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Characterization of the effects of Ca^{2+} on the intramitochondrial Ca^{2+}-sensitive dehydrogenases within intact rat-kidney mitochondria

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The regulatory properties of the Ca^{2+}-sensitive intramitochondrial enzymes (pyruvate dehydrogenase phosphate phosphatase, NAD^+-isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase) in extracts of rat kidney mitochondria were found to be essentially similar to those described previously for other mammalian tissues; in particular each enzyme could be activated severalfold by Ca^{2+} with half-maximal effects ($K_{0.5}$ values) of about 1 μM and effective ranges of approx. 0.1–10 μM Ca^{2+}. In intact mitochondria prepared from whole rat kidneys incubated in a KCl-based medium containing respiratory substrates, the amount of active, nonphosphorylated pyruvate dehydrogenase could be increased severalfold by increases in extramitochondrial $[Ca^{2+}]$; these effects could be blocked by ruthenium red. Similarly, Ca^{2+}-dependent activations of NAD^+-isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase could be demonstrated in intact, fully coupled, rat kidney mitochondria by either following O_2 uptake (in the presence of ADP) and NAD(P)H reduction (in the absence of ADP) on presentation of non-saturating concentrations of either *threo*-D_s-isocitrate or 2-oxoglutarate, respectively, under appropriate conditions, or for the latter enzyme only, also by following $^{14}CO_2$ production from 2-oxo[1-^{14}C]glutarate (in the absence or presence of ADP). Effects of Na^+ (as a promoter of egress) and Mg^{2+} (as an inhibitor of uptake) on Ca^{2+}-transport by rat kidney mitochondria could be readily demonstrated by assaying for the Ca^{2+}-sensitive properties of the intramitochondrial Ca^{2+}-sensitive dehydrogenases within intact rat kidney mitochondria. In the presence of physiological concentrations of Na^+ (10 mM) and Mg^{2+} (2 mM), activation of the enzymes was achieved by increases in extramitochondrial $[Ca^{2+}]$ within the expected physiological range (0.05–5 μM) and with apparent $K_{0.5}$ values in the approximate range of 300–500 nM. The implications of these results on the role of the Ca^{2+}-transport system of kidney mitochondria are discussed.

Introduction

There are three exclusively intramitochondrial dehydrogenases in mammalian tissues that occupy key regulatory sites in oxidative metabolism which can be activated severalfold by increasing the concentration of free Ca^{2+} within the range 0.1–10 μM, and with $K_{0.5}$ values (half-maximally effective concentrations) for Ca^{2+} of about 1 μM in each case. They are the pyruvate (PDH), NAD^+-

Abbreviations: PDH, the pyruvate dehydrogenase complex; PDH_a, the active, non-phosphorylated form of the pyruvate dehydrogenase complex; NAD-ICDH, NAD^+-linked isocitrate dehydrogenase (EC 1.1.1.41); OGDH, the 2-oxoglutarate dehydrogenase complex; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; $K_{0.5}$ value, the concentration of effector required for half-maximal response; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetracetic acid.

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isocitrate (NAD-ICDH) and 2-oxoglutarate (OGDH) dehydrogenases (for reviews, see Refs. 1–3). Ca^{2+} activates PDH indirectly by causing increases in the amount of active, non-phosphorylated PDH (PDH_a) through its activation of PDH phosphate phosphatase [4]. Ca^{2+} activates NAD-ICDH and OGDH more directly by causing marked decreases in their respective K_m values for *threo*-D_s-isocitrate and 2-oxoglutarate [5,6]. Regulation of these enzymes by Ca^{2+} has been demonstrated in extracts from all vertebrate tissues so far examined, but appears not to be evident in extracts of invertebrate tissues [7]. There are already reports which give brief descriptions of the Ca^{2+} sensitivity of PDH phosphate phosphatase in extracts of both porcine [4] and bovine [8] kidney mitochondria, and of the Ca^{2+} sensitivity of both NAD-ICDH [5] and OGDH [1] in extracts of rat kidney mitochondria. In addition, there are more detailed studies on the regulation of OGDH by Ca^{2+}, and by adenine nucleotides, using preparations from bovine kidney [9,10]. The present work also confirms that these enzymes in extracts of rat kidney mitochondria exhibit essentially similar regulatory properties, especially with respect to Ca^{2+}, to those in extracts of other mammalian tissues.

There are many studies now on intact, fully coupled, mitochondria from a variety of different mammalian tissues, including rat heart [11–13], brain [14], liver [15,16], skeletal muscle [17,18] and adipose tissue [19], and within permeabilized pig lymphocytes [20], which have demonstrated that these intramitochondrial Ca^{2+}-sensitive dehydrogenases can be activated by increases in the extramitochondrial concentration of Ca^{2+} within the expected physiological range, i.e., about 0.05–5 μM [21], and with $K_{0.5}$ values for extramitochondrial Ca^{2+} of about 0.3–0.5 μM. This effective range is achieved by incubation in the presence of physiological concentrations of extramitochondrial Mg^{2+} (which inhibits mitochondrial Ca^{2+} uptake) and Na^+ (which promotes mitochondrial Ca^{2+} egress) [22]. In the absence of either or both of these effector ions of Ca^{2+} transport, the observed $K_{0.5}$ values and effective ranges for extramitochondrial Ca^{2+} are (additively) decreased by magnitudes dependent on the magnitudes of their respective effects on Ca^{2+} transport by the type of mitochondria being investigated (see Refs. 1–3). Thus in this way the Ca^{2+}-sensitive properties of these enzymes can be used as probes for estimating changes in the matrix concentration of Ca^{2+} and hence for investigating features such as mitochondrial Ca^{2+} transport.

These studies on intact mammalian mitochondria led to the important proposal that hormones and other agents which cause increases in cytoplasmic [Ca^{2+}] may, as a result, also bring about increases in intramitochondrial [Ca^{2+}] and hence elicit stimulations of mitochondrial oxidative metabolism through the activation of these key Ca^{2+}-sensitive dehydrogenases [1–3,23]. The ability to assay for the Ca^{2+}-sensitive properties of these enzymes within intact rat heart and liver mitochondria has allowed strong evidence to be obtained in support of this view [24,25]. Such a mechanism may thus explain how such hormones bring about increases in oxidative metabolism and ATP utilisation, but often do not cause observable decreases in, or often in fact increases in, cellular ATP/ADP and NADH/NAD^+ concentration ratios (see Ref. 3). Similarly, it is most likely that, as in other mammalian tissues, changes in the cytoplasmic concentration of Ca^{2+} will be an important means whereby hormones and other extracellular agents bring about changes in various processes within the cells of the kidney [26–28], and it is important therefore to characterise the effects of Ca^{2+} on the Ca^{2+}-sensitive intramitochondrial dehydrogenases within intact kidney mitochondria.

Tullson and Goldstein [29] have previously demonstrated that both PDH and OGDH could be activated by increases in the extramitochondrial concentration of Ca^{2+} when located within intact, but uncoupled, rat kidney mitochondria. In addition, results similar to a small part of the present work have been described briefly in abstract form [30].

Materials and Methods

All chemicals and biochemicals were obtained from the sources listed previously [15]. Mitochondria were prepared from whole kidneys of decapitated male or female fed Wistar rats

(250–400 g) by slight modification of the method described by McCormack [31] for rat liver. The sex or weight of the rats, within this range, did not affect the results obtained, and essentially similar results could be obtained if anaesthetised rats were used instead. Briefly, the preparation procedure involved the rapid disruption of the kidneys with a Polytron homogenizer in ice-cold sucrose-based pH-buffered (7.4) medium containing 2 mM EGTA and 1% (w/v) albumin (8 ml per kidney), and then included a Percoll-purification step in the isolation procedure exactly as described previously for the liver [31]. The mitochondria obtained appeared to be well coupled [32] (results not shown).

Mitochondria were extracted for the assay of PDH phosphate phosphatase activity as described previously [33], and the enzyme assayed as described in Ref. 19, and results calculated as in Ref. 15. Mitochondria were extracted and then extracts were assayed for NAD-ICDH activity and for OGDH activity exactly as described previously [5,6], respectively. A unit of enzyme activity is defined as the amount that catalyses the conversion of 1 μmol of substrate per min at 30 °C.

Mitochondria were incubated (0.5–2 mg protein per ml) at 30 °C in a basic medium consisting of 125 mM KCl, 20 mM Tris (pH 7.0 or 7.3, as indicated) and 5 mM potassium phosphate, except that when 10 mM NaCl was present (as indicated) only 115 mM KCl was used, together with additions as indicated in figure and table legends. Potassium salts were used throughout unless otherwise stated. Samples (usually 1 ml) in which PDH activity was to be assayed were rapidly sedimented (10000 for 20 s) and quickly frozen in liquid N_2 before extraction and assay for both the amount of PDH_a and the total amount of PDH present, as described before [34]; results are generally given as the percentage of total enzyme existing as PDH_a. NAD-ICDH activity was assayed for within the intact kidney mitochondria by following *threo*-D_s-isocitrate dependent O_2 uptake as described by Marshall et al. [19] for white adipose tissue mitochondria, or by following *threo*-D_s-isocitrate induced NAD(P)H production as described by McCormack [15], except that a Perkin-Elmer LS-5 fluorimeter ($\lambda_{ex} = 340$ nm; $\lambda_{em} = 420$ nm) was used to detect NAD(P)H [13]. OGDH activity was also assayed for within the intact kidney mitochondria by these two techniques described for NAD-ICDH, except that 2-oxoglutarate rather than *threo*-D_s-isocitrate was presented to elicit the responses, and also by the additional technique of following $^{14}CO_2$ production from 2-oxo[1-^{14}C]glutarate as described previously [31].

Ca-EGTA and Sr-EGTA buffers were prepared and used (at 5 mM ligand unless otherwise stated), and free metal ion concentrations were calculated, as has been described before [5,34]. Mitochondrial protein was measured by the method of Gornall et al. [35]. Calculations were performed as described before [15] and statistical significance has been assessed by using the Student's *t* test.

Results and Discussion

In extracts of the kidney mitochondria which had undergone the 'Percoll-purification' step (see experimental section [31]), the total activity of PDH and the V_{max} activities of NAD-ICDH and OGDH (as munits per mg protein) were found to be 112 ± 9 (22), 90 ± 6 (12) and 88 ± 8 (12), respectively; values are means $\pm$ S.E.M. for the number (in parentheses) of different mitochondrial preparations. These values were not affected by the treatments described below, and are similar to those values published previously for heart [11] and adipose tissue [19,33], except that in the latter there is somewhat more PDH presumably due to this enzyme's role in lipogenesis as well as oxidation in adipose tissue.

Table I shows that the regulatory properties of PDH phosphate phosphatase, NAD-ICDH and OGDH that were exhibited in extracts of rat kidney mitochondria were essentially similar to those described previously for extracts of rat heart, brown and white adipose tissue, and liver mitochondria [11,15,19,33]. It should be noted that Ca^{2+} could stimulate the activities at least four-fold in each case (not shown in full). Sr^{2+}, as has been shown with the enzymes from other tissues, also mimics the action of Ca^{2+} on the kidney enzymes, but at approx. 10-fold higher concentrations of Sr^{2+} than Ca^{2+}.

The present results confirm and, to varying degrees, extend the previous work on porcine and bovine kidney PDH phosphate phosphatase [4,8]

TABLE I

SUMMARY OF THE KINETIC PROPERTIES OF THE Ca^{2+}-SENSITIVE INTRAMITOCHONDRIAL ENZYMES IN EXTRACTS OF RAT KIDNEY MITOCHONDRIA

Details for the preparation of mitochondria and extracts, and the enzyme assays and calculations are given in Materials and Methods. Assays were at pH 7.0 unless indicated with other conditions as shown. Results are given as $K_{0.5}$ or K_m values ± S.D. for the numbers (in parentheses) of degrees of freedom, based on observations made on samples from at least three different preparations of mitochondria.

Enzyme	Parameter (units)	Other conditions	Parameter value
PDH phosphate phosphatase	$K_{0.5}$ for Ca^{2+} (μM)	-	0.96 ± 0.14 (24)
	$K_{0.5}$ for Sr^{2+} (μM)	-	14.5 ± 1.4 (18)
NAD-ICDH	$K_{0.5}$ for Ca^{2+} (μM)	0.1 mM-*threo*-D_s-isocitrate	1.60 ± 0.18 (26)
	$K_{0.5}$ for Sr^{2+} (μM)	0.1 mM-*threo*-D_s-isocitrate	20.3 ± 2.5 (18)
	K_m for *threo*-D_s-isocitrate (mM)	$[Ca^{2+}] < 1$ nM	0.46 ± 0.06 (36)
		$[Ca^{2+}] \approx 30$ μM	0.09 ± 0.01 (36)
OGDH	$K_{0.5}$ for Ca^{2+} (μM)	0.1 mM 2-oxoglutarate	0.95 ± 0.11 (24)
		0.1 mM 2-oxoglutarate plus 1 mM ADP	0.18 ± 0.03 (20)
	$K_{0.5}$ for Sr^{2+} (μM)	0.1 mM 2-oxoglutarate	10.1 ± 1.1 (18)
	K_m for 2-oxoglutarate (mM)	$[Ca^{2+}] < 1$ nM	3.05 ± 0.27 (36)
		$[Ca^{2+}] < 1$ nM, 1 mM-ADP	0.61 ± 0.05 (18)
		$[Ca^{2+}] < 1$ nM, 1 mM-ATP	22.40 ± 1.90 (18)
		$[Ca^{2+}] < 1$ nM (pH 7.2)	7.41 ± 0.65 (18)
		$[Ca^{2+}] \approx 30$ μM	0.33 ± 0.06 (24)
		$[Ca^{2+}] \approx 20$ μM, 1 mM-ADP	0.06 ± 0.01 (18)
		$[Ca^{2+}] \approx 20$ μM, 1 mM-ATP	0.98 ± 0.06 (18)
		$[Ca^{2+}] \approx 20$ μM (pH 7.2)	0.78 ± 0.07 (18)

and rat kidney NAD-ICDH [5] and OGDH [1]. For NAD-ICDH activity, extractions and assays were carried out in the presence of 1 mM ADP; in the absence of ADP, Ca^{2+} no longer activated the kidney enzyme, as has been found previously for the enzyme from other sources [5]. Kidney NAD-ICDH was also found to be pH sensitive (in the same manner as OGDH, Table I) as has been found previously for the pig heart [36] and rat liver [15] enzymes (results not shown). Rat kidney OGDH was found to be sensitive to Ca^{2+}, Sr^{2+}, pH, ADP and ATP in much the same manner as has previously been described for both the pig and rat heart [6,11] and rat liver [15] enzymes (Table I).

Activation of the pyruvate dehydrogenase complex in intact rat kidney mitochondria by Ca^{2+}

When intact mitochondria isolated from rat heart [11,12], skeletal muscle [17,18], white adipose tissue [19] or brain [14], or within permeabilised pig lymphocytes [20], are incubated in KCl-based medium containing respiratory substrates (e.g., 2-oxoglutarate with L-malate), the effects of changes in intramitochondrial Ca^{2+} (as the result of changes in extramitochondrial Ca^{2+}) on PDH phosphate phosphatase can be assayed for indirectly by measuring increases in steady-state PDH_a content; this is because PDH_a kinase activity is likely to be constant under such conditions [11,33], although there is a report that heart PDH_a kinase may also be inhibited by micromolar Ca^{2+} [37]. These circumstances also proved to be satisfactory for the demonstration of the effects of Ca^{2+} on PDH phosphate phosphatase in intact rat kidney mitochondria (Fig. 1).

Pyruvate is an inhibitor of PDH_a kinase [38] and its presence can therefore lead to amplification of the effects of Ca^{2+} on PDH phosphate phosphatase in kidney mitochondria (Fig. 1) as has been shown previously for other mitochondria, e.g., liver [15] where its presence was necessary to make the effects of Ca^{2+} evident at all. The pres-

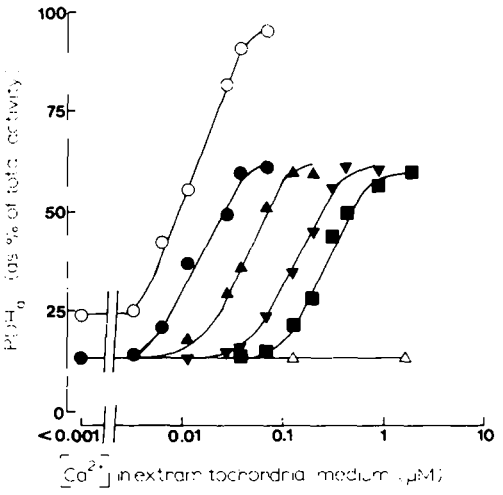

Fig. 1. Effects of Ca^{2+} on the amount of PDH_a in intact rat kidney mitochondria incubated under various conditions. Mitochondria were incubated for 4 min at 30 °C and pH 7.3 and at 0.5–1 mg protein per ml, in 1 ml of KCl-based medium (see Materials and Methods) containing 2 mM 2-oxoglutarate and 0.2 mM L-malate together with appropriate amounts of EGTA-Ca buffers (at 5 mM-EGTA) to give the concentrations of Ca^{2+} shown, and the following other additions: ●, none; ○, 0.25 mM pyruvate; ▲, 10 mM NaCl; ▼, 2 mM $MgCl_2$; ■, 10 mM NaCl plus 2 mM-$MgCl_2$; △, 1 μM ruthenium red. The total activity of PDH was unaffected by the treatments used (see text for value). Results shown are means of values obtained from at least three different preparations of mitochondria; S.E.M. values were all within 15% of the values shown.

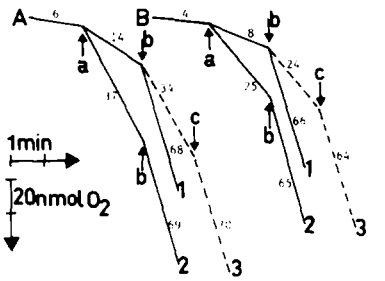

Fig. 2. Effects of Ca^{2+} on O_2 uptake by fully coupled rat kidney mitochondria incubated with non-saturating concentrations of (A) 2-oxoglutarate or (B) *threo*-D_s-isocitrate. Mitochondria (equivalent to 0.5 mg protein) were incubated at 30 °C in 1 ml of KCl-based medium (see Materials and Methods) containing 2 mM ADP and either (a) 0.5 mM malonate or (b) 0.5 mM hydroxymalonate, together with either 2 mM EGTA (lines 1 and 3, less than 1 nM Ca^{2+}) or 2 mM EGTA plus 1 mM $CaCl_2$ (line 2, approx. 50 nM Ca^{2+} free). Additions at the arrows, as indicated, were as follows: for A, lines 1 and 2, a, 100 μM 2-oxoglutarate; b, 10 mM 2-oxoglutarate: for lines 3, a, 100 μM 2-oxoglutarate b, 2 mM EGTA plus 2 mM $CaCl_2$ (resultant free Ca^{2+} approx. 50 nM); c, 10 mM 2-oxoglutarate: for B, lines 1 and 2, a, 100 μM *threo*-D_s-isocitrate; b, 2 mM *threo*-D_s-isocitrate: for line 3, a, 100 μM *threo*-D_s-isocitrate b, 2 mM EGTA plus 2 mM $CaCl_2$; c, 3 mM *threo*-D_s-isocitrate. The values given on the traces indicate nmol of O_2 taken up per min.

ence of pyruvate (0.25 mM) did not affect the $K_{0.5}$ values for Ca^{2+} which were obtained. The addition of Na^+ and/or Mg^{2+} to the incubations of the kidney mitochondria produced the expected increases in the $K_{0.5}$ values for Ca^{2+} (Fig. 1, Table II). The effects of Na^+ appeared to be saturating at 10 mM (results not shown), and in

TABLE II

THE $K_{0.5}$ VALUES FOR EXTRAMITOCHONDRIAL Ca^{2+} IN THE ACTIVATION OF THE Ca^{2+}-SENSITIVE INTRAMITOCHONDRIAL DEHYDROGENASES WITHIN INTACT RAT KIDNEY MITOCHONDRIA INCUBATED UNDER VARIOUS CONDITIONS

$K_{0.5}$ values (±S.D.) were derived from observations made on at least three different preparations of mitochondria and of the type illustrated in Figs. 1–4, the parameter values from which were collected together where appropriate.

Additions to mitochondrial incubations	Calculated $K_{0.5}$ values (nM) for extramitochondrial Ca^{2+} in the activation of:		
	pyruvate dehydrogenase	NAD^+-isocitrate dehydrogenase	2-oxoglutarate dehydrogenase
None (control)	26 ± 1 (64)	31 ± 2 (30)	22 ± 1 (86)
10 mM Na^+	76 ± 8 (38)	not determined	104 ± 8 (60)
10 mM Na^+, 300 μM diltiazem	32 ± 2 (32)	not determined	28 ± 2 (32)
2 mM Mg^{2+}	161 ± 11 (56)	189 ± 15 (32)	145 ± 8 (32)
2 mM Mg^{2+}, 10 mM Na^+	340 ± 18 (60)	410 ± 26 (40)	370 ± 18 (80)

kidney mitochondria appeared also to be of slightly lesser magnitude than in heart, skeletal muscle [17,18] and white adipose tissue [19] mitochondria but of greater magnitude than in liver mitochondria [15]. In the absence of Na^+ and Mg^{2+}, the $K_{0.5}$ values for kidney are similar to those for heart, skeletal muscle and white adipose tissue and lower than those for liver (see above references). In the presence of Na^+ and Mg^{2+} the $K_{0.5}$ values for the mitochondria from all the tissues are all rather similar and the effective extramitochondrial Ca^{2+} range coincides with that expected to occur in the cytosol of mammalian cells (see Ref. 3). The above also suggests that Mg^{2+} is a very potent effector of the Ca^{2+} transport system of rat kidney mitochondria. Fig. 1 also shows that the effects of Ca^{2+} on rat kidney PDH can be blocked by ruthenium red which is a potent inhibitor of mitochondrial Ca^{2+} uptake [39], thus providing strong evidence that they were due to increases in matrix $[Ca^{2+}]$. Table II also shows that the effects of Na^+ can be diminished by diltiazem which is an inhibitor of the mitochondrial Na^+/Ca^{2+} exchanger egress systems [25,40]. Diltiazem (300 μM) had no effects on the $K_{0.5}$ values obtained in the absence of Na^+ (results not shown).

Essentially similar results to those shown in Fig. 1 could be obtained if 5 mM succinate or else 5 mM glutamate with 0.5 mM L-malate replaced the 2-oxoglutarate and L-malate as respiratory substrates (not shown). The effects of Ca^{2+} on PDH_a in the intact mitochondria could be mimicked by Sr^{2+}, except that approx. 10-fold higher concentrations of Sr^{2+} were required in each case (see Table II).

The results shown in Fig. 1 were obtained with kidney mitochondria incubated for 4 min; over the concentration ranges of Ca^{2+} used, essentially similar results were obtained after 8 min incubation indicating that steady-state amounts of PDH_a were present (not shown). This also suggests that there were also steady-state relationships between extra- and intra-mitochondrial $[Ca^{2+}]$ under these conditions, i.e., there was 'cycling' of Ca^{2+} across the mitochondrial inner membrane, but after the steady state had been reached, no net change of $[Ca^{2+}]$ in either compartment (see Refs. 1, 2 and 41). Under such circumstances the mitochondrial ATP content (mean ± s.e.m. of 8.4 ± 0.8 for 82 observations) was not altered by Ca^{2+} over the concentration ranges used in Fig. 1. However, if extramitochondrial $[Ca^{2+}]$ was raised above the ranges shown, then there was an apparent time-dependent diminution of mitochondrial ATP content as has been noted previously for both heart [11,12] and liver [15] mitochondria (results not shown). Such diminution presumably reflects the saturation of the Ca^{2+} egress mechanisms by matrix $[Ca^{2+}]$ and subsequent net Ca^{2+} accumulation (to a massive extent) by the mitochondria leading to generalised perturbation of mitochondrial function (see Refs. 1, 11 and 15). In the presence of Na^+ and Mg^{2+} (Fig. 1), this only appears to occur when the extramitochondrial $[Ca^{2+}]$ exceeds the expected physiological range [21]. Additionally, it should be noted that for Ca^{2+} accumulation to occur, i.e., to have egress pathway saturation, in the presence of Na^+, requires that the total Ca content of the mitochondria be in excess of approx. 10–15 nmol per mg protein depending on the source of the mitochondria (see Refs. 1 and 4). This was also found with the kidney mitochondria used in the present study (results not shown). In contrast, the total Ca content of the kidney mitochondria over the ranges of Ca^{2+} where the enzymes exhibited Ca^{2+}-sensitive properties (as in Fig. 1) was found to be from 0–0.3 up to about 3–5 nmol per mg protein with a content of 1.6 ± 0.2 (observations on ten separate preparations of mitochondria) found to be the mean ± S.E.M. value at the half-maximally activated points on the curves (results not shown). This is again in agreement with the total Ca contents of both heart [42] and liver [43] mitochondria as the Ca^{2+}-sensitivity of the enzymes is exhibited. Such values also agree well with estimates of total Ca content for mitochondria in situ from rat heart, liver and kidney obtained from both subcellular fractionation studies (Refs. 44 and 43, and McCormack, J.G., unpublished results, respectively) and from studies using X-ray probe microanalysis of 'snap-frozen' pieces of intact tissue (Refs. 45, 46 and 47, respectively). Indeed, using the latter approach, it can be demonstrated that values of total mitochondrial Ca content in excess of 10 nmol per mg protein are only observed in obviously damaged cells whose other ion balances have also been deranged [45–47].

Assay of the Ca^{2+}-sensitive properties of the 2-oxoglutarate dehydrogenase complex and the NAD^+-linked isocitrate dehydrogenase within intact rat kidney mitochondria

Studies using the oxygen electrode

The Ca^{2+}-sensitive properties of OGDH within intact mitochondria from rat heart [11] and both brown [33] and white [19] adipose tissue can be readily demonstrated by simply measuring the O_2 uptake induced by adding 2-oxoglutarate at sub-saturating concentrations to the mitochondria when the restrictions imposed on this by respiratory chain activity are overcome either by using uncoupled mitochondria or by adding an excess of ADP. Indeed, Tullson and Goldstein [29] previously have demonstrated the Ca^{2+}-sensitive properties of OGDH within intact rat kidney mitochondria which were uncoupled with FCCP. Thus under such conditions the activity of OGDH appears to be the rate-limiting step for the oxidation of added 2-oxoglutarate. This approach was initially found to be more difficult with rat liver mitochondria [15]; however, subsequently Johnson and Brand [16], principally due to performing experiments at 37°C instead of 30°C, were able to demonstrate increased rates of oxidation of sub-saturating concentrations of 2-oxoglutarate in the presence of excess ADP in rat liver mitochondria. The Ca^{2+}-sensitive properties of NAD-ICDH have also been demonstrated in this way within intact mitochondria from both rat white [19] and brown [33] adipose tissue; however, again this approach proved difficult for this enzyme in the liver [15], and also completely unsuccessful in the heart owing to the low activity of the tricarboxylate carrier in heart mitochondria [48].

Experiments performed using the oxygen electrode and incubations in the presence of FCCP (1 μM) allowed the demonstration of the effects of Ca^{2+} on OGDH within intact but uncoupled rat kidney mitochondria which were similar to those found previously [29], and also allowed the similar demonstration of the effects of Ca^{2+} on NAD-ICDH (results not shown); the observed $K_{0.5}$ values for Ca^{2+} were close to 1 μM in each case, i.e., suggesting that there is free equilibrium of intra- and extra-mitochondrial Ca^{2+} under such conditions (see Ref. 33). Fig. 2 shows that the Ca^{2+} sensitivity of both OGDH and NAD-ICDH can also be readily demonstrated by incubating fully coupled mitochondria in the presence of excess ADP. The effects of Ca^{2+} were also evident in mitochondria incubated in the absence or presence of L-malate (0.25 mM) (results not shown); however, in these two cases there were some problems with linearity or high background rates, respectively. The most clear effects of Ca^{2+} were found when malonate (0.5 mM) or hydroxymalonate (0.5 mM) were used as non-metabolisable transport partners for 2-oxoglutarate or *threo*-D_s-isocitrate respectively (Fig. 2). Clear effects of Na^+ and/or Mg^{2+} on the $K_{0.5}$ values for extramitochondrial Ca^{2+}, as the result of their effects on Ca^{2+} transport by rat kidney mitochondria, could also be demonstrated (see Table II). It should be noted that neither the K_m nor the V_{max} value for succinate oxidation by the kidney mitochondria was affected by Ca^{2+} in the concentration ranges used; this was employed as a control (see Ref. 11).

Studies using 2-oxo[1-^{14}C]glutarate

This technique has been used previously with both rat heart [24] and liver [15,21] mitochondria. Fig. 3 shows that the Ca^{2+}-sensitive properties of OGDH within intact, fully coupled, rat kidney mitochondria can also be demonstrated by measuring the production of $^{14}CO_2$ from 2-oxo[1-^{14}C]glutarate at non-saturating concentrations of 2-oxoglutarate. As with heart [24], but unlike liver [15], clear effects of Ca^{2+} were evident in the presence of ADP as well as in its absence (Fig. 3), though again similarly to the heart [34] the presence of ADP did not appear to affect the calculated $K_{0.5}$ values for extramitochondrial $[Ca^{2+}]$ (see Fig. 3). Ruthenium red again blocked all effects of increasing extramitochondrial $[Ca^{2+}]$ within the ranges used (Fig. 3), and effects of Na^+ and Mg^{2+} were again evident (Fig. 3; Table II). The results shown in Fig. 3 were obtained by incubating the mitochondria in the presence of L-malate; similar results were also obtained in its absence or in the presence of malonate (0.5 mM), except that slightly lower or higher rates were obtained, respectively, (not shown) under all conditions. However, no effects of Ca^{2+} were evident at a saturating concentration of 2-oxoglutarate

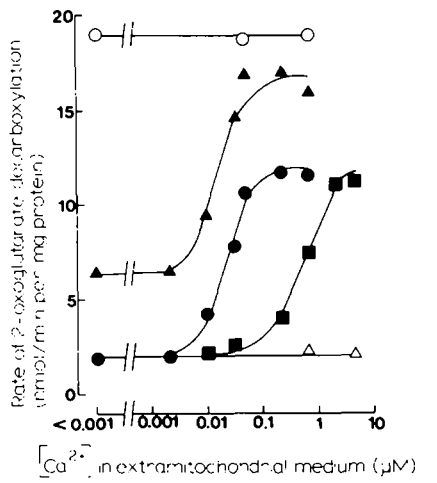

Fig. 3. Effects of increases in extramitochondrial Ca^{2+} on the rate of decarboxylation of 2-oxoglutarate by intact, fully coupled, rat kidney mitochondria incubated under various conditions. Mitochondria were incubated at 30 °C and approx. 0.5 mg protein/ml in 100 μl of KCl-based media (see Materials and Methods) containing 0.5 mM L-malate and the concentrations of Ca^{2+} shown (obtained by using EGTA-Ca buffers as in Fig. 1) together with other additions as follows: ● and ○, none; ▲, 2 mM-ADP; ■, 10 mM NaCl plus 2 mM $MgCl_2$; △, 1 μM ruthenium red. The incubation media were contained in a small test-tube within a sealed vial (the latter containing 0.5 ml of 2-phenylethylamine). After 4 min pre-incubation, 50 μM (●, ▲, ■, △) or 5 mM (○) 2-oxoglutarate was added in a volume of 5 μl in each case and containing suitable amounts of 2-oxo[1-^{14}C]glutarate, and followed 2.5 min later by 50 μl of 20% (v/v) $HClO_4$. Full details of the method are given in Ref. 31. Values shown are means of values obtained from observations on three different preparations of mitochondria; S.E.M. values were all within 15% of the values shown. Rates of $^{14}CO_2$ production under the conditions used appeared to be linear over a 4 min period (not shown); similar results to those shown were also obtained if a 2 min pre-incubation period was used instead.

(Fig. 3). [1-^{14}C]glutamate could also be used to demonstrate effects of Ca^{2+} on OGDH (see Ref. 15). Unfortunately, NAD-ICDH could not be studied satisfactorily using this technique because of reasons discussed before [15].

Studies using fluorimetry to monitor NAD(P)H production

This technique has also been used successfully previously with both rat heart [13,34] and liver [15] mitochondria (a dual-wavelength spectrophotometer can also be used (see Ref. 15). These experiments are best performed in the absence of ADP to allow sufficient NAD(P)H formation (see Ref. 15). Fig. 4 shows that the Ca^{2+} sensitivity of both OGDH and NAD-ICDH can be conveniently studied within intact, fully coupled, kidney mitochondria in this way. Fig. 5 shows that by using the Ca^{2+}-sensitive properties of OGDH as a probe for matrix Ca^{2+} using this approach, then the effects of ruthenium red, Mg^{2+}, Na^+ and diltiazem on the Ca^{2+}-transport system of the inner membrane of kidney mitochondria can be readily demonstrated. The data from this technique used in the calculation of the $K_{0.5}$ values was derived from measurements of the overall changes in steady-state levels of NAD(P)H reduction induced by Ca^{2+} rather than the initial rates of reduction (see Ref. 15), although similar values were obtained using either parameter. Ca^{2+}-dependent increases in NAD(P)H due to OGDH and NAD-ICDH activations could also be observed in the absence of malonate or hydroxymalonate, respectively (but results were more variable), or on presentation of non-saturating concentrations of glutamate or citrate, respectively. However, no effects of Ca^{2+} were evident on NAD(P)H produced due to the presentation of non-saturating or saturating concentrations of L-malate (not shown).

It should be noted that, as all of the various $K_{0.5}$ values for extramitochondrial Ca^{2+} in the activations of OGDH and NAD-ICDH within the kidney mitochondria using the different techniques were very similar, these have all been aggregated together to give the values in Table II. It must also be noted that, although perhaps unlikely, the observed effects of Ca^{2+} on O_2 uptake or NAD(P)H production induced by *threo*-D_s-isocitrate which have been allocated to its effects on NAD-ICDH, could theoretically be due instead to effects on OGDH as 2-oxoglutarate is of course the product of NAD-ICDH and also NADP-ICDH (see Ref. 5). Attempts to resolve this question by trying to use arsenite as a specific inhibitor of OGDH within the kidney mitochondria proved unsuccessful (see Ref. 15) (not shown).

General Discussion and Conclusions

The major conclusion of the present paper is that the three Ca^{2+}-sensitive intramitochondrial

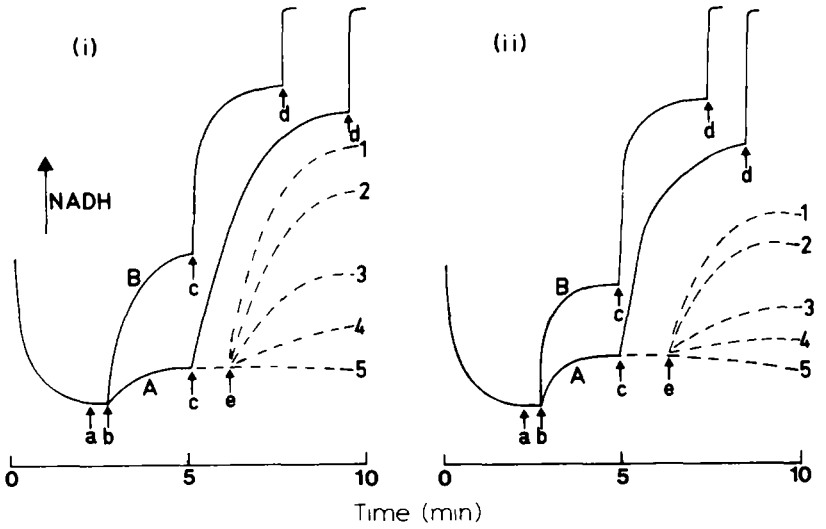

Fig. 4. Effects of Ca^{2+} on the production of NAD(P)H induced by (i) 2-oxoglutarate and (ii) *threo*-D_s-isocitrate in intact, fully coupled, rat kidney mitochondria. Mitochondria were incubated (with stirring in a cuvette) at 30 °C and pH 7.2 and approx. 1 mg protein per ml, and for the times shown, in 2 ml of KCl-based medium (see Materials and Methods) containing initially 2 mM-EGTA and either (i) 0.25 mM malonate or (ii) 0.25 mM hydroxymalonate. In both (i) and (ii) at arrow a either (A) 2 mM EGTA or (B) 2 mM EGTA plus 2 mM $CaCl_2$ (resultant-free $[Ca^{2+}]$ approx. 50 nM) was added, except for the broken traces where no addition was made at arrow a (or at c). The other additions to the continuous traces were at the arrows, as follows: (i) b, 100 μM 2-oxoglutarate; c, 5 mM 2-oxoglutarate; d, 0.5 μg of rotenone; in (ii), b, 100 μM *threo*-D_s-isocitrate; c, 5 mM *threo*-D_s-isocitrate; d, 0.5 μg of rotenone. For the unbroken traces in both (i) and (ii) the addition at arrow e were: 1, 3 mM EGTA plus 3 mM $CaCl_2$ (resultant-free $[Ca^{2+}]$, approx. 80 nM); 2, 1.5 mM EGTA plus 1.5 mM $CaCl_2$ (≈ 40 nM) 1 mM EGTA plus 1 mM $CaCl_2$ (≈ 25 nM); 0.5 mM EGTA plus 0.5 mM $CaCl_2$ (≈13 nM); 5, 2 mM EGTA. Results from a typical experiment are shown, but data from at least three such experiments has been incorporated into the values given in Table II. Note also that 2-oxoglutarate itself absorbed slightly at the excitation wavelength used; however, this has been omitted for clarity of presentation.

dehydrogenases within intact, fully coupled, rat kidney mitochondria incubated with physiological concentrations of extramitochondrial Na^+ [49] and Mg^{2+} [50] can be activated by increasing the extramitochondrial concentration of Ca^{2+} within the expected physiological range, i.e., approx. 0.05–5 μM [21]. This therefore lends further support to the proposal based on observations made with other types of mammalian mitochondria (see Introduction) that calcium ions are likely to be important regulators of intramitochondrial metabolism through their effects on these key enzymes. This will of course include oxidative metabolism and ATP production, but also as well other key metabolic pathways such as gluconeogenesis or urea synthesis. The present work thus also substantiates the proposal [1–3] that under normal physiological conditions in kidney and other mammalian cells, the Ca^{2+}-transport systems of the mitochondrial inner membrane exist

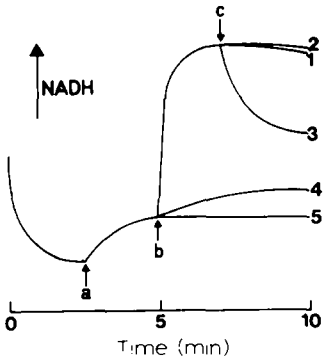

Fig. 5. Effects of Na^+, Mg^{2+}, diltiazem and ruthenium red on the activation of 2-oxoglutarate dehydrogenase by Ca^{2+} within intact, fully coupled rat kidney mitochondria. Mitochondria were incubated initially exactly (with 2 mM EGTA) as in Fig. 4, but with the further additional presence of 1 μM ruthenium red in trace 5 and 2 mM $MgCl_2$ in trace 4, and then further additions were made, at the arrows, as follows: a, 100 μM 2-oxoglutarate; b, 3 mM EGTA plus 3 mM $CaCl_2$ (resultant-free $[Ca^{2+}] \approx 80$ nM); and the different additions at c were, 1, 10 mM KCl (control); 3, 10 mM NaCl; 2, 10 mM NaCl plus 300 μM diltiazem. A typical experiment is shown (see also Fig. 4 legend).

primarily to relay changes in cytoplasmic [Ca^{2+}] to the mitochondrial matrix, rather than, conversely, to buffer or set cytoplasmic [Ca^{2+}] (see, e.g., [22,51–53]). As has been mentioned already, the most recent estimates of in situ mitochondrial total calcium content [47] give comparatively low values, which are more compatible with those over which Ca^{2+} regulation of the dehydrogenases is observed (see text above) than with those over which Ca-buffering behaviour is evident (see Ref. 15). Therefore it is perhaps very likely that hormones and other agents which lead to increases in cytosolic [Ca^{2+}] in kidney cells, e.g., parathyroid hormone [26] or calcitonin [54], will also thus bring about increases in intramitochondrial [Ca^{2+}] and hence activation of these enzymes, though perhaps different hormones will bring about such increases in different cell types within the kidney (see Ref. 55).

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