Changes in the Response of Mitochondrial Calcium Transport to Exogenous Phosphate during Development in Flight Muscle of the Sheep Blowfly *Lucilia cuprina*

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(Received 8 June 1977)

1. Ca^{2+} transport by mitochondria isolated from flight muscle of the sheep blowfly *Lucilia cuprina* does not occur in the absence of added P_i . Maximum rates of transport are attained when about 2.5 mM-phosphate is present. 2. As mitochondria develop, high but not low phosphate concentrations begin to inhibit Ca^{2+} transport markedly; those isolated from 2-day-old flies, for example, are inhibited by about 75% by 20 mM-phosphate. Maximum rates of transport, i.e. those measured in the presence of 2.5 mM-phosphate, begin to decline only when the fly is about 3 days old. 3. Mitochondrial phosphate transport activity does not change during development of the blowfly, but the endogenous concentration of the anion does. At emergence it is about 6 nmol/mg of protein, increases to about 17 nmol/mg of protein at 2–3 h and then rapidly declines to reach less than 5 nmol/mg of protein after 2 days of adult life. 4. Studies on the effect of phosphate on oxidation of α -glycerophosphate in the absence and presence of ADP reveal a lack of inhibition by high phosphate concentrations indicating that the anion does not influence Ca^{2+} transport by preventing the generation of the proton electrochemical gradient across the inner membrane. 5. It is concluded that the molecular assembly in the inner membrane of *Lucilia* mitochondria responsible for transporting Ca^{2+} is fully developed at emergence and remains so for at least 2–3 days of adult life. The possibility exists that Ca^{2+} -transport activity in these mitochondria is controlled at least in part by P_i .

An earlier study from this laboratory provided evidence for a Ca^{2+} -transport system in mitochondria from flight muscle of the sheep blowfly *Lucilia cuprina* (Bygrave *et al.*, 1975). The study also showed that the ability of *Lucilia* mitochondria to transport Ca^{2+} depends critically on the stage of mitochondrial development; Ca^{2+} transport was shown to be minimal in pre-emerged adults, maximal by 1–2 h after emergence, and thereafter rapidly declined to reach less than 20% of the maximum by 2–3 days after emergence.

The potential of this system for obtaining information on aspects of mitochondrial Ca^{2+} transport has induced us to examine further the relation between Ca^{2+} transport and mitochondrial development. Since P_i has been long recognized as a major effector of mitochondrial Ca^{2+} transport (see Lehninger *et al.*, 1967; Bygrave, 1977), we investigated in some detail its influence on Ca^{2+} transport during development. The experiments reported here reveal that when low concentrations (about 2.5 mM) of P_i are used instead of

high concentrations (25 mM), i.e. those that had been previously used (Bygrave *et al.*, 1975), transport activity remains near maximal for up to about 3 days after emergence. By contrast, phosphate-transport activity does not change during development, but the endogenous concentrations of the anion are high immediately after emergence and gradually fall to very low values by about 4 days after emergence. Other experiments reported here further reveal that high phosphate concentrations have little inhibitory effect on oxidation of respiratory substrates by *Lucilia* mitochondria. The mechanism by which high phosphate concentrations inhibit Ca^{2+} transport therefore is unlikely to involve the oxidation-reduction or ATP synthetase components of the inner mitochondrial membrane.

Experimental

Maintenance of flies

Flies were obtained from the Division of Entomology, CSIRO, Canberra. Rearing and maintenance procedures have been fully documented (Bygrave *et al.*, 1975).

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Isolation of mitochondria

Mitochondria were isolated from the flight muscle of flies exactly as described previously (Bygrave *et al.*, 1975). Control experiments using measurements of respiration and Ca^{2+} transport showed that the mitochondria remained intact for the duration of the experiment, which usually lasted 60–90 min (cf. Slack & Bursell, 1976a).

Mitochondrial protein concentration was measured by the biuret method (Szarkowska & Klingenberg, 1963) after solubilization with deoxycholate; KCN was added subsequently to allow corrections to be made for non-biuret colour and turbidity (Szarkowska & Klingenberg, 1963).

Ca^{2+} transport

Transport of Ca^{2+} was measured by the EGTA–Ruthenium Red-quench technique (Reed & Bygrave, 1974). The reaction medium contained, in a final volume of 1.0 ml: 100 mM-KCl, 25 mM- α -glycerophosphate and various concentrations of K_2HPO_4 as indicated. The final pH and temperature were 7.4 and 25°C respectively. Mitochondria (1.0 mg of mitochondrial protein) were added, and after a 1 min preincubation, 50 μM - Ca^{2+} (containing approx. 0.3 μCi of $^{45}\text{Ca}^{2+}$) was added to initiate the reaction. Further details are indicated in legends to Figures.

Phosphate transport

Transport of P_i was measured in a medium similar to that used for Ca^{2+} transport. The reaction medium initially contained in a final volume of 1.0 ml: 100 mM-KCl, 1 mM-Hepes,* 25 mM- α -glycerophosphate and 4 mg of mitochondrial protein. The final pH was 7.4 and the temperature 4°C. After preincubation for 1 min, a small volume of P_i (see Figure legends) was added to initiate the reaction. At intervals of 15, 30 and 45 s, a sample (250 μl) of the mixture was rapidly transferred to an Eppendorf tube containing 200 μM -*N*-ethylmaleimide to stop the reaction (see Coty & Pederson, 1974). The suspension was centrifuged (Eppendorf Microfuge for 2 min at 12000g), the supernatant carefully removed and 250 μl of 10% (w/v) trichloroacetic acid added to the mitochondrial pellet. The tube was agitated thoroughly and a further 250 μl of 10% trichloroacetic acid added. The mixture was left for 1–1½ h at 4°C, after which time it was again centrifuged as above. A sample (200 μl) of the supernatant was then assayed for P_i by the procedure of Baginski *et al.* (1967). A zero-time control was run with each incubation and the values were subtracted from the timed values. Thus the phosphate-transport values obtained in this work reflect movement of the anion into the mitochondria independent of the amount already present. The endogenous concentrations of P_i in the mitochondria were measured by a similar

procedure, but with the omission of the initial incubation involving uptake of exogenous phosphate.

No attempts were made in the Ca^{2+} - and P_i -transport experiments to estimate the error introduced into these experiments by carry-over of Ca^{2+} or P_i from the incubation medium by the medium adhering to the mitochondrial pellet.

Measurement of mitochondrial respiration

Respiration was measured exactly as previously described (Bygrave *et al.*, 1975). States 3 and 4 respiration refer to the rates of respiration observed in the presence and absence of ADP respectively (Chance & Williams, 1956).

Materials

All materials were obtained from sources previously mentioned (Bygrave *et al.*, 1975).

Reproducibility of data

Each of the experiments shown was carried out at least twice and usually three times. Agreement between experiments was within 10%.

Results

Influence of P_i concentration on Ca^{2+} transport by mitochondria isolated at different stages of development

In previous experiments from this laboratory (Bygrave *et al.*, 1975) Ca^{2+} transport by mitochondria was measured in the presence of only a single concentration (25 mM) of P_i . In the present experiments, transport was measured at different concentrations of the permeant anion and at various stages of mitochondrial development. The results (see Fig. 1) are presented in terms of both the initial rate of Ca^{2+} transport and the amount of Ca^{2+} present in the mitochondria after 5 min of incubation at 25°C (see the Experimental section). The good agreement between the two sets of data is evident.

The following features are noteworthy. (i) Little Ca^{2+} -transport activity occurred in the absence of added phosphate at all stages of development. (ii) In general, concentrations of phosphate as low as about 2.5 mM were sufficient to induce maximal or near-maximal Ca^{2+} -transport activity for any stage of mitochondrial development. (iii) In the developmental stage extending from 1 day before to 6 h after emergence, near-maximal activity was observed over a broad phosphate concentration range (2.5–25 mM); it was not until the phosphate concentration exceeded about 25 mM that any decline in activity became apparent. (iv) Mitochondria from 1-day-old and older flies exhibited a Ca^{2+} -transport activity that reached a peak sharply at 2.5 mM-phosphate. Concentrations greater or less than this resulted in consider-

Fig. 1. Effect of P_i concentration on Ca^{2+} transport by mitochondria isolated from *Lucilia* mitochondria at different developmental stages

Identical reaction media were used to measure Ca^{2+} transport (see the Experimental section) in the presence of different phosphate concentrations as shown. Each reaction vessel also contained 1.0mg of mitochondrial protein in a total volume of 1.0ml. The temperature was 25°C. Graphs (a)–(h) show data for mitochondria isolated from 1-day pre-emergence, 12-h pre-emergence, 1–2h-, 6h-, 1-day-, 2-day-, 3-day- and 5-day-old flies respectively. ○, Initial rate of Ca^{2+} transport (nmol of Ca^{2+} transported/min per mg of protein); ●, Ca^{2+} transported at 5 min (nmol/mg of protein).

ably less Ca^{2+} transport; this was seen especially with mitochondria from 2-day-old flies.

The presence of *N*-ethylmaleimide in the reaction mixture completely prevented any stimulation by phosphate of Ca^{2+} transport (results not shown). This indicates that the anion needs to enter the mitochondria via the phosphate/hydroxyl antiporter before it stimulates mitochondrial Ca^{2+} transport (Coty & Pederson, 1974). Moreover, energy-dependent accumulation of phosphate by *Calliphora* thorax mitochondria was demonstrated by Hansford & Chappell (1968).

The data in Fig. 2 are derived from those in Fig. 1 and show the relation between Ca^{2+} transport and age of the mitochondria for two different phosphate concentrations, 25 mM and 2.5 mM. The derived curve, obtained with the higher phosphate concentration, shows that Ca^{2+} transport was maximal at emergence and thereafter rapidly declined to reach about 30% of maximum activity after 1 day. By contrast, Ca^{2+} -transport activity with 2.5 mM-phosphate, was low before emergence, but maximal over the period extending from emergence to about 3 days of adult life. Mitochondrial Ca^{2+} -transport activity did not begin to decline significantly until the fly was about 5 days old.

Fig. 2. Maximum rates of Ca^{2+} transport during development measured at fixed phosphate concentrations. Ca^{2+} -transport activity in the presence of (○) 25 mM- or (●) 2.5 mM-phosphate are replotted from data in Fig. 1.

Phosphate transport by mitochondria isolated at different stages of development

Initial-rate measurements showed that P_i transport was maximal at 25 mM- P_i and did not change significantly during development (Fig. 3, upper curve).

P_i concentration in mitochondria during development

Data in Fig. 3 (lower curve) show the endogenous concentration of phosphate in mitochondria from flight muscle at different stages of development. Immediately before emergence there was a slight decrease in phosphate concentration to about 6–7 nmol/mg of protein. This was followed by a pronounced increase to about 17–18 nmol/mg of protein by about 3 h. Thereafter the endogenous phosphate concentration rapidly declined to values of about 6–7 nmol/mg of protein by 1 day. At 3 days after emergence the endogenous phosphate concentration in mitochondria had declined to 2–3 nmol/mg of protein.

The upper curve of Fig. 3 reflects the finding that

Fig. 3. Phosphate transport and phosphate content of mitochondria during development

Maximum rates of transport measured at 25 mM- P_i are presented as a function of age of the blowfly ($\circ$). Phosphate content of mitochondria was determined (see the Experimental section) at different stages of development ($\bullet$).

phosphate-transport activity in mitochondria does not change with development.

Effect of phosphate concentration on energy transduction in mitochondria

In view of the likelihood that the phosphate-transport system in these mitochondria is not responsible for the variation with age of Ca^{2+} -transport activity, it was therefore decided to study the possibility that high phosphate concentrations were influencing energy transduction and thereby interrupting the driving force for Ca^{2+} transport.

Data in Fig. 4(a) show the effect of phosphate concentration on State-3 respiration with pyruvate together with malate as substrate in mitochondria from 1 h- and 1-day-old flies. Maximum rates of respiration were observed at about 5 mM-phosphate, but further increases in the phosphate concentration did not alter the rates of respiration.

Data in Fig. 4(b) show the effect of phosphate concentration on State-3 and -4 respiration of α -glycerophosphate in mitochondria from 2-day-old flies. As in the experiment of Fig. 4(a), maximum rates of respiration were seen at about 5 mM-phosphate. Further increasing the phosphate concentration to 25 mM (State 3) or 15 mM (State 4) had no inhibitory effect. Although some inhibition of State-3 respiration was seen at 30 mM-phosphate, no direct correlation exists between the effects of the anion on respiration of α -glycerophosphate on the one hand and Ca^{2+} transport on the other.

Discussion

The effects of P_i on Ca^{2+} transport in mature mitochondria such as those isolated from liver and

Fig. 4. Influence of phosphate concentration on mitochondrial energy transduction during development

State-3 rates of respiration (a) were measured at different phosphate concentrations with 4 mM-pyruvate plus 2 mM-malate as substrate with 1 h-old ($\circ$) or 1-day-old ($\bullet$) fly mitochondria. (b) State-3 ($\bullet$) and State-4 ($\circ$) respiration and Ca^{2+} transport (Δ) were measured in the presence of 25 mM- α -glycerophosphate. State-IV respiration was measured after addition of ADP, which was added at a concentration of 100 μ M.

heart tissue of adult rats are quite fully documented, at least in qualitative terms. Much evidence supports the conclusion that the anion increases both the initial rate of Ca²⁺ transport and the capacity of the mitochondria to accumulate Ca²⁺ (reviewed by Bygrave, 1977). A second important influence of the anion on these mitochondria relates to the possible role it plays in controlling Ca²⁺ transport, either by providing an environment in the internal space of mitochondria that induces inward Ca²⁺ movement (Lehninger, 1974) or by influencing the effect of other metabolites such as phosphoenolpyruvate that influence, directly or indirectly, Ca²⁺ movement across the inner mitochondrial membrane (see, e.g., Chudapongse & Haugaard, 1973).

The present study shows that P_i also has a marked effect on Ca²⁺ transport in *Lucilia* mitochondria. In contrast with Ca²⁺ transport in mammalian mitochondria, which can occur in the absence of added phosphate, that in *Lucilia* mitochondria does not take place unless the anion is present in the incubation system; maximum rates of Ca²⁺ transport are seen in the presence of about 2 mM-phosphate. A similar dependence of Ca²⁺ transport on added phosphate was seen previously with mitochondria isolated from flight muscle of *Phormia* (Carafoli *et al.*, 1971).

Perhaps the most significant findings revealed in the present work, however, are that the degree of stimulation of transport by phosphate depends on the developmental stage at which the mitochondria are isolated and that Ca²⁺ transport in mitochondria isolated from adult flies older than about 6 h is markedly inhibited by phosphate concentrations greater than about 2 mM (Figs. 1 and 2). An important implication of this latter point is that our earlier conclusion, based on measurements of Ca²⁺-transport activity in the presence of only 25 mM-phosphate (Bygrave *et al.*, 1975), is erroneous to the extent that such activity does not decline markedly soon after emergence, but rather remains maximal for up to about 3 days of adult life when consideration is given to the observation that high phosphate concentrations are inhibitory (Fig. 2).

Whether or not the effects of P_i that bring about changes in mitochondrial Ca²⁺ transport as seen here have physiological significance remains to be determined; the high phosphate concentrations required to inhibit transport appear to be excessive at first sight. However, pertinent to this point are the studies by Hansford & Chappell (1968) and Hansford (1972), which revealed that NAD⁺-linked isocitrate dehydrogenase of *Calliphora* mitochondria exhibited an absolute requirement for phosphate, with maximum rates attained only when the concentration exceeded about 30 mM. Moreover, quite high phosphate concentrations are needed for maximum rates of ADP-stimulated pyruvate oxidation (Hansford & Chappell, 1968; Hansford, 1972; the present work;

but see Slack & Bursell, 1976b). Also pertinent is the observation of Sacktor & Hurlbut (1966) that at the onset of contraction of flight muscle of the blowfly *Phormia regina*, the concentration of P_i in the muscle increases rapidly.

The search for the mechanism by which high concentrations of the anion inhibit Ca²⁺ transport so far has been only partially successful. Our data indicate that neither the activity of the phosphate carrier itself nor the 'driving force' for Ca²⁺ transport, as revealed by measurements of respiration in the absence and presence of ADP, is involved. Nonetheless there may be a degree of significance to the observation that the endogenous concentration of phosphate in the mitochondria changes during development in a way that is not dissimilar to that of the mitochondrial Ca²⁺-transport activity (cf. Figs. 2 and 3). In any case our data would support the proposition that the molecular components in the inner membrane of mitochondria from *Lucilia* flight muscle responsible for transporting Ca²⁺ develop full activity by emergence and retain it for at least several days of adult life.

We gratefully acknowledge support for this work by the Australian Wool Trust Fund and are grateful to Mrs. A. A. Daday for carrying out the experiment described in Fig. 3.

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