THE MITOCHONDRIAL PYRUVATE CARRIER, ITS EXCHANGE PROPERTIES AND ITS REGULATION BY GLUCAGON

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1. Introduction

Earlier reports [1,2] have demonstrated the presence of a pyruvate carrier in rat liver mitochondria. The evidence was pH dependence of uptake and efflux of pyruvate suggesting exchange with hydroxide anions, saturation kinetics and the existence of a specific inhibitor, α-cyano-4-hydroxy-cinnamic acid. It was reported that phosphate and citrate anions would not exchange for pyruvate [1] but later there has been disagreement as to whether dicarboxylate anions could exchange with pyruvate across the mitochondrial membrane (cf. [3,4]). Because of this and because of the potential relevance of pyruvate/dicarboxylate exchange to the regulation of gluconeogenesis we have reinvestigated this point using a recently published technique [5]. This technique, which uses α-cyano-3-hydroxy-cinnamic acid as an 'inhibitor-stop', has the merit (in comparison with other methods) of measuring initial rates of uptake along a controlled pH gradient at temperatures near to physiological. Our results suggest that such a pyruvate/dicarboxylate exchange does occur and that it probably involves the pyruvate carrier only. Using the same technique we have also investigated the possibility suggested by others [6–8] that glucagon stimulation of gluconeogenesis in the liver in situ or in liver cells incubated in vitro may involve stimulation of pyruvate entry into the mitochondria. The suggestion was based on indirect evidence, namely increased rates of pyruvate decarboxylation and carboxylation by isolated mitochondria from liver exposed to high concentrations of glucagon. We have no shown directly that there is in fact a concomitant stimulation of initial pyruvate transport rate. Furthermore this effect does not depend on prior accumulation of dicarboxylate anions within the mitochondria. This is relevant because accumulation of dicarboxylate anions in liver mitochondria after glucagon injection into rats has been reported [9].

2. Methods and materials

Methods of preparation of mitochondria and for studying uptake and efflux of pyruvate by mitochondria were as in our earlier publication [5]. Two types of mitochondrial incubation media were used in the present work: Medium 1 contained 250 mM sucrose, 5 mM Hepes, 1 mM EGTA, 3 mM ascorbate, 0.05 mM tetramethyl-phenylenediamine, 5 μg/ml rotenone and 1 μg/ml antimycin A. Medium 2 contained 125 mM KCl, 20 mM Hepes, 2 mM $MgCl_2$, 2 mM arsenite, 5 μg/ml rotenone, 1 μg/ml antimycin A and ascorbate/tetramethylphenylenediamine as above. The pH of both media was 7. Additions to media are indicated in tables and legends. Mitochondrial pH gradient and membrane potential were determined by published methods [10]. The possibility of significant loss of pyruvate during mitochondrial uptake or efflux experiments or transformation of malate to pyruvate when the former ion was present was tested by incubating mitochondria in the medium used in these studies with addition of 1 mM pyruvate or malate. After 5 min the mitochondrial protein was precipitated with 2% perchloric acid and pyruvate was assayed [11].

For experiments involving glucagon injection a matched pair of rats was used for an experiment on a single day. After anaesthetization with ether and nembutal (60 mg/kg i.p.) and a further 20 min the rats were injected i.p. with either saline or glucagon and after an additional 30 min blood was sampled from the tail vein. The animal was killed by cutting the neck vessels and the liver removed. Measurement of the rates of [1-^{14}C]pyruvate decarboxylation was as described by Adam and Haynes [6].

Plasma glucose was measured by glucose oxidase kit (Boehringer, London).

Glucagon was from Eli Lilly, Basingstoke, U.K. Nembutal was from Abbott Laboratories Ltd., Queenborough, Kent, U.K. Sources of other materials were given earlier [5].

3. Results and discussion

3.1. *Pyruvate recovery*

Under the conditions used for experiments measuring pyruvate uptake or efflux there was no detectable loss of pyruvate during 5 min incubation of mitochondria when 1 mM pyruvate was initially present nor gain of pyruvate when 1 mM malate was initially present (3 determinations, limit of detection 40 nmol per ml). In earlier work [5] no radioactivity from [2-^{14}C]pyruvate appeared in other mitochondrial metabolites.

3.2. *Rates of pyruvate uptake and efflux in various conditions*

Applying the usual concept of membrane carriers

Table 1
Effect of various anions on initial rates of [2-^{14}C]pyruvate uptake by mitochondria

Anion tested (K^+ salts)	Procedure	Concentration of tested anion in preincubation medium (mM) during 2 min before addition of [2-^{14}C]pyruvate	Concentration of tested anion in incubation medium (mM) after addition of [2-^{14}C]pyruvate	Initial rate as % of control for each mitochondrial preparation
Succinate	a	1	1	27 ±* 1 (3)
	b	0	1	25 ±* 8 (3)
	c	1	0.05	158 ±* 4 (3)
Malate	a	1	1	36.0 ±* 8.6 (3)
	b	0	1	26.4 ±* 6 (4)
	c	1	0.05	132 ±* 12 (5)
Citrate	a	1	1	57.8 ±* 5.5 (4)
	b	0	1	82.9 ±* 6.8 (3)
	c	1	0.05	91.5 ±* 2.4 (3)
Phosphate	a	1	1	41.7 ±* 10.2 (3)
	b	0	1	33.0 ±* 8.3 (3)
	c	1	0.05	84.9 ±* 4.7 (3)

For procedures a and b medium 2 was used for both incubations. Mitochondrial concentration was 1 mg protein/ml in both cases. For procedure c medium 1 was used for preincubation and the mitochondrial concentration then was 20 mg/ml while medium 2 was used for incubation when mitochondrial concentration was 1 mg protein/ml. In every experiment we determined a control rate of [2-^{14}C]pyruvate uptake in the absence of all the other anions to be tested.
The temperature of all media was 27°C. The control rate of pyruvate uptake averaged for all experiments was 27.52 ± 1.55 [19] nmoles/mg protein/min. Numbers in brackets are numbers of mitochondrial preparations.

*p <0.05 for % differences from control rates.

to the special case of the mitochondrial membrane one can predict that to establish that another anion travels on the pyruvate carrier we should (1) show competition by the anion for uptake of pyruvate (2) show counter-transport i.e. stimulation of pyruvate movement in one direction by movement of the other anion in the other direction (3) eliminate alternative explanations for the data e.g. effects on pH gradient or use of different carriers. Table 1 (procedures a and b) shows that exposure of mitochondria to succinate, malate, citrate or phosphate at a concentration of 1 mM before and/or during incubation with 1.4 mM [2-^{14}C]pyruvate resulted in significant inhibition of initial rates of pyruvate uptake. However, results of procedure c in table 1 show that preincubation with one of the anions malate, succinate, phosphate or citrate at a concentration of 1 mM followed by 20 times dilution of the anion during the period of exposure to [2-^{14}C] pyruvate results in a clear distinction in the effects of the dicarboxylate anions from that of citrate or phosphate. Preincubation with dicarboxylate anions resulted in a significant stimulation of initial rate of [2-^{14}C]pyruvate uptake whereas preincubation with citrate or phosphate anions still led to inhibition of initial rate of [2-^{14}C]pyruvate uptake. Mowbray [3] has also noted stimulation of the rate of pyruvate uptake by the presence of low concentrations of malate in the incubation medium. We have attempted to determine how far alterations of mitochondrial pH gradient may account for the above results. We observed (data not shown) that pyruvate and malate anions each at a concentration of 1 mM did not disturb the mitochondrial pH gradient (or membrane potential) set up by the ascorbate/tetramethylphenylenediamine couple but there was a small decrease in the pH gradient due to the presence of 1 mM phosphate. However, such measurements made in the steady state do not exclude the possibility of a transient fall in the pH gradient on exposure to two permeant anions and hence inhibition of the anions uptake which depends on this gradient. It is possible therefore that the results of procedures a and b are at least partly explicable on the basis of just such a transient fall in the mitochondrial pH gradient. This may also occur to a smaller extent in the cases involving preincubation with citrate or phosphate in procedure c since the small quantity of these anions unavoidably carried over to the incubation medium may not be insignificant compared to the K_M of the respective carriers. However, the stimulation of [2-^{14}C]pyruvate uptake due to preincubation with the dicarboxylate anions could be an example of counter-transport of incoming pyruvate for previously accumulated malate or succinate. This interpretation is supported by the data of table 2 in which efflux of [2-^{14}C]pyruvate from pre-loaded mitochondria was stimulated by external pyruvate, malate or oxaloacetate but not by citrate or phosphate anions at the same concentration. Since butylmalonate (an inhibitor of the dicarboxylate carrier; [12,13] did not prevent the effect of external pyruvate or malate we suggest that the malate/pyruvate exchange involved the pyruvate carrier only. Butylmalonate alone encouraged efflux of [2-^{14}C] pyruvate but in view of its high concentration (10 mM) the effect was relatively slight. Mowbray [3] reported similar behaviour of butylmalonate in experiments on efflux of pyruvate from pre-loaded mitochondria. Since also α-cyano-3-hydroxycinnamic acid which is unlikely to block the dicarboxylate carrier [5] blocked the [2-^{14}C]pyruvate/unlabelled pyruvate and [2-^{14}C]pyruvate/malate exchanges we conclude that pyruvate anions are unable to utilize the dicarboxylate anion carrier.

3.3. *Effects of glucagon injection on pyruvate transport and decarboxylation by isolated mitochondria*

Experiment 1 in table 3 shows that glucagon pretreatment of rats led to significant stimulation of the rate of production of $^{14}CO_2$ from [1-^{14}C]pyruvate by isolated liver mitochondria. The effect is somewhat smaller than that reported by Adam and Haynes [6] as also the absolute values for the rates. Experiment 2 shows that there is a concomitant and quantitatively similar stimulation of the initial rate of pyruvate uptake by the mitochondria. A possible mechanism for this latter effect might involve accumulation in vivo of counter ions in the mitochondrial matrix. One such counter-ion might be palmitoyl-l-carnitine due to mobilization of free fatty acids by glucagon. Mowbray [3] has shown that extra-mitochondrial DL-palmitoyl carnitine can encourage efflux of previously accumulated mitochondrial pyruvate. Another counter ion might be malate since Parilla et al. [9] have recently shown this ion to accumulate in the liver mitochondrial matrix after glucagon injection into rats. A third possible counter ion is

Table 2
Effect of various anions on rate of [2-^{14}C]pyruvate efflux from mitochondria

Anions added to incubation medium	Pyruvate content of mitochondria at zero time (nmoles per mg of protein)	Pyruvate content of mitochondria after 10 s incubation (nmoles per mg of protein)	% of pyruvate remaining after 10 s
None	13.27 ± 1.42 (4)	13.23 ± 1.41 (4)	98.7 ± 1.7 (4)
1 mM pyruvate	12.19 ± 1.34 (6)	7.35 ± 1.50* (6)	61.2 ± 1.02^{+} (6)
1 mM pyruvate + 10 mM α-cyano-3-hydroxycinnamate	11.7 ± 1.14 (3)	11.02 ± 2.68 (3)	99.6 ± 9.4 (6)
10 mM α-cyano-3-hydroxycinnamate	11.79 ± 1.58 (4)	12.35 ± 1.96 (4)	104.3 ± 4.2 (4)
1 mM malate	12.04 ± 1.28 (6)	7.11 ± 1.23** (6)	59.5 ± 11.2^{+} (6)
1 mM oxaloacetate	13.97 ± 1.6 (3)	9.92 ± 0.38 (3)	72.5 ± 6.2† (3)
1 mM succinate	13.2 ± 1.22 (3)	8.18 ± 1.16* (3)	64.6 ± 14.5 (3)
1 mM malate + 10 mM α-cyano-3-hydroxycinnamate	10.23 ± 0.44 (3)	10.58 ± 0.17 (3)	98.4 ± 8.8 (3)
1 mM malate + 10 mM butylmalonate	10.65 ± 0.10 (3)	6.85 ± 1.08* (3)	64.3 ± 10† (3)
1 mM pyruvate 10 mM butylmalonate	10.65 ± 0.10 (3)	5.56 ± 1.19** (3)	52.1 ± 1.02^{+} (3)
10 mM butylmalonate	10.65 ± 0.10 (3)	7.83 ± 0.32** (3)	73.5 ± 2.82† (3)
1 mM citrate	12.25 ± 1.42 (2)	11.86 ± 1.49 (2)	97.9 ± 2.1 (2)
1 mM phosphate	13.83 ± 0.33 (3)	13.2 ± 0.2 (3)	95.5 ± 1.71 (3)

Mitochondria (0.8 mg/protein) which had accumulated [2-^{14}C]pyruvate were suspended in 5 ml of medium 2 (see Methods) with or without stated additions, at 20°C. Mitochondria were separated from the medium by filtration after 10 s. Data are means ± S.E.M. and in parenthesis the numbers of mitochondrial preparations. *p <0.05, **p <0.01; for difference of mean mitochondrial [2-^{14}C]pyruvate content before and after 10 s incubation.
†p <0.05; ^{+}p <0.01 for difference from 100 of mean percentage residual mitochondrial [2-^{14}C]pyruvate after 10 s incubation.

pyruvate itself due to increased glycolytic flux after glucagon injection. In experiment 3 we have attempted to minimize contributions from these factors by starving the rats for 24 h which abolished the glucose mobilization due to glucagon and would be expected to increase the palmitoyl-l-carnitine content in the control mitochondria. Inclusion of malate in the preincubation medium would also be expected to minimize any pre-existing difference in the concentration of this ion between the two mitochondrial populations. Since the magnitude of the glucagon effect on pyruvate uptake in experiment 3 was unchanged it appears that mitochondrial accumulation in vivo of either or all of the three anions—pyruvate, palmitoyl-l-carnitine or malate did not in fact contribute to the effect observed subsequently in vitro. It still remains possible that another so far unidentified counter ion was responsible. A difference in the pH gradient of the isolated mitochondria from glucagon injected rats compared to controls (under our conditions) seems unlikely since utilization of all physiological substrates was blocked during preincubation and the only energy source provided was the ascorbate/tetramethylphenylenediamine couple. Whatever the precise mechanism of this effect of glucagon on mitochondrial pyruvate transport it appears to involve the mobility of the pyruvate carrier in the mitochondrial membrane (V_{max}) rather than its affinity for pyruvate (K_M) (table 4). The K_M value obtained was higher than that reported by

Table 3
Rates of pyruvate uptake and decarboxylation by liver mitochondria isolated from rats 30 min after i.p. injections of saline or glucagon

Expt. No.	Treatment of rat	Rate of $^{14}CO_2$ production from [1-^{14}C]pyruvate by isolated mitochondria in nmoles/mg/min	% Effect of treatment on pyruvate decarboxylation	Initial rate of [2-^{14}C]pyruvate uptake in nmoles/mg/min	% Effect of treatment on rate of pyruvate uptake	Plasma glucose of rats at time of removal of liver (mM)
	Saline injection control	2.72 ± 0.11 (4)	–	–	–	8.6 ± 0.06 (4)
1	Glucagon (300 μg/kg) injection	3.66** ± 0.23 (4)	134† ± 8.5 (4)	–	–	16.4** ± 0.10 (4)
	Saline injection control	–	–	25.12 ± 0.40 (3)		9.6 ± 0.6 (3)
2	Glucagon (300 μg/kg) injection	–	–	34.32$^{\neq}$ ± 0.80 (3)	137^{+} ± 5.4 (3)	16.1* ± 2.3 (3)
	Saline injection control	–	–	24.8 ± 5.8 (3)		7.0 ± 0.050 (3)
3	Glucagon (300 μg/kg) injection	–	–	36.8$^{\neq}$ ± 6.3 (3)	155.7† ± 15.5 (3)	6.6 ± 0.04 (3)

For experiment 1 treatment of rats, method of preparation and incubation of mitochondria is given in Materials and methods. For experiment 2 method used was as given for procedure b in legend to table 1 except that no anion other than [2-^{14}C]pyruvate was added after 2 min incubation. For experiment 3 method used was as given for procedure c in the legend to table 1, malate being present at concentrations of respectively 1 mM and 0.05 mM before and after the addition of [2-^{14}C]pyruvate. The rats had been starved for 24 h. Numbers of mitochondrial preparations are given in brackets.
*p <0.05; **p <0.01; comparing means of data from treated and control rats.
$^{\dagger}p$ <0.05; ^{+}p <0.01; for difference from 100 of percentage effects comparing mitochondria from treated rats with mitochondria from control rats.
$^{\neq}p$ <0.01 for differences calculated on a paired 't' test.

Table 4
Effect of glucagon injection on kinetic characteristics of pyruvate transport in subsequently isolated mitochondria

Treatment of rat	K_M (mM)	V_{max} (nmoles/mg/min)
Control	0.84 ± 0.09 (4)	43.10 ± 3.80 (4)
Glucagon	1.17 ± 0.13 (4)	74.83** ± 1.99 (4)

K_M and V_{max} were calculated from linear regression plots of pyruvate concentration/rate of pyruvate uptake versus pyruvate concentration (cf. [14]) for 4 separate experiments in which the method used was as given in legend to table 3, experiment 3 except that the rats were unstarved.

**P <0.01 for difference from control.

others [15]. The presence of 0.05 mM malate in our incubation media may have contributed to the higher value. In any case it is doubtful if absolute significance should be attached to K_M values for transport under in vitro conditions which depart widely from the in vivo state. Yamazaki has also deduced from measurements of oxygen and calcium uptake by mitochondria that glucagon pretreatment of rats leads to increased mobility of other mitochondrial carriers [16].

An effect of insulin treatment of rats on calcium transport into or out of isolated liver mitochondria has recently been described [17]. Taken along with our present observations it would seem that mitochondrial transport systems must be considered as likely sites for hormonal regulation of metabolism.

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