated Ca^{2+} fluxes in mitochondria is discussed.

Uniport of Ca^{2+}; Oxaloacetate; NAD(P)-transhydrogenase (EC 1.6.1.1); Mitochondria; Rat liver

1. INTRODUCTION

The control of mitochondrial/cytosolic Ca^{2+} distribution has been intensively investigated during the last two decades [1]. The importance of the reversible Ca^{2+}-uniporter as a Ca^{2+} efflux pathway in coupled mitochondria has been questioned on the basis of thermodynamic considerations (see refs. in [1]). Since the work of Lehninger's group, some metabolites such as OA and acetoacetate are known to induce the Ca^{2+} release either by a putative Ca^{2+}-efflux mechanism [2] or due to a damage of mitochondrial integrity [3,4]. Here, by taking precautions against non-mediated Ca^{2+} efflux [3], we demonstrate the inward operation of mitochondrial Ca^{2+}-uniporter due to the presence of OA. Evidence is presented that the energy-linked cation uptake is primarily driven by the OA-promoted transhydrogenation of NADPH by NAD^+ in mitochondria.

2. MATERIALS AND METHODS

Rat-liver mitochondria were prepared by a conventional method [5] and protein was assayed with the biuret procedure. Mitochondria (2.5 mg protein/ml) were incubated at 20°C in the basic medium containing 0.1 M sucrose, 75 mM KCl, 2.6 mM $MgCl_2$, 10 mM HEPES (pH 7.4) and oligomycin (2 μg/mg protein). Uptake of Ca^{2+} and changes in the membrane potential were recorded by dual-wavelength photometry with 50 μM arsenazo III (662–692 nm) [6] and 8 μM safranine (523–554 nm) [7], respectively. Redox changes in mitochondrial PN were monitored by fluorescence at 450 nm with an excitation wavelength at 366 nm. Enzymatic determinations of PN in acid mitochondrial extracts were carried out as described in [8].

*Corresponding author. Fax: (095) 939 3955.

Abbreviations: OA, oxaloacetate; PN, pyridine nucleotides; RR, ruthenium red; FCCP, carbonylcyanide-4-trifluoromethoxyphenylhydrazone; HEPES; 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid

3. RESULTS

The trace 4 in Fig. 1a shows the dye absorbance change that followed Ca^{2+} addition into the OA-supplemented mitochondrial suspension. Just like the respiratory-supported reactions (traces 1–3), the slow dye response in the presence of OA are prevented by FCCP or RR (trace 5). Despite the low rate (20 nmol Ca^{2+}/min per mg of protein), OA-dependent reaction proceeds up to the disappearance of all Ca^{2+} added and there is a small fraction of the cation that was accumulated and then released under OA-free conditions (trace 6). The time course of the OA-supported Ca^{2+} uptake was not changed when the contaminating pyruvate (40 μM) was carefully removed by introduction of a lactate dehydrogenase-containing trap into the assay mixture (not shown). The OA-dependent reaction as well as pyruvate-supported one are blocked specifically by arsenite (Fig. 1b, traces 4 and 3). As seen in Fig. 1c (trace 4), OA is the only substrate which allows the non-respiring mitochondria to slowly take up some amount of cation. The net uptake of Ca^{2+} is resumed by the addition of ADP or compounds none of which were active by themselves without respiration (Fig. 1c, traces 1–3). The rate values of Ca^{2+} accumulation are listed in Table I, which also shows that low arsenite inhibits Ca^{2+} uniport with the only exception being the OA plus isocitrate-supported reaction. Using safranine as a probe [7] we observed arsenite-sensitive membrane energization in response to the addition of OA (results not presented).

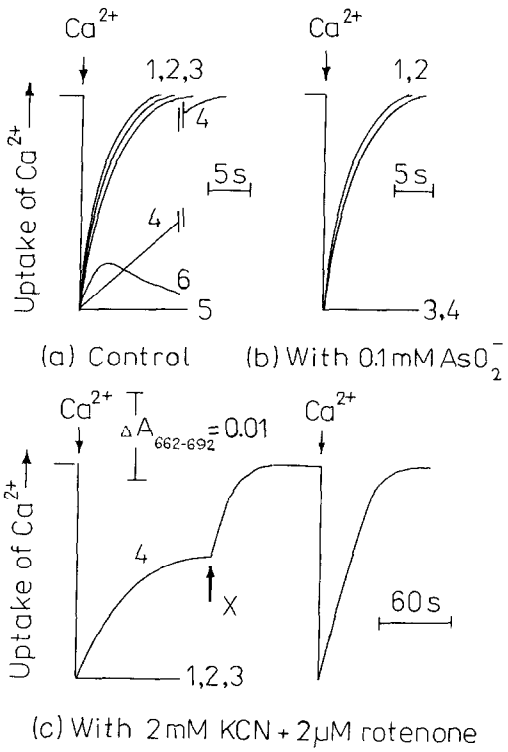

Fig. 1 The effect of arsenite and cyanide plus rotenone on the mitochondrial uptake of Ca^{2+}. Rat liver mitochondria were preincubated for 2 min with or without inhibitors in basic medium (see Section 2) containing succinate (trace 1), isocitrate (trace 2), pyruvate (trace 3) or OA (trace 4) each at 2 mM and then reactions were initiated by the addition of 50 μM Ca^{2+}. In panel (a) trace 5, 1 μM FCCP or 0.1 μM RR was also present in each substrate-containing incubation (1–4); trace 6, no addition to basic medium was made. In panel (c), the addition of 0.2 mM ADP is indicated by symbol X; the addition of any reductant (1, 2 or 3) instead of ADP was almost equally effective. The measured uptake rates in the presence of OA (traces 4) are equal to 20 and 3–4 nmol of Ca^{2+}/min per mg of protein in panels (a) and (c), respectively. Note the shortened time scale in panel (c).

Thus, it is reasonable to assume that the creation of the potential and Ca^{2+} uptake by oligomycin-treated mitochondria under non-respiring conditions may result from the outward H^+-pumping in the course of PN transhydrogenation. As one can see in Fig. 2, there is an appreciable fraction of PN that was steadily reduced after the OA-promoted decay in fluorescence had been completed (trace 1). This fraction clearly increases (traces 2–5) under conditions which were found to restore a high Ca^{2+}-capacity of mitochondria (see Fig. 1c). Another point concerns the effect of arsenite, which is active as an oxidant of the OA-inaccessible PN (Fig. 2) and as an inhibitor of the OA-dependent uptake of Ca^{2+} (Table I) under the same sets of conditions. In all cases the arsenite effect is reversed excessively by the addition of isocitrate (Fig. 2, traces 1–4, 6). As evidenced from data in Table II, it is NADPH but not NADH, that accounts for all intermediate fluorescence levels in the presence of OA. Thus, the metabolism of OA in mitochondria maintains the stable NADPH/$NADP^+$ ratio as being in excess of that NADH/NAD^+ thereby providing the NADPH transhydrogenation by NAD^+.

4. DISCUSSION

This study clearly demonstrates that liver mitochondria when incubated in the OA-supplemented assay mixture are capable of net Ca^{2+} accumulation which is supported by the PN transhydrogenation in non-respiring conditions at least. In the experiments not shown here we observed that the OA-dependent potential creation and Ca^{2+} uptake both were inhibited by millimolar ammonium chloride due to a non-energy-linked transhydrogenation via the glutamate dehydrogenase reaction [9,10]. In principle, the coupling between the Ca^{2+}-uniporter and NAD(P)-transhydrogenase is hardly surprising [11], but the question arises by which means OA keeps the mass action ratio of the enzyme (Table II) apart from the equilibrium [12] without $NADP^+$-specific reductant in the medium [13]. The answer comes from the experiment with ADP (Fig. 1c), which in accordance with the earlier observation [14] strongly indicates the key role of the OA-decarboxylation step in the overall reaction. This point is further evidenced by the effect of succinate, due apparently to GDP/ADP supply by back substrate phosphorylation. As would be rationalized in metabolic terms (cf. [15]), the ADP requirement is shown to be bypassed without loss of the arsenite effect (Table I), when pyruvate was substituted for ADP or succinate. Finally, the contribution of OA appears to be minimized to rapid NAD^+ production when that of NADPH was brought about by the arsenite-insensitive [13] isocitrate dehydrogenation. Thus, the sufficiency of OA to bring up NADPH for transhydrogenation can be consistently explained by self-supported forward flux of the compound through the TCA cycle. When the OA- promoted redox activity of loop

Table I

Effects of ADP and reductants on the OA-dependent uptake of Ca^{2+} by respiratory-arrested mitochondria with or without arsenite

Metabolite(s)	Uptake of Ca^{2+}, nmol/min per mg of protein	
	Without arsenite	With arsenite
OA only	3–4	0
OA + ADP	10	< 1
OA + succinate	12	0
OA + pyruvate	8	0
OA + isocitrate	10	9

Rat liver mitochondria were preincubated for 2 min in cyanide and rotenone containing basic medium with the metabolite(s) listed (see legend to Fig. 1c for concentrations) and then reactions were started by an addition of 50 μM Ca^{2+}. Where indicated, 0.1 mM sodium arsenite was also present during the preincubation. The same results have been obtained with using of myxythiazol as a respiratory inhibitor.

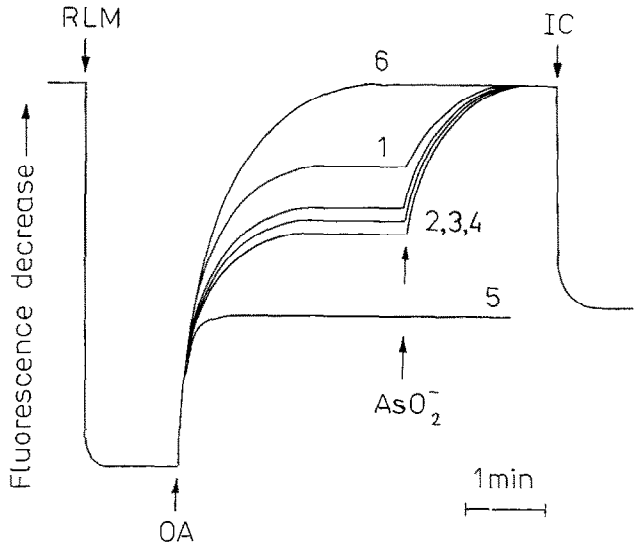

Fig. 2. OA-dependent changes in PN fluorescence of non-respiring mitochondria under different conditions. Rat liver mitochondria (RLM) were added to cyanide plus rotenone containing basic medium (trace 1) supplied with 0.2 mM ADP (trace 2), succinate (trace 3), pyruvate (trace 4), or isocitrate (trace 5) each in 2 mM. Trace 6, 0.1 mM sodium arsenite was initially present. Where indicated by arrows, 2 mM OA, 0.1 mM arsenite and 2 mM isocitrate (IC) were introduced into the assay mixture.

O with $\rightarrow H^+/2e = 2$ [13] is paralleled by slow respiration [14] with $\rightarrow H^+/2e = 10$ [16], a six-fold increase in the cation uptake rate would be predicted. In fact, it was invariably observed that rotenone and/or cyanide declined the rate of the reaction approximately by factor 6. The experiments described were carried out in the presence of Mg^{2+}, added to prevent the onset of membrane permeabilization [3] and that is why the pattern of Ca^{2+} movement is inconsistent with the 'Ca^{2+}-releasing' effect of OA [4]. So, this definition now seems to be correct only under conditions where Ca^{2+} accumulation per se is deleterious to mitochondria. The same is true for acetoacetate, which has been recently reported to be responsible for the reuptake of Ca^{2+} by mitochondria [17]. In summary, taking into account the low cytoplasmic OA level [9], we don't rely on Ca^{2+} release [2], which is in need of rotenone [4], and Ca^{2+} uptake (present paper) as physiologically relevant processes. However, the revealed coupling between the PN transhydrogenation and Ca^{2+} uniport should be a rather flexible one due to the full reversibility of both the partial reactions. In other words, the back H^+-pumping activity of the enzyme may presumably be considered as a suitable charge- compensating device for outwards Ca^{2+} uniport in mitochondria, thus stimulating the operation of a putative $Ca^{2+}/2H^+$-antiporter.

Table II

Mitochondrial pyridine nucleotides content at different conditions

Conditions	NAD^+	NADH	$NADP^+$	NADPH
OA only	3.79	0.59	2.86	2.45
OA + ADP	3.82	0.67	2.50	2.84
OA + succinate	3.93	0.64	2.03	3.18
OA + pyruvate	3.60	0.61	1.98	2.82
OA + isocitrate	2.57	1.11	0.14	4 41
OA + arsenite	3.63	0.16	5.03	0.23

Samples for enzymatic determinations [8] were taken from incubations identical to those of experiments in Fig. 2 after the steady states were attained in the presence of OA. Values reported are the averages of 2–4 separate determinations and are expressed as nmol of PN per mg of protein.

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