DEVELOPMENT OF MITOCHONDRIAL CALCIUM TRANSPORT ACTIVITY IN RAT LIVER

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1. Introduction

A fundamental property of mitochondria isolated from a range of tissues and species is an ability to efficiently accumulate Ca^{2+} [1,2]. That this process plays an important physiological role in the control of cell Ca^{2+} in vivo [3,4] has received impetus from studies showing that Ca^{2+} transport in liver mitochondria might be a target for hormone action in vivo [5,6], that Ca^{2+} transport in tumour mitochondria may to some extent be deranged [7–9] and that Ca^{2+} transport in mitochondria isolated from flight-muscle of the blowfly changes markedly with development [10].

These latter findings in particular, led us to consider the possibility that mitochondrial Ca^{2+} transport changes also during development of mammalian tissue. Some evidence for this was provided in the reports of Nakazawa et al. [11] and Pollak [12] in which the appearance of energy-linked functions in mitochondria was examined during development of rat liver. It was shown that Ca^{2+}-induced H^+ efflux [11] and Ca^{2+}-induced respiration [11,12] was considerably dampened in foetal mitochondria.

We have extended these observations by (a) examining mitochondrial Ca^{2+} transport over a broad span of development, (b) employing techniques that directly measure movement of the ion into the matrix space of the organelle and (c) characterising aspects of the Ca^{2+} transport system in mitochondria from foetal liver. Our data show that Ca^{2+} transport activity in liver mitochondria undergoes major changes around the time of birth; quite low activities exist 2–3 days before birth and near-maximal activities are expressed within 1–2 days of adult life especially following preincubation of the mitochondria in vitro with Mg.ATP.

Abbreviations: Hepes: 2-(*N*-2-hydroxyethylpiperazin-*N*-yl) ethane sulphonic acid; EGTA: ethanedioxybis-(ethylamine) tetra-acetic acid

2. Experimental

Wistar strain albino rats were used in this study. Generally 1 male rat was housed with 3 female rats for a period of 24 h. Young rats were killed by decapitation while older rats were first stunned and then killed by cervical dislocation.

Preparation of mitochondria: Livers from foetal and neonatal rats were rapidly excised and pooled in ice-cold homogenising medium (250 mM sucrose, 2.5 mM Hepes and 0.5 mM EGTA (pH 7.4), with KOH). The livers were minced with scissors and washed several times to remove excess blood. Those from foetal and young animals were homogenised by hand (10% homogenate) in a Thomas B glass homogeniser using 5 up-and-down strokes of a Teflon pestle. These were then diluted to 5% prior to centrifugation. Adult livers were homogenised in a Thomas C glass homogeniser using one passage of a motor-driven pestle. All preparations were centrifuged (Sorvall RC-2B) at 750 × *g* for 10 min to remove cell debris and nuclei. The supernatant from this step was centrifuged at 13 500 × *g* for 10 min and the pellet obtained resuspended in 10–30 ml (depending on age) of the above medium but minus EGTA. This suspension was centrifuged at 750 × *g* for 10 min and the resulting supernatant further centrifuged at 13 500 × *g* for 10 min. The pellet obtained was finally resuspended in the sucrose-Hepes medium to

give a final protein concentration of between 10 and 60 mg/ml, the low concentrations being unavoidable in preparations from prenatal animals. Mitochondrial protein was estimated using a modified biuret procedure [13] with bovine serum albumin as standard. Acceptor control ratios were usually about 9 with succinate as substrate for adult mitochondria but genarally were quite low for foetal and neonatal mitochondria as observed previously [11,12]. Ca^{2+} transport measurements: The EGTA-Ruthenium Red-quench technique [14] was employed to measure Ca^{2+} transport. Details of incubation conditions are contained in the legends to figures.

3. Results

3.1. *Ca^{2+} transport in mitochondria isolated from foetal and adult liver*

The rates of Ca^{2+} transport obtained with mitochondria isolated from adult and 1 day prenatal liver are compared in fig.1 both in terms of the basal (control) rate and following a preincubation with 2 mM Mg.ATP (see below). Activity in the mitochondria from the younger liver is considerably less than in that from adult liver. On the other hand, preincubation with Mg.ATP, which greatly improves several energy-linked functions in foetal liver mitochondria [11,12], leads to a significant enhancement also in the ability of these mitochondria to transport Ca^{2+}, an effect that occurs to only a marginal extent in mitochondria from adult liver (see below).

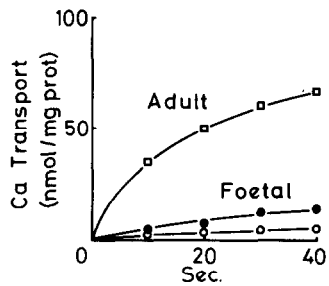

Fig.1. Ca^{2+} transport by mitochondria isolated from adult and foetal livers. Reaction mixtures contained: 250 mM sucrose, 2.5 mM Hepes buffer (pH 7.4), 2 mM succinate and where indicated 2 mM Mg.ATP. Mitochondria were added and the preincubation allowed to proceed for 1 min at which time 100 nmol Ca^{2+} (containing 0.2 μCi $^{45}Ca^{2+}$) was added. At the times indicated 100 μl samples were rapidly transferred into Eppendorf tubes containing 50 μl of ice-cold EGTA (0.5 mM) and Ruthenium Red (200 μM). After centrifuging (Eppendorf Microfuge for 2 min), a 50 μl sample of the supernatant was counted for radioactivity. Reaction vol., 1.0 ml; reaction temp., 25°C. Adult mitochondria (□); 1 day prenatal mitochondria without (○) and with (●) 1 min preincubation with 2 mM Mg.ATP.

3.2. *Ca^{2+} transport in mitochondria during liver development*

Data in fig.2 were collected from a number of individual experiments in which Ca^{2+} transport in mitochondria isolated from rat liver at the ages indicated was measured as shown in fig.1 with and without a preincubation involving Mg.ATP. The amount of Ca^{2+} transported at 60 sec was used as a measure of transport ability although initial rate measurements gave qualitatively similar data (latter data not shown). At the time of birth there exists a pronounced increase in Ca^{2+} transport in control mitochondria that levels off after about 2 days and thereafter gradually increases to attain adult capacities. Ca^{2+} transport following a 1 min preincubation with Mg.ATP is greater throughout development (fig.2). Particularly evident is that the stimulation is more marked in perinatal mitochondria than in those from adult animals. When account of this is taken, it is clear that Ca^{2+} transport in mitochondria from 1–2 day old rat livers has already reached the rates seen in adult liver. In numerous other experiments, measurement of respira-

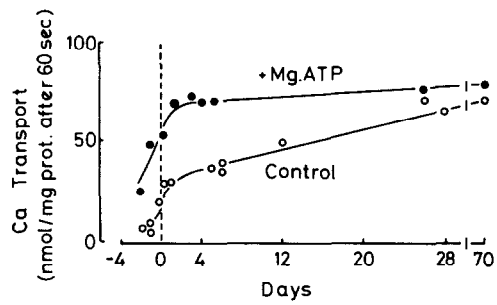

Fig.2. Ca^{2+} transport by mitochondria isolated from livers at different stages of development. Ca^{2+} transport was measured as described in fig.1 in mitochondria isolated from livers at the indicated age with (●) and without (○) a 1 min preincubation.

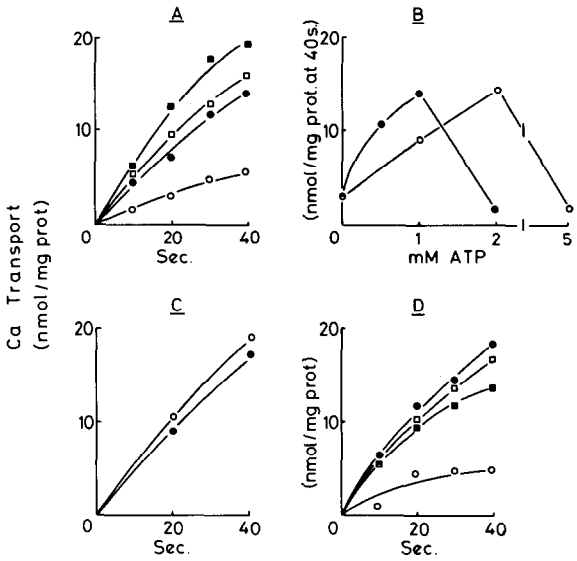

Fig.3. Properties of the Mg.ATP effect on foetal mitochondria. Reactions were carried out as described in fig.3 with mitochondria from livers of 1 day prenatal animals and with the following variations: Graph A. The time of preincubation of mitochondria with 2 mM Mg.ATP was control (○), 5 sec (●), 2 min (□), 5 min (■). Graph B. The concentration of ATP was varied with 1 mM (●) or 2 mM Mg^{2+} (○) during a fixed preincubation time of 1 min. Graph C. Atractyloside (40 μM) and oligomycin (5 μg/ml) together were present (●) or absent (○) during the 1 min preincubation with 2 mM Mg.ATP. Graph D. ATP (●) was replaced by 2 mM ADP (□) or 2 mM GTP (■). No nucleotide present (○). Mg^{2+} was present at 2 mM.

tion revealed that the perinatal mitochondria were reasonably intact as judged from acceptor control ratio values.

3.3. *Properties of Mg.ATP induction of Ca^{2+} transport*

Data in fig.3 summarise aspects of the effect of Mg.ATP on Ca^{2+} transport in 20–21 day foetal (i.e., 1–2 day prenatal) mitochondria. The effect is time-dependent (fig.3A) with about 100% stimulation induced after only 5 sec exposure of mitochondria to the complex before addition of Ca^{2+}. Thereafter the degree of stimulation slowly increases further to approach about 200% after 5 min of preincubation.

Maximal effects of Mg^{2+} and ATP are produced when they are present at equimolar concentrations (fig.3B). Other experiments have shown that little difference in the magnitude of the response occurs between 1 and 5 mM Mg.ATP.

Data in fig.3C reveal that when atractyloside and oligomycin (compounds that prevent transport of ATP into mitochondria and hydrolysis of ATP via ATPase, respectively) are present during the preincubation, little alteration in the stimulatory effect is observed.

Finally, data in fig.3D indicate that ADP and GTP have a similar effect to ATP in the presence of equimolar amounts of Mg^{2+} (cf. ref. [12]); GDP produced a similar effect to the other nucleotides (data not presented). No other nucleotides were tested.

4. Discussion

This work clearly reveals by direct measurement, that mitochondrial Ca^{2+} transport undergoes significant changes during liver development. The most pronounced change occurs in the perinatal period when the activity increases approximately 3–5-fold, depending on the absence or presence of Mg.ATP, between 1–2 days before and 1–2 days after birth. At this time also the pre-incubation effects of Mg.ATP on Ca^{2+} transport were particularly prominent, so much so that by as early as 1–2 days after birth, a mitochondrial Ca^{2+} transport activity, virtually indistinguishable from that of adult mitochondria, is expressed (fig.2).

Although our data reveal that mitochondrial Ca^{2+} transport increases several-fold over the perinatal period, they do not permit the conclusion that this reflects exclusively an increase in Ca^{2+} carrier activity. It is likely that a proportion of the increase is attributable to an enhanced ability of the mitochondria to generate and maintain the energy for Ca^{2+} transport (see below). Thus it is possible that the Ca^{2+} carrier complex itself is fully functional by 1–2 days before birth, but that the inadequacy at this time of the mitochondrial energy transducing system, prevents full expression of its activity.

The characteristics of the Mg.ATP effect on mitochondrial Ca^{2+} transport (fig.3) together with its near absence in adult mitochondria (fig.2), lead us like previous workers [11,12,15], to believe that the stimulation involves interactions of the chelate with a yet unidentified site(s) on the external surface of the

inner mitochondrial membrane and/or is in some way related to the low adenine nucleotide content of foetal liver mitochondria [16]. As pointed out by Pollak [12] the latter observation in turn may be critical in a maturation process that leads eventually to an H^+ impermeable membrane [17] which then allows for efficient energy transduction including mitochondrial Ca^{2+} transport. The role of purine nucleotides in making brown fat mitochondria less permeable to H^+ [18] also may be of some relevance to these ideas.

As mentioned in the Introduction, recent work has provided evidence that mitochondrial Ca^{2+} transport might be a site for insulin [5] and glucagon [6] action in vivo. It seems quite significant in this regard that that at the time of birth marked changes in the plasma levels of both insulin [19] and glucagon [20] occur in the rat. These interrelationships clearly merit further study.

Finally, an informative comparison may now be initiated between the present study on rat liver and previous studies from this laboratory on Ca^{2+} transport in mitochondria of the developing sheep blowfly *L. cuprina* [10,21]. In the latter system, Ca^{2+} transport not only exhibits (kinetic) properties similar in many respects to those of mammalian mitochondria, but it too undergoes a pronounced increase in activity at the time of emergence. It seems reasonable to suggest from this analogy (see also ref. [1]) that Nature at least, regards mitochondrial Ca^{2+} transport to be of sufficient importance in cell physiology and development, to warrant its being conserved in the course of evolution.

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