KINETIC EVIDENCE FOR CALCIUM-ION AND PHOSPHATE-ION TRANSPORT SYSTEMS IN MITOCHONDRIA FROM EHRLICH ASCITES TUMOUR CELLS

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1. Introduction

Calcium ions accumulated by tightly-coupled mitochondria from Ehrlich ascites tumour cells, fail to uncouple oxidative phosphorylation [1]. As a consequence, a number of energy-linked reactions in these mitochondria also are unaffected by high concentrations of Ca^{2+} [2–4]. Especially significant is the finding that aspects of this resistance to Ca^{2+} are seen as well in mitochondria isolated from several ascitic and solid tumours but not in those from rapidly-dividing cells of regenerating rat liver [5].

The purpose of the present study was two-fold; first to establish by a kinetic analysis [6] whether a Ca^{2+} transport system exists in the ascites tumour mitochondria similar to that in mitochondria from normal mammalian cells (cf. [7]) and second, to determine if any properties of this transport system might be sufficiently abnormal as to contribute to the observed resistance of the tumour mitochondria to the accumulated Ca^{2+}.

2. Experimental

Ehrlich ascites tumour cells were maintained and propagated as described elsewhere [8]. Mitochondria

were isolated from these cells by a modification [3] of the method of Wu and Sauer [9].

Initial rates of Ca^{2+} transport were measured using the EGTA-Ruthenium red** procedure described by Reed and Bygrave [10,11]. In experiments measuring the dependence of initial velocity on the concentration of free Ca^{2+}, the latter values were established accurately using Ca^{2+}/nitrilotriacetic acid buffers (11).

Initial rates of phosphate transport were measured by the method of Coty and Pedersen [12] except that inorganic phosphate was measured by the procedure of Baginski et al. [13].

Materials were obtained from the sources indicated previously [1–3,5]. All experiments were carried out at least three times with a high degree of reproducibility.

3. Results

The following points were established in preliminary experiments: (a) the initial rate of Ca^{2+} transport bore a linear relation to the concentration of mitochondria present indicating that neither the capacity of the carrier nor that of the mitochondria were rate-limiting under the experimental conditions employed, (b) complete inhibition of Ca^{2+} transport occurred at an EGTA concentration of about 1 mM (K_i approx. 80 μM) (see Experimental). Concentrations of EGTA as high as 30 mM ie. that employed to quench Ca^{2+} transport, had no adverse effect on the normal functioning of the mitochondria. (c) The presence of 2 mM succinate was a suitable energy source for Ca^{2+} transport; in the presence of 1 μM CCCP the initial rate of Ca^{2+} transport was reduced by approximately 90%.

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** *Abbreviations*: EGTA, ethanedioxy-bis-(ethylamine)-tetraacetic acid; Hepes, 2-(N-2-hydroxyethyl-piperazin-N'-yl) ethanesulphonic acid; CCCP, carbonyl cyanide m-chlorophenylhydrazone; p-MB, p-chloromercuribenzoate.

3.1. Time course of Ca^{2+} transport and influence of Ca^{2+} concentration

The time course of Ca^{2+} transport by ascites tumour mitochondria is shown in fig.1A. In the absence of added phosphate (lower curve) a steady increase in Ca^{2+} in the EGTA-inaccessible space (see [10]) occurs which is still continuing after 60 sec. The presence of 0.5 mM phosphate greatly increases the rate of transport and diminishes considerably the time (to about 30 sec) at which the mitochondria have transported all the available Ca^{2+}.

Data in fig.1B show the influence of Ca^{2+} concentration on the initial rate of Ca^{2+} transport in the absence and presence of added phosphate. In this experiment the concentration of free Ca^{2+} was controlled with the buffer nitrilotriacetic acid (see [11]). The

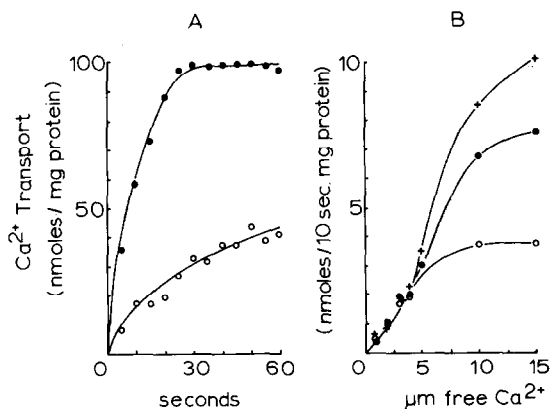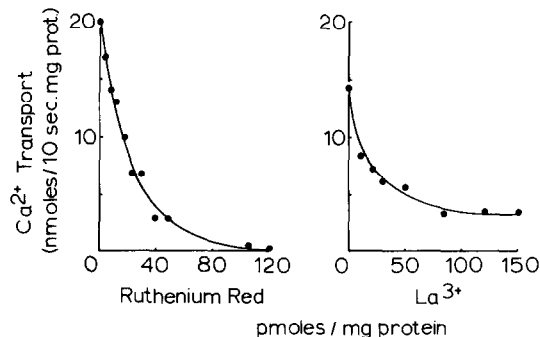Fig.2. Effect of Ruthenium red and La^{3+} concentrations on the initial rate of Ca^{2+} transport. The incubation media contained 250 mM sucrose, 2.5 mM Hepes, 2 mM succinate and other additions as indicated. The final vol was 0.2 ml. Mitochondria (0.2 mg protein) were added and after 1 min. 100 μM $^{45}Ca^{2+}$ added. The incubation mixtures were quenched after 10 sec. by the addition of Ruthenium red plus EGTA. After centrifuging this mixture the supernatant was measured for radioactivity. Incubation temp., 10°C.

Fig.1. Effect of time and Ca^{2+} concentration on transport of Ca^{2+} by ascites tumour mitochondria. In each experiment the incubation media contained 250 mM sucrose, 2.5 mM Hepes (pH 7.4) and 2 mM succinate. For fig.1A the total vol was 2.0 ml. Mitochondria (4 mg protein) were preincubated for 1 min. prior to the addition of 100 μM $^{45}Ca^{2+}$. At the times shown 100 μl portions were removed and added to Eppendorf centrifuge tubes containing 2 μM Ruthenium red and 30 mM EGTA. The mitochondria were separated by centrifuging and 50 μl of the supernatant assayed for radioactivity. Incubation temp., 10°C. (○), Phosphate absent; (●), Phosphate (0.5 mM) present. For fig.1B, incubations also contained 10 mM nitrilotriacetic acid in a total vol of 0.2 ml. Mitochondria (0.2 mg protein) were preincubated for 1 min. prior to adding Ca^{2+} in a (total) concentration range from 0.12 mM to 1.8 mM. Incubations were for 10 sec at 10°C. The reactions were rapidly quenched with the Ruthenium red plus EGTA, the mitochondria collected on Gelman filters (0.45 μM pore size), washed with 10 ml of cold 250 mM sucrose plus 2.5 mM Hepes, dried and counted for radioactivity. (○), Phosphate absent; (●) 50 μM Phosphate; (+) 200 μM Phosphate.

following features of the data are noteworthy: the curve relating Ca^{2+} concentration and initial velocity is sigmoidal in shape (cf. [6,14,15]), the K_M for free Ca^{2+} is approx. 5 μM (cf. [6,14,15]), the system exhibits saturation kinetics and finally, added phosphate increases the maximum velocity several-fold (see also fig.3 below).

3.2. Influence of inhibitors of Ca^{2+} transport

The effect of increasing concentrations of the Ca^{2+} transport inhibitors Ruthenium red [16] and La^{3+} [17] on the initial rates of Ca^{2+} transport in tumour mitochondria are shown in fig.2. Each compound prevents Ca^{2+} transport when present at quite low concentrations. With Ruthenium red, inhibition is complete at approx. 80–100 pmol per mg protein; the K_i is approx. 15 pmol per mg protein. La^{3+} is also a potent inhibitor of Ca^{2+} transport by the tumour mitochondria (K_i approx. 12 pmol per mg protein) but the inhibition remains only about 75% effective even at a concentration of 150 pmol La^{3+} per mg protein. The values obtained for the K_i for each inhibitor are similar to those found with rat liver and blowfly flight-muscle mitochondria [10,18].

3.3. Influence of concentration of permeant anions on the initial rate of Ca^{2+} transport
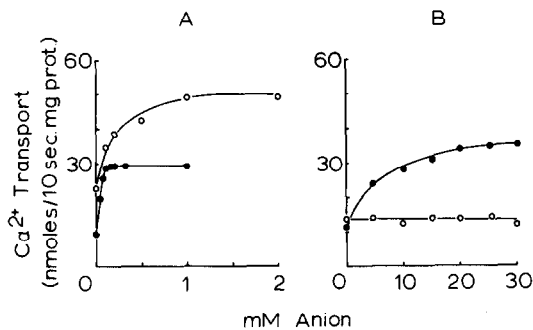
Data in fig.3A show how the initial rate of Ca^{2+}

Fig.3. Effect of anion concentration on the initial rate of Ca^{2+} transport. In fig.3A the reaction was carried out exactly as described for fig.2 but with liver (o) or tumour (•) mitochondria in the presence of increasing concentrations of inorganic phosphate. In fig.3B the reaction was carried out as in fig.2 but with the tumour mitochondria and the indicated concentrations of acetate (•) or thiocyanate (o).

transport changes with increasing concentrations of added phosphate. Since comparable data for rat liver mitochondria are not available in the literature, this is also provided. In the absence of added phosphate, the initial rate of transport in the tumour is about 50% of that observed with rat liver mitochondria. Addition of only quite low concentrations of phosphate markedly stimulates Ca^{2+} transport in the tumour mitochondria the K_M for phosphate is approx. 60 μM and the maximal stimulation is about 3-fold. With rat liver mitochondria there is still a pronounced stimulation of Ca^{2+} transport (see also [6]) but the affinity for phosphate is less (K_M approx. 220 μM) and the maximal stimulation is about 2-fold.

Addition of acetate to the tumour mitochondria, like phosphate, stimulates the initial rate of Ca^{2+} transport. However as found with rat liver mitochondria [6] much higher concentrations of the anion are required. This difference is most likely attributable to the absence of a specific transport system in mitochondria for acetate. The apparent K_M for acetate is approx. 6 mM or some 100-fold greater than for phosphate; Ca^{2+} transport is again stimulated about 3-fold. Thiocyanate on the other hand has no effect on the initial rate of Ca^{2+} transport. Like acetate, this anion enters the mitochondria by diffusion but unlike acetate and phosphate, it penetrates as the dissociated species SCN^- [19] and not as a neutral weak acid.

3.4. Transport of phosphate by mitochondria from Ehrlich ascites tumour cells

Since the initial rate of Ca^{2+} transport was found to be stimulated by exceptionally low concentrations of inorganic phosphate (see fig.3) it was of interest to examine the kinetics of phosphate transport by these mitochondria. Data in fig.4A show first that inorganic phosphate is present in the tumour mitochondria (ie. that at zero time) at a concentration similar to that in rat liver mitochondria [12] and second that the anion is rapidly accumulated by the tumour mitochondria in a process that is inhibited by the phosphate transport inhibitor p-MB [20]. Fig.4B shows the results of an experiment in which the initial rate of phosphate transport was measured as a function of phosphate concentration. The non-linear relationship and saturability observed are seen also with mitochondria from rat liver ([12], R.F.W.T. and F.L.B., unpublished). As well the affinity for phosphate (K_M approx. 0.8 mM) is significantly less that found with rat liver mitochondria (K_M approx. 1.8 mM; 12, R.F.W.T. and F.L.B., unpublished).
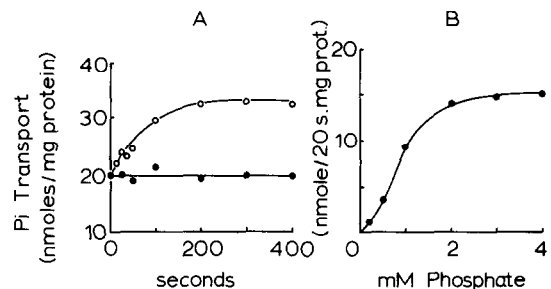

Fig.4. Effect of time and phosphate concentration on the transport of phosphate by ascites tumour mitochondria. Reaction mixtures contained 250 mM sucrose, 2.5 mM Hepes (pH 7.4) and mitochondria at a concentration of 5 mg protein per ml. In experiment 4A mitochondria were preincubated for 1 min. in the absence (o) or presence (•) of 200 μM p-MB prior to the addition of 4 mM phosphate. At the times indicated, 200 μl of the reaction mixture were removed and added to Eppendorf tubes containing p-MB at a final concentration of 200 μM. The tubes were centrifuged and the pellets washed with sucrose-Hepes-p-MB, dissolved in 0.5 ml of 10% trichloroacetic acid and phosphate determined. Total vol, 2.0 ml. Temp., 0°C. In fig.4B phosphate was added at the concentrations indicated and the net amount of phosphate accumulated after 20 sec was determined.

4. Discussion

The kinetic information obtained in this study provides strong evidence that a specific transport system for Ca^{2+} exists in mitochondria isolated from Ehrlich ascites tumour cells. Moreover all of the features of this transport system examined were found to be identical with those seen in most mammalian mitochondria. These included the high affinity for Ca^{2+}, the non-linear relation between initial rates of Ca^{2+} transport and Ca^{2+} concentration, saturability, high sensitivity to Ruthenium red and La^{3+} and stimulation by phosphate and acetate (summarised in [21]). The kinetic data also provide evidence for the existence in the tumour mitochondria of a specific transport system for phosphate similar to that found in rat liver mitochondria (cf. [12]). Of particular interest was the finding that the ascites tumour mitochondria have a higher affinity for phosphate than rat liver mitochondria both for stimulating Ca^{2+} transport (fig.3) and for its own transport (fig.4).

The most important deduction to be made from this study is that because the properties of the Ca^{2+} transport system in liver and tumour mitochondria are practically similar, the resistance of the tumour mitochondria to uncoupling by Ca^{2+} must involve factors other than the entry of Ca^{2+} into the mitochondria. As the mechanism(s) underlying such uncoupling in rat liver mitochondria are still largely unknown, so are the factors which allow tumour mitochondria to resist this ion. However a fruitful answer to this question will be provided more readily by studying the interaction of Ca^{2+} with both ascites tumour and liver mitochondria.

Acknowledgement

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