Evidence that adrenaline activates key oxidative enzymes in rat liver by increasing intramitochondrial [Ca^{2+}]

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The effects of intramitochondrial Ca^{2+} on the activities of the Ca^{2+}-sensitive intramitochondrial enzymes, (i) pyruvate dehydrogenase (PDH) phosphate phosphatase, and (ii) oxoglutarate dehydrogenase (OGDH), were investigated in intact rat liver mitochondria by measuring (i) the amount of active PDH (PDH_a) and (ii) the rate of decarboxylation of α-[1-^{14}C]oxoglutarate (at non-saturating [oxoglutarate]), at different concentrations of extramitochondrial Ca^{2+}. In the presence of Na^+ and Mg^{2+}, both PDH and OGDH could be activated by increases in extramitochondrial [Ca^{2+}] within the expected physiological range (0.05 – 5 μM). When liver mitochondria were prepared from rats treated with adrenaline, and then incubated in Na-free media containing EGTA, both PDH and OGDH activities were found to be enhanced. Evidence is presented that the activation of these enzymes by adrenaline is brought about by a mechanism involving increases in intramitochondrial [Ca^{2+}].

Liver *Adrenaline* *Mitochondrial metabolism* *Intramitochondrial [Ca^{2+}]* *Pyruvate dehydrogenase* *Oxoglutarate dehydrogenase*

1. INTRODUCTION

In mammals there are three intramitochondrial enzymes that occupy key regulatory sites in oxidative metabolism which can be activated by Ca^{2+} in the range 0.1–10 μM. They are pyruvate dehydrogenase (PDH), phosphate phosphatase [1] (whose activation increases amounts of active PDH (PDH_a)), NAD^+-isocitrate dehydrogenase [2] and oxoglutarate dehydrogenase (OGDH) [3]. Studies on rat heart [4–6], skeletal muscle [7,8] and adipose tissue [9] and pig lymphocytes [10] have demonstrated that these enzymes can be activated within mitochondria (incubated with physiological concentrations of Mg^{2+} and Na^+) by increases in extramitochondrial [Ca^{2+}] within the expected physiological range [11]. This led to the proposal [12,13] that hormones and other agents which increase cytoplasmic [Ca^{2+}] [11] may thereby increase intramitochondrial [Ca^{2+}] and so activate these enzymes. Strong evidence to support this view has recently been obtained in studies on β-adrenergic agonist activation of rat heart PDH and OGDH [14,15].

In liver it is known that several hormones which increase cytoplasmic [Ca^{2+}] (such as adrenaline, vasopressin, angiotensin and glucagon [16–18]) also cause increases in PDH_a [19,20]. There is also indirect evidence that OGDH is activated [21–24]. However, other workers have argued that liver intramitochondrial [Ca^{2+}] is too high to regulate these enzymes [25–27] and moreover, that mitochondrial calcium is decreased by these Ca-mobilising hormones [28–31].

The present study outlines methods whereby the Ca^{2+}-sensitive properties of PDH phosphate phosphatase and OGDH can be investigated within liver mitochondria. These techniques were used (i) to study the effects of changes in extramitochondrial [Ca^{2+}] (especially within the physiological

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range) on the enzymes activities and (ii) to examine the possibility that the increases in liver PDH_a which can be brought about by the administration of adrenaline in vivo are the result of the hormone causing increases in intramitochondrial $[Ca^{2+}]$.

2. MATERIALS AND METHODS

For the experiments shown in fig.1, mitochondria were prepared from livers of fed female rats (approx. 250 g) which had been anaesthetised with 0.25 ml Sagatal, by modifying the method used by Belsham et al. [32] to prepare adipose tissue mitochondria. For one rat, about ten 1 g (approx.) pieces of liver were each homogenised in 5 ml of (ice-cold) 250 mM sucrose, 20 mM Tris (pH 7.4), 2 mM EGTA and 1% albumin (largely defatted) (medium A) by disruption with a precooled polytron probe (two bursts of 2 s at Mark 4). The homogenate was diluted with medium A to about 100 ml and centrifuged in two tubes at 1000 × *g* for 90 s; the mitochondria were then sedimented from the supernatant at 10000 × *g* (5 min). Each pellet was suspended in 6 ml of medium A with no albumin (medium B), 1.4 ml of Percoll was added and mitochondria sedimented at 10000 × *g* (10 min). Mitochondria were washed once in medium B and then suspended in medium B to give 40–80 mg protein/ml. Approx. 150 mg protein was obtained from each liver, corresponding (on the basis of total PDH activity) to a yield of 25–35%.

The procedure used for the experiments described in table 1 (effects of adrenaline) was as above except that only three (approx. 1 g) pieces of liver (from different lobes) were rapidly removed and quickly homogenised (taking 10–15 s per rat) in 8 ml of medium A. Also in these experiments (table 1) a small piece of tissue from a fourth lobe was rapidly frozen in liquid N_2 for subsequent analysis of tissue PDH_a content.

Table 1

The activation, as a result of adrenaline administration in vivo, of rat liver PDH and OGDH in subsequently prepared and incubated mitochondria; evidence that a rise in intramitochondrial $[Ca^{2+}]$ is involved

Sample	Additions to basic incubation media	(a) PDH_a (as % of total activity) in liver tissue or mitochondria from rats injected with			(b) Rate of decarboxylation of α-[1-^{14}C]oxoglutarate at 50 μM oxoglutarate (as % of rate at 2 mM oxoglutarate) by mitochondria from rats injected with		
		Saline (control)	Adrenaline	(*n*)	Saline (control)	Adrenaline	(*n*)
Frozen tissue	–	5.6 ± 1.2	14.5 ± 2.9[a]	(3)	–	–	–
Freshly prepared mitochondria	–	17.9 ± 3.9	27.7 ± 4.5	(5)	–	–	–
Incubated mitochondria	10 mM KCl (control)	12.6 ± 1.3	20.3 ± 2.3[a]	(6)	8.4 ± 0.9	13.0 ± 1.0[b]	(9)
	10 mM NaCl	13.4 ± 1.8	13.9 ± 1.2[c]	(5)	7.9 ± 0.8	6.9 ± 1.0[d]	(9)
	10 mM KCl, 300 μM Diltiazem	10.8 ± 1.2	16.9 ± 1.8[a]	(3)	9.5 ± 0.7	18.9 ± 2.8[a]	(4)
	10 mM NaCl,300 μM Diltiazem	13.8 ± 2.0	22.2 ± 3.0[a]	(5)	9.5 ± 0.9	16.4 ± 1.9[b]	(7)
	4 mM EGTA·Ca (final $[Ca^{2+}]$ ≈400 nM)	48.9 ± 4.6	50.4 ± 6.4	(3)	34.8 ± 2.0	32.3 ± 2.9	(7)

Anaesthetised rats were injected intraperitoneally with either 0.3 ml saline (control) or 0.25 mg adrenaline (tartrate) in 0.3 ml saline. After 2 min, mitochondria and tissue were prepared and then analysed as described in section 2. Mitochondria were incubated as described in section 2, in Medium C containing 0.5 mM EGTA and other additions as indicated. Results are given as means ± SE for (*n*) rats. [a] $P \leqslant 0.05$ and [b] $P \leqslant 0.01$ for the effects of adrenaline and [c] $P \leqslant 0.05$ and [d] $P \leqslant 0.001$ for the effects of Na^+ when compared to the appropriate control values by the Student's *t*-test

Mitochondria were incubated (at 1–2 mg protein/ml and 30°C) in 125 mM KCl, 20 mM Tris (pH 7.3) and 5 mM K phosphate (medium C). When PDH was to be measured 2 mM oxoglutarate, 0.2 mM malate and 1 mM pyruvate (K-salts) were also present and after 5 min mitochondria (in 1 ml of medium) were harvested and analysed for both PDH_a and total PDH content as in [33] (as were tissue samples), and results are given as % of total enzyme existing as PDH_a; total PDH was unchanged by the treatments used and was between 40 and 70 munits/mg of mitochondrial protein or between 1.7 and 2.4 units/g wet wt of tissue (where a unit forms 1 μmol acetyl CoA/min at 30°C). For OGDH, after a 5 min preincubation, 50 μM (non-saturating) or 2 mM (saturating) oxoglutarate containing a suitable amount of α-[1-^{14}C]oxoglutarate was added to 100 μl of mitochondrial suspension in a small test-tube (surrounded by 0.5 ml 2-phenylethylamine) within a sealed scintillation vial, followed 2.5 min later by 50 μl of 20% perchloric acid. The rate of $^{14}CO_2$ production at 50 μM oxoglutarate is expressed as a % of that at 2 mM oxoglutarate: this latter rate was unchanged by the treatments used and was between 6 and 9 nmol/min per mg protein; similar results to those shown were obtained if 0.5 mM malate was present throughout (not shown). It should also be noted that essentially similar results for both enzymes were obtained if mitochondrial incubations were continued for a further 5 min (not shown). Any other additions are indicated in the figure and table legends.

The sources of all the materials used are given in [15].

3. RESULTS

3.1. *Activation of PDH and OGDH in intact liver mitochondria by increases in extramitochondrial [Ca^{2+}]*

When intact rat heart [4,5], skeletal muscle [7,8] or adipose tissue [9] mitochondria are incubated (at 25 or 30°C) in KCl-based medium containing phosphate and respiratory substrates (e.g., oxoglutarate with malate) the effects of intramitochondrial [Ca^{2+}] on PDH phosphate phosphatase can be assayed indirectly by following increases in steady-state PDH_a content as the extramitochondrial [Ca^{2+}] is increased. With intact liver mitochondria (fig.1) it was found to be also necessary to add some pyruvate (1 mM) to inhibit PDH_a kinase (see [33]) otherwise PDH_a values were very low and the effects of Ca^{2+} were small and difficult to observe. This is analogous to the situation with heart mitochondria from starved or diabetic rats [33] and presumably means that in the absence of pyruvate the kinase activity is far greater than that of the phosphatase [33]. It should be noted that mitochondrial ATP content was not altered by Ca^{2+} in the PDH experiments of fig.1 (not shown).

Ca^{2+} activates OGDH from liver and other mammalian tissues by decreasing its K_m value for oxoglutarate [3,12]. The Ca^{2+}-sensitive properties of OGDH within liver mitochondria (incubated in the absence of ADP) can be demonstrated by following the production of $^{14}CO_2$ from a non-saturating concentration of α-[1-^{14}C]oxoglutarate (fig.1).

Fig.1 shows that in the presence of Mg^{2+} (which inhibits mitochondrial Ca^{2+} uptake [25]) and Na^+ (which stimulates egress [25]), both PDH and OGDH can be activated within liver mitochondria by increases in extramitochondrial [Ca^{2+}] within the expected physiological range [11]. These effects of Ca^{2+} can be blocked by ruthenium red, a potent inhibitor of Ca^{2+} uptake [25]. In the absence of Na^+ and Mg^{2+} the activation curves are shifted to the left (fig.1) as with heart [4] or adipose tissue [9] but the shift is less, presumably because in liver mitochondria Na^+-dependent egress of Ca^{2+} is slower, and Na^+-independent egress faster, compared to these others [25].

3.2. *Enhancement of PDH and OGDH activities in liver mitochondria prepared from adrenaline-treated rats; evidence that increases in intramitochondrial [Ca^{2+}] are involved*

The ability to assay the Ca^{2+}-sensitive properties of PDH and OGDH within intact liver mitochondria allowed a test to be made of the hypothesis [12,13] that hormones which increase cytoplasmic [Ca^{2+}] in liver (see [16–18]) could thereby increase intramitochondrial [Ca^{2+}] and so cause the increases in PDH_a which are known to be brought about by these same hormones [19,20]. This was done by following the procedures developed to demonstrate that adrenaline increases in-

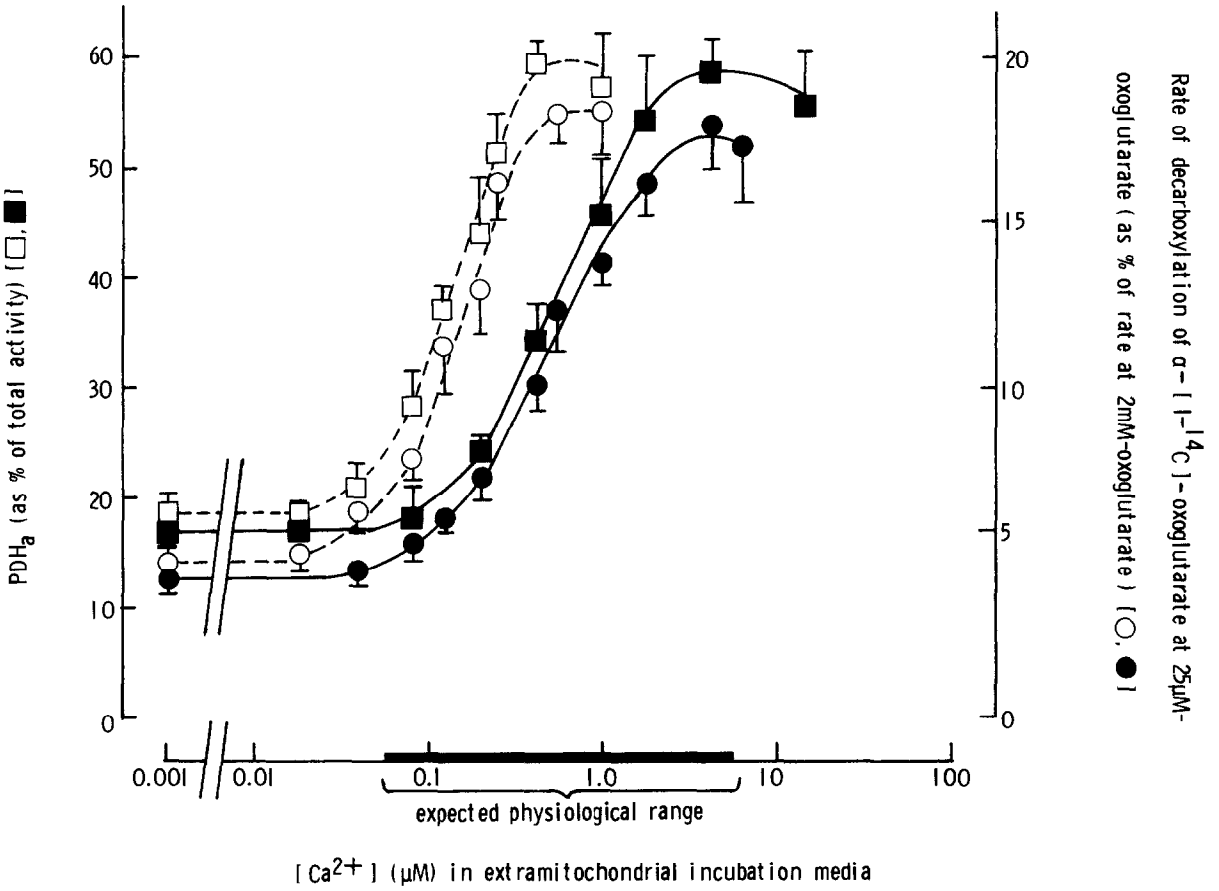

Fig.1. The effects of different extramitochondrial [Ca^{2+}] on (□, ■) the PDH_a content of, and (○, ●) the rate of $^{14}CO_2$ production from a non-saturating concentration of α-[1-^{14}C]oxoglutarate by rat liver mitochondria in the absence (□, ○) or presence (■, ●) of 10 mM NaCl and 1 mM $MgCl_2$. Mitochondria were incubated as described in section 2 (and above) together with the required amounts of EGTA·Ca buffers (at 5 mM EGTA) to give the indicated [Ca^{2+}]. Each point and error bar is a mean ± SE of observations made on at least three different preparations of mitochondria. Calculated $K_{0.5}$ values (nM) ± SD for the effects of Ca^{2+} (for the number of degrees of freedom in parentheses) were (□) 112 ± 40 (26), (○) 96 ± 14 (26), (■) 499 ± 69 (44) and (●) 586 ± 92 (29).

tramitochondrial [Ca^{2+}] in rat heart [15] and involves the rapid preparation of mitochondria from control and treated tissues. However, as this approach relies on assays of Ca^{2+}-sensitive enzymes within subsequently incubated mitochondria as indicators for changes in intramitochondrial [Ca^{2+}], it is first of all necessary to assess its feasibility by examining the behaviour of PDH and OGDH within mitochondria loaded in vitro with Ca^{2+} and then 're-prepared' (again as in [15]). The results of these experiments, which are not given here, were as follows. Briefly, if liver mitochondria were incubated (as in fig.1) with enough Ca^{2+} to cause about 70% of its maximal effect on PDH or OGDH and re-isolated, it was found that these Ca^{2+}-dependent activations persisted through the 're-preparation' of the mitochondria and their subsequent incubation (in media containing EGTA at 30°C), providing that Na^+ was not present. These persistent activations were rapidly lost (by 5 min) if Na^+, or enough extramitochondrial [Ca^{2+}] to saturate the Ca^{2+}-dependent effects on the enzymes, was added to the incubations. Moreover, the effects of Na^+ could be blocked by Diltiazem which is known to inhibit Na/Ca exchange in heart mitochondria [34]. In a similar

series of preliminary experiments (again based on those in [15]) liver mitochondria were re-isolated after loading with ^{45}Ca under conditions where about 70% of the Ca^{2+}-dependent stimulation of PDH and OGDH was observed. When these mitochondria were incubated subsequently, it was found that very little ^{45}Ca was lost at 0°C (< 10% over 2 h, re. mitochondrial preparation) or at 30°C (< 10% in 5 min), providing Na^+ was absent. At 30°C, Na^+ rapidly reduced mitochondrial ^{45}Ca content (by about 50% in 5 min) unless Diltiazem was also present.

The effects of the in vivo administration of adrenaline on the activities of PDH and OGDH within subsequently prepared and incubated liver mitochondria are presented in table 1. In the absence of Na^+, both activities were increased about 2-fold in mitochondria from adrenaline-treated rats compared to controls, even after 5 min incubation at 30°C in the presence of EGTA. These effects were lost if Na^+ was present, but not when Diltiazem was also present, suggesting that Na^+ acts by stimulating the efflux of Ca^{2+} from the mitochondria. The effects could be lost by increasing extramitochondrial $[Ca^{2+}]$ to about 400 nM, presumably because this leads (in the absence of Na^+ and Mg^{2+}) to maximal Ca^{2+}-dependent activation of these enzymes (see fig.1). It should be noted that, under the conditions used, the mitochondrial ATP and NAD(P)H contents are unchanged or even increased as the result of adrenaline treatment (not shown).

4. DISCUSSION

The results of the present study are consistent with increases in liver intramitochondrial $[Ca^{2+}]$ being responsible for the activations of PDH and OGDH by adrenaline and may thus form part of the mechanism whereby adrenaline and other Ca-mobilising hormones stimulate oxidative metabolism in the liver (see [35]). The results are also in accord with the recent proposals that Ca-mobilising hormones stimulate the production of *myo*-inositol 1,4,5-trisphosphate which then releases Ca^{2+} from pools in the endoplasmic reticulum [36,37]. However, they are not in accord with the measurement of a decrease in the total calcium content of mitochondrial fractions caused by these hormones and the consequent hypothesis that they increase cytoplasmic $[Ca^{2+}]$ by eliciting mitochondrial Ca^{2+} release by an unknown mechanism [27–31,38]. A possible explanation for these latter observations is that mitochondrial fractions were contaminated with the hormonally-sensitive reticulum fraction which will have lost Ca^{2+} following hormone treatment. It is of interest that when ^{45}Ca is used to assess the calcium content of mitochondrial fractions, an increase is observed following hormone treatment [39,40]. The present study should avoid contamination problems because exclusively intramitochondrial parameters have been measured, although there remains the possibility that hormones may alter relationships between free and total Ca pools in the mitochondria. Finally, the present results further support the concept that under normal physiological conditions the Ca^{2+}-transport systems of the mitochondrial inner membrane exist primarily to relay changes in cytoplasmic $[Ca^{2+}]$ to the mitochondrial matrix [12,13] rather than, conversely, to 'buffer' or 'set' cytoplasmic $[Ca^{2+}]$ [25,41].

In female rats, adrenaline probably brings about its effects on liver by acting through both α- (i.e., Ca^{2+}-mediated) and β- (i.e., cyclic AMP-mediated) adrenergic receptor stimulation [42]. However, it is clear that adrenaline does cause a rise in cytoplasmic $[Ca^{2+}]$ in the liver of female rats [42] and it is also becoming evident that β-receptor stimulation may also lead to increases in cytoplasmic $[Ca^{2+}]$ [16,42]. At any rate, essentially similar results to those given in table 1 were obtained with male rats (about 200–250 g) (not shown) where adrenaline appears to act virtually exclusively through α-receptors [42].

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