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Maintenance of energy-linked functions in rat liver mitochondria

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EGTA and EDTA are compared with respect to their ability to preserve ATP-requiring reactions in rat liver mitochondria. The presence of EGTA sustains the high ATP requirement of citrulline synthesis. EDTA does not, even with excess Mg^{2+}. Carboxylation of pyruvate, which has a lower ATP demand, is not influenced by the type of chelating agent. In mitochondria stored for 24 h at 4°C, EGTA is more effective than EDTA in preventing loss of these energy-linked functions.

Introduction

It is usual to include EGTA or EDTA in the isolation and/or incubation medium of mitochondria. One of the actions of chelating agents in liver mitochondria is to prevent P_i-induced swelling. They are considered to act by binding membrane-bound Ca^{2+}, thus preventing activation of Ca^{2+}-dependent phospholipase(s) [1] and the loss of functional integrity associated with mitochondrial swelling. The similarity in binding constants of EGTA and EDTA for Ca^{2+} [2] has led to indiscriminate use of the two compounds, so that there is no consistency in concentration or stages of the procedure at which the chelating agent is present, in spite of potential differences that may result, for example, from binding of Mg^{2+} by EDTA with release of K^+ [3]. Although EGTA and EDTA at similar concentrations appear to have indistinguishable effects on gross morphology and adenine nucleotide content of liver mitochondria, there appear to have been no attempts to make any sort of systematic comparison of the two with respect to metabolic processes. We now report differences between EGTA and EDTA which came to light because of our inability to reproduce the results of Yamazaki and Graetz [4] and Cohen et al. [5], who found that rates of citrulline synthesis in uncoupled liver mitochondria supplied with exogenous ATP are considerably higher than in coupled mitochondria in which endogenous ATP provides the energy source. If true, this would imply that there are constraints on the synthesis of citrulline in coupled mitochondria that are unrelated to the concentration of *N*-acetylglutamate, the activator of carbamoyl-phosphate synthase that is generally considered to control synthesis of citrulline [6]. One, apparently minor, difference between our incubation conditions and those of Yamazaki and Graetz [4] and Cohen et al. [5] was that their incubations contained EDTA, whereas we used EGTA. This proved to be the explanation for the discrepant results. Liver mitochondria isolated and incubated in the presence of EDTA appear to have a defect in re-phosphorylation of endogenous ADP and/or AMP, which manifests itself when demand for ATP is high and which is not overcome by excess Mg^{2+}.

Materials and Methods

Fed male rats (200–250 g) were killed by cervical dislocation and their livers were removed into ice-cold 0.3 M mannitol containing 5 mM Hepes adjusted to pH 7.4 with KOH. The liver was divided into three equal parts. Mitochondria were isolated by the rapid method described by Kun et al. [7] in three different media: 0.3 M mannitol/5 mM Hepes; 0.3 M mannitol/5 mM Hepes/1 mM EGTA; and 0.3 M mannitol/5 mM Hepes/1 mM EDTA, all at pH 7.4. Incubation, or storage of the suspensions, was in the same medium.

Citrulline synthesis. For 'coupled' conditions final concentrations were: 10 mM NH_4Cl, 5 mM succinate, 10 mM ornithine, 10 mM KH_2PO_4/K_2HPO_4 (pH 7.4) and 20 mM $KHCO_3$. 'Uncoupled' conditions included, in addition, 2 mM ATP, 0.4 μM carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) and 6 μg/ml oligomycin. Stock solutions of the latter two compounds were dissolved in ethanol, pipetted into the

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flask and evaporated to dryness before adding the other components.

Pyruvate carboxylation. Final concentrations 10 mM KH_2PO_4/K_2HPO_4 (pH 7.4), 5 mM pyruvate and 20 mM $KHCO_3$.

Incubations were in 25 ml Erlenmeyer flasks. Substrates (excluding $KHCO_3$) were made up to 1.6 ml with the respective mannitol/Hepes medium to give a final concentration of 0.23 M mannitol and 0.78 mM EDTA or EGTA in the incubation. Flasks were equilibrated in a shaker bath to 37° C before addition of 0.2 ml mitochondrial suspension, followed after 1 min by 0.2 ml 0.2 M $KHCO_3$ containing about 6000 dpm $NaH^{14}CO_3$ per μmol. Flasks were gassed immediately with O_2/CO_2 (95 : 5%), stoppered and incubated for exactly 5 min. Then 0.2 ml 60% $HClO_4$ was added, flasks were restoppered immediately and transferred to a hood. A portion of acid supernatant (0.5 ml) was transferred to a scintillation vial, heated to 70° C for 60 min to eliminate unreacted HCO_3^- before scintillation counting. The remaining acid supernatant was neutralized with KOH for assay of metabolites.

For the experiments in Table IV, the flask contents were made up to 1.7 ml with the respective mannitol medium and the reaction was started with 0.3 ml mitochondrial suspension. The incubation time was 5 min in O_2.

A loss of counts from $NaH^{14}CO_3$ was noticed in the vial containing the scintillation fluid in routine use (Optiphase 'Safe'; Fisons, Loughborough, U.K.). This occurred when the sample of freshly prepared 0.2 M $KHCO_3/NaH^{14}CO_3$ was counted to determine specific radioactivity of the starting material for each experiment. The problem was overcome by adding alkali (0.5 ml 1 M NaOH) to the vial before the sample; dpm were then stable, and more than 20% higher than when alkali was omitted.

A sample of each fresh mitochondrial suspension was deproteinized and neutralized for assay of adenine nucleotides. The remaining suspension was stored in a stoppered tube for 24 h at 4° C when the procedure was repeated.

Mitochondrial protein, corrected for reaction of the reagent with mannitol [8], was determined on each suspension by the biuret method. The protein content ranged from 15–20 mg/ml in the separate experiments.

ATP [9], ADP and AMP [10] were determined enzymically, using either a Zeiss spectrophotometer or an Aminco DW-2 double-beam spectrophotometer. Citrate [11], malate [12] and fumarate, after addition of fumarase to the cuvette on completion of the malate assay, were also determined. The citrate assay was modified by omitting lactate dehydrogenase so that there was no interference from residual pyruvate; the modified assay gave a quantitative recovery of a citrate standard, showing negligible decarboxylation of oxaloacetate to pyruvate when formed by citrate lyase.

Enzymes and co-enzymes were obtained from Boehringer (London, U.K.); mannitol (Analar) was from BDH Chemicals (U.K.) and $NaH^{14}CO_3$ was from Amersham International (U.K.).

Results

Synthesis of citrulline: EGTA versus EDTA

The capacity of isolated mitochondria to synthesize citrulline gives an assessment of the efficiency of energy coupling because of the high rates that are required to correlate with maximum rates of urea synthesis in the intact hepatocyte (approx. 4 μmol/min per g wet wt.) [13] at 2 ATP/mol citrulline synthesized.

In preliminary experiments we compared the methods of Charles et al. [14] and McGivan et al. [15] on which subsequent methods have been based. Under so-called coupled conditions succinate was superior to glutamate as an energy source, added ATP and Mg^{2+} had no effect, and the highest rates were obtained when a final concentration of 0.2 M mannitol, rather than KCl, constituted the bulk phase. Conditions that gave maximum rates in the EGTA-containing suspension (see Materials and Methods) were chosen for comparison of citrulline synthesis in the different mitochondrial preparations. Uncoupled conditions, in which added ATP provided the energy source, included FCCP and

TABLE I

Synthesis of citrulline: EGTA vs. EDTA

Liver mitochondria were isolated and incubated in mannitol/Hepes medium in the presence or absence of chelating agents as described in Materials and Methods. Suspensions were re-assayed for capacity to synthesize citrulline after 24 h at 4° C. Rates are expressed as nmol/min per mg mitochondrial protein (means ± S.E. for five livers).

Mitochondria	Condition	Source of ATP	Mannitol	Mannitol/EGTA	Mannitol/EDTA
Fresh	'coupled'	endogenous	9.86 ± 2.9	46.9 ± 3.9	32.0 ± 3.3
Fresh	uncoupled	exogenous	22.0 ± 5.6	46.9 ± 3.7	50.0 ± 3.9
24 h at 4° C	'coupled'	endogenous	2.10 ± 0.44	39.2 ± 5.2	18.8 ± 4.0
24 h at 4° C	uncoupled	exogenous	5.30 ± 2.16	41.9 ± 3.3	41.4 ± 4.8

oligomycin which Yamazaki and Graetz [5] had found gave higher rates.

The relative rates of citrulline synthesis under different conditions are shown in Table I. Three batches of mitochondria were prepared from each liver. Incubation conditions were identical apart from the presence of EDTA or EGTA. As expected, mitochondria without a chelating agent swell, rapidly lose adenine nucleotides and are not able to maintain a high rate of citrulline synthesis. In these mitochondria, synthesis is partly restored when exogenous ATP is supplied. With EGTA, rates are high and identical, whether endogenous or exogenous ATP is the energy source. However, if EGTA is replaced by EDTA, the capacity for citrulline synthesis is lower in what are taken to be coupled mitochondria, but is restored by added ATP in the uncoupled condition.

The overall picture is the same after storage of mitochondria for 24 h at 4°C under the three different conditions. Activity is well maintained by the suspension in EGTA, irrespective of the source of ATP. The EDTA suspension appears to have lost activity, as measured under coupled conditions, but shows the same rate of citrulline synthesis in the uncoupled state as is found in the EGTA-suspension. These data show that there is little or no loss of carbamoyl-phosphate synthase or ornithine carbamoyl transferase during storage of mitochondria with 1 mM EGTA or EDTA for 24 h. However, mitochondria prepared and incubated in the presence of EDTA appear to have a defect in the re-phosphorylation of endogenous ADP that is more pronounced after storage of the suspension for 24 h at 4°C. The low activity in the suspension without a chelating agent, even when ATP is supplied, may be the consequence of the loss of structural integrity that destroys the spatial organization of carbamoyl-phosphate synthase and ornithine carbamoyl transferase [16].

Carboxylation of pyruvate: EGTA versus EDTA

Incorporation of $H^{14}CO_3^-$ into acid-stable products with pyruvate as sole substrate was measured only under conditions in which endogenous adenine nucleotide was the energy source in the three types of mitochondrial preparation; the so-called coupled conditions. As expected, incorporation was low in the absence of any chelating agent. However, fresh mitochondria prepared and incubated in the presence of EGTA or EDTA formed the same amount of acid-stable product. Incorporation was about 30% lower on storage of the EGTA suspension for 24 h at 4°C, but 50% lower after storage of the suspension in EDTA. Malate, fumarate and citrate were measured after incubation. The amount of citrate remained the same after incubation of the fresh and stored suspensions, though the amount formed in the presence of EDTA was only about one-third of that formed with EGTA, which suggests a difference in activation state of pyruvate dehydrogenase. The amount of citrate, however, represented at most 50% of the amount of malate formed in the fresh suspension. Malate was the major product with some fumarate. Others who have found citrate as a major product, included ATP in the assay system [17,18] and so the results are not directly comparable with ours. Since the ATP needed in the experiments of Table II for carboxylation of pyruvate is less than 50% of that required for the amounts of citrulline formed in the experiments of Table I, there is no great demand on the ATP-regeneration system; instead, NADH, presumably formed via pyruvate dehydrogenase, is diverted from the electron transport chain to reduce oxaloacetate to malate.

TABLE II

Carboxylation of pyruvate: EGTA vs. EDTA

Pyruvate (5 mM) as substrate under 'coupled' conditions. For details see Materials and Methods and Table I. Rates are expressed as nmol/min per mg mitochondrial protein (means ± S.E. for five livers).

Mitochondria	Mannitol	Mannitol /EGTA	Mannitol /EDTA
Fresh	4.34 ± 1.17	36.1 ± 1.5	35.0 ± 2.0
24 h at 4°C	2.02 ± 0.37	25.5 ± 3.2	16.6 ± 2.3

Losses of adenine nucleotides after 24 h at 4°C

The adenine nucleotide content of freshly isolated liver mitochondria is unaffected by the presence of chelating agent in the isolation medium (see also Ref. 8). A large proportion of the total is lost from suspensions stored without EDTA or EGTA, and 32% is lost from suspensions stored with either chelating agent (Table III). The lost adenine nucleotides appear as adenosine [8].

The distribution of endogenous ATP at the end of the citrulline synthesis experiments of Table I was as follows (nmol/mg protein, mean ± S.E. with total adenine nucleotides in parentheses): EGTA suspen-

TABLE III

Adenine nucleotide contents: EGTA vs. EDTA after 24 h at 4°C

Liver mitochondria isolated and stored in 0.3 M mannitol/5 mM Hepes (pH 7.4) and with 1 mM EGTA or 1 mM EDTA. Details are given in Materials and Methods. Values are nmol/mg mitochondrial protein (means ± S.E.) for the five suspensions prepared under each condition for the experiments in Tables I and II.

Mitochondria	Total adenine nucleotide content		
	mannitol	mannitol /EGTA	mannitol /EDTA
Fresh	13.0 ±0.7	13.8 ±0.7	14.2 ±0.5
24 h at 4°C	4.04 ± 0.65	8.62 ± 0.7	8.78 ± 0.65

TABLE IV

Adenine nucleotide distribution and oxidation of succinate: EGTA vs. EDTA

Succinate (5 mM) and P_i (10 mM) present under all conditions. $MgCl_2$ (5 mM) was added where indicated. For details see Materials and Methods. Values for adenine nucleotides are given as nmol/mg mitochondrial protein (means ± S.E. of five livers) and for product formation from succinate as nmol/min per mg protein (means ± S.E. of four livers).

Mitochondria	Additions	ATP	ADP	AMP	Total	Fumarate + malate
Mannitol/EGTA	–	8.47 ± 0.96	2.39 ± 0.51	0.55 ± 0.04	11.4 ± 0.6	35.7 ± 1.2
Mannitol/EGTA	Mg^{2+}	9.30 ± 0.81	2.42 ± 0.35	0.35 ± 0.03	12.1 ± 0.7	35.6 ± 1.3
Mannitol/EDTA	–	5.52 ± 0.05	5.07 ± 0.43	2.21 ± 0.39	12.8 ± 0.5	55.9 ± 4.3
Mannitol/EDTA	Mg^{2+}	10.2 ± 0.4	1.75 ± 0.31	0.35 ± 0.12	12.3 ± 0.2	44.0 ± 1.8

sions, fresh 6.61 ± 0.62 (11.09 ± 0.77), 24 h at 4°C 3.5 ± 0.5 (5.51 ± 0.53); EDTA suspensions, fresh 3.50 ± 0.71 (11.51 ± 1.05), 24 h at 4°C 1.62 ± 0.40 (5.62 ± 0.78). If the assumption is made that the adenine nucleotides remain in the matrix, then nucleotide content is not a reliable indicator of metabolic survival when 84% of capacity to synthesize citrulline is maintained by the 62% remaining after storage of the suspensions.

Succinate oxidation: EGTA versus EDTA

The results so far indicate that fresh or stored mitochondria are less stable in mannitol medium containing EDTA than in that containing EGTA. The question is: is there a defect in energy coupling in EDTA relative to EGTA and can it be reversed by Mg^{2+}. Measurements of the products of succinate oxidation (fumarate and malate) show that succinate is oxidized significantly faster by mitochondria prepared and incubated in EDTA than in EGTA: 55.9 versus 35.76 nmol/min per mg protein; $P = < 0.005$ (Table IV). At the same time, the higher rate of succinate oxidation is not able to maintain the adenine nucleotides as ATP, although there is no loss of total (see Table IV). Under these conditions EDTA produces uncoupling. The presence of 5 mM $MgCl_2$ appears to reverse these effects of EDTA. However, in six experiments there was no increase of citrulline synthesis by 5 mM $MgCl_2$ in either EDTA- or EGTA-containing suspensions, in spite of a shift of adenine nucleotides in favour of ATP in the EDTA condition; the patterns of adenine nucleotides were virtually identical to those in Table IV. In the uncoupled EDTA-containing suspensions, 5 mM $MgCl_2$ produced a decrease of 39.5 ± 2.54% in the rate of citrulline synthesis in six experiments.

Discussion

There has been little or no systematic comparison of the relative merits of EGTA and EDTA in stabilizing liver mitochondria so that biosynthetic processes are maintained. The implication is that the two are virtually interchangeable if EDTA-containing suspension are supplemented with excess Mg^{2+}. Certainly, there is no consistency, whether in concentration or stage of the procedure at which the chelating agent is included. The present work shows differences between the two that are evident when demand for ATP is high. The differences are more pronounced after storage of suspensions at 4°C. Limited conditions of incubation have been examined to allow simultaneous comparisons of mitochondria from the same liver. However, the conditions chosen gave rates comparable with the highest published for citrulline synthesis [19] and carboxylation of pyruvate [20] under coupled conditions in mitochondria from normal fed rats. Under these conditions EGTA is more effective than EDTA in preserving energy-linked function.

Although synthases require a divalent metal ion, i.e., Mg^{2+}, and carbamoyl-phosphate synthase requires also free Mg^{2+} [6], depletion of Mg^{2+} by EDTA does not appear to explain the results. Firstly, under uncoupled conditions in which added ATP is the energy source, the same high rate of citrulline synthesis occurs in EDTA- and EGTA-containing suspensions and it is unlikely that FCCP and oligomycin could protect against chelation of Mg^{2+} by EDTA. Secondly, added $MgCl_2$ (5 mM) does not affect the rate of citrulline synthesis in the 'coupled' condition and inhibits by 40% in the uncoupled condition. Thirdly, pyruvate carboxylation, which also requires MgATP, is not inhibited by EDTA.

These experiments raise questions on the molecular mechanisms in stabilization of mitochondria. The protective effects of EDTA or EDTA depend on the continuous presence of a chelating agent [8]. This argues against chelation of Ca^{2+} as the primary effect. There may be freely reversible associations with positively charged groups on the inner membrane which hinder the morphological changes, by analogy with the stabilization of mitochondria by polyamines from their electrostatic association with the anionic components of protein and of phospholipid headgroups (see Ref. 21). Modulation of the surface charge could influence membrane-bound enzymes. In our experience, liver

glutaminase which is loosely associated with the inner membrane, is a very sensitive indicator of mitochondrial morphology; activity is decreased in relation to the extent by which EDTA, EGTA, certain local anaesthetic agents, sulphydryl compounds, or spermine inhibit P_i-induced increases in matrix volume. EDTA is reported to stimulate glutaminase [22], but the mitochondrial suspensions already contained EGTA. Although glutaminase activity is relatively low in mitochondria-containing chelating agents, it is higher in EDTA than in EGTA (Lund, P. and Wiggins, D., unpublished experiments). Furthermore, Azzi and Azzone [23] observed that 'low amplitude' swelling of liver mitochondria containing EDTA occurred on addition of P_i. The effect was much smaller in EGTA. These different effects of EDTA and EGTA on mitochondrial morphology, as measured by light scattering and activity of glutaminase, may or may not be related to chelation of Mg^{2+}.

The experiments cast some light on the phenomenon of mitochondrial ageing, which is generally defined as a loss of energy-linked functions. It can occur very rapidly under the influence of various swelling agents [24], or more slowly on storage at low temperature [25], It is curious that the capacity for citrulline synthesis, with its high energy demand, is maintained in EGTA-containing suspensions in spite of the loss of over 30% of total adenine nucleotides that occurs after 24 h at 4°C. Adenine nucleotide content is obviously not a reliable indicator of metabolic survival.

In view of the data presented in this paper we suggest that more importance should be given to the concentration and form of chelating agents for experiments in liver mitochondria.

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