Review Letter

ON THE ROLE OF THE CALCIUM TRANSPORT CYCLE IN HEART AND OTHER MAMMALIAN MITOCHONDRIA

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1. Introduction

Much attention has been focussed recently on the transfer of Ca^{2+} across the inner membrane of mammalian mitochondria and within the last year or so this Journal has published two other review letters on the topic [1,2]. The elegant studies which formed the basis of these letters have shown that the calcium-transport system in mammalian mitochondria consists of separate influx and efflux components. These components taken together constitute a calcium-transport cycle which determines the distribution of Ca^{2+} across the inner mitochondrial membrane (fig.1). The main purpose of this letter is to suggest a role for this cycle. In the past, it appears to have been assumed rather generally amongst biochemists interested in Ca^{2+}-transport in mitochondria that the system is important in the regulation of cytoplasmic Ca^{2+} [3–6]. We will argue in this article that this emphasis is probably misplaced. We will summarise evidence that Ca^{2+} enhances the activity of three key intramitochondrial dehydrogenases and that $[Ca^{2+}]$ at 0.1–10 μM is potentially an important regulator of oxidative metabolism within mammalian mitochondria. If this view is correct then the function of the calcium-transport system in the inner mitochondrial membrane should be considered primarily as a means of determining the intramitochondrial (rather than extramitochondrial) $[Ca^{2+}]$ concentration in the same sense that the system in the plasma membrane is usually viewed as a means of determining the cytoplasmic rather than the plasma $[Ca^{2+}]$. There are, in fact, a number of similarities between the systems in the plasma and mitochondrial membranes and these are brought out in fig.1. This figure also illustrates the transfer of calcium across the endoplasmic reticulum membrane which may be important in the short-term regulation of cytoplasmic $[Ca^{2+}]$ in many tissues and certainly in muscle (see [5]).

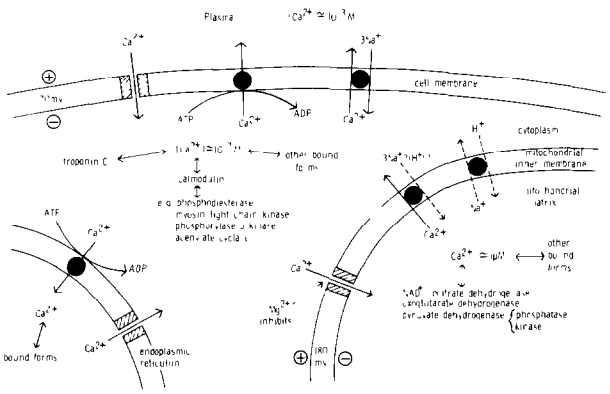

Fig.1. Calcium-transporting systems in the cell, endoplasmic reticulum and mitochondrial membranes of mammalian cells. It should be noted that calcium efflux from the mitochondria of heart, white and brown adipose tissue and many other mammalian tissues is stimulated by Na^+ probably because efflux occurs by exchange of 3 Na^+ for Ca^{2+} [1,2]. However, efflux from liver, kidney and smooth muscle mitochondria is not stimulated by Na^+ and efflux probably occurs by direct exchange of Ca^{2+} with 2 or more H^+ [1,2].

2. Recognition of the sensitivity of three key intramitochondrial dehydrogenases to Ca^{2+}

The activity of the pyruvate dehydrogenase com-

Abbreviations: PDH and PDHP, the nonphosphorylated and phosphorylated forms of the pyruvate dehydrogenase complex; NAD–ICDH, NAD^+-linked isocitrate dehydrogenase (EC 1.1.1.41); OGDH, the oxoglutarate dehydrogenase complex; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone

'Ca^{2+}' refers exclusively to free calcium ions

plex from mammalian sources may be regulated both by end-product inhibition (through increases in the concentration ratios of both NADH/NAD$^+$ and acetyl-CoA/CoA) and by phosphorylation [7,8]. Interconversion of the inactive phosphorylated form (PDHP) and the active dephosphorylated form (PDH) is catalysed by an ATP-requiring kinase and a phosphatase. Studies on the properties of these interconverting enzymes have led to the recognition of a number of effectors of potential physiological importance. The pioneering studies of Lester Reed and his colleagues identified ADP and pyruvate as inhibitors of the kinase and Mg^{2+} as a necessary activator of the phosphatase [9,10]. Subsequently, it has been found that the kinase is also activated by high concentration ratios of NADH/NAD$^+$ and acetyl-CoA/CoA implying that inactivation by phosphorylation may be increased under conditions of end-product inhibition [11,12]. In studies carried out in Bristol with Philip Randle it was realised that EGTA greatly inhibited the activity of PDHP phosphatase and this led to the demonstration that PDHP phosphatase from the mitochondria of a number of mammalian tissues was activated many-fold by Ca^{2+} in the presence of saturating [Mg^{2+}] [13]. Using Ca-EGTA buffers the [Ca^{2+}] which gave half-maximum activation ($k_{0.5}$) was found to be close to 1 μM [13] and later studies showed that Sr^{2+} will cause an equivalent activation but that 10-times greater concentrations were needed (fig.2a) [14,15]. In addition PDH kinase may be inhibited by Ca^{2+} with $k_{0.5}$ a little below 1 μM. This latter effect can be viewed as potentially reinforcing the effects of Ca^{2+} on PDHP phosphatase but to date it has only been reported for the pig heart enzyme [11].

Our attention was drawn to NAD–ICDH initially by reports that Ca^{2+} may inhibit the activity of this enzyme by increasing the apparent K_m for *threo*-D_s-isocitrate [17,18]. However, these studies were carried

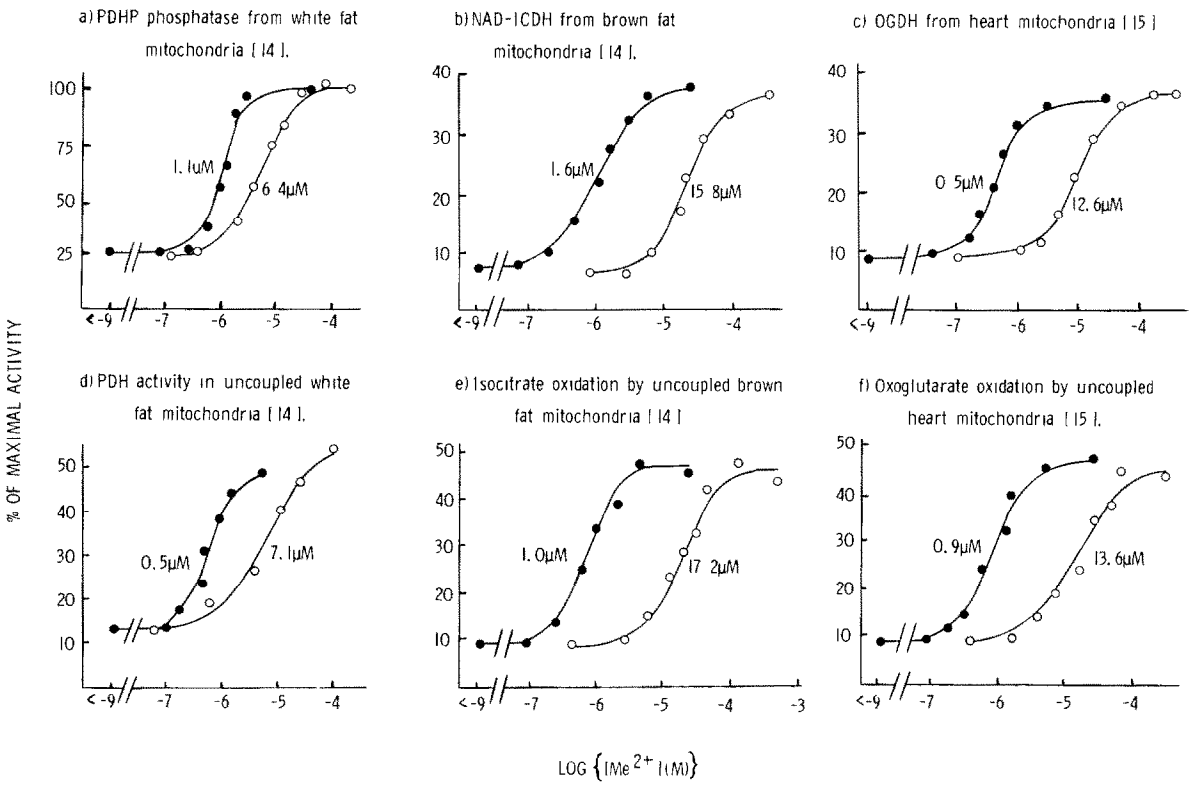

Fig.2. Sensitivity of pyruvate dehydrogenase phosphate phosphatase, NAD$^+$-linked isocitrate dehydrogenase and oxoglutarate dehydrogenase to Ca^{2+} (•) and Sr^{2+} (○) in rat mitochondrial extracts (a–c) and within intact uncoupled mitochondria (d–f). Ca-EGTA or Sr-EGTA buffers were used throughout to regulate [Ca^{2+}] or [Sr^{2+}]. The [isocitrate] in (b,e) and oxoglutarate in (c,f) was 0.1 mM. The numbers adjacent to the traces are the appropriate calculated $k_{0.5}$ values. Full details may be obtained from references given in the panel headings.

out in the presence of Mn^{2+} at concentrations greatly in excess of the [EGTA] in the Ca-EGTA buffers employed and the tight binding of Mn^{2+} by EGTA was neglected. On re-examining the sensitivity of this enzyme to Ca^{2+} in the presence of Mg^{2+} (rather than Mn^{2+}), we found that μM levels of Ca^{2+} caused a very marked decrease in K_m for isocitrate instead of an increase (fig.2b) [19].

Subsequent studies on the sensitivity of other intramitochondrial enzymes to Ca^{2+} revealed that OGDH was also activated by Ca^{2+} [20]. The activation was closely analogous to that of NAD–ICDH; namely, that Ca^{2+} greatly decreased the K_m for oxoglutarate without any change in the V_{max} (fig.2c). The $k_{0.5}$ for the activation by Ca^{2+} of both enzymes is close to 1 μM and thus similar to PDHP phosphatase. Moreover, again as with PDHP phosphatase, activation was also observed with Sr^{2+} (although higher concentrations were required) but not with Mg^{2+} and probably not with Mn^{2+}. As indicated in table 1, similar Ca^{2+}-sensitivity has been found for the three dehydrogenases from all mammalian tissues so far studied. In contrast, we have been unable to demonstrate activation of NAD–ICDH and OGDH from potato and blow-fly flight muscle mitochondria or of OGDH or NADP–ICDH from *Escherichia coli* (unpublished).

The very similar sensitivity of PDHP phosphatase, NAD–ICDH and OGDH from mammalian mitochondria to Ca^{2+} suggests that a common calcium-binding subunit may be involved which remains bound to the enzymes during extraction and purification. However, we have been unable to detect the presence of calmodulin (or calcium-dependent regulator protein) but the possibility of the presence of a related protein has not been ruled out [20].

Activation by Ca^{2+} is not the only means of potential regulation that PDH, NAD–ICDH and OGDH share. The activity of all three enzymes is also enhanced by increases in the ADP/ATP and NAD^+/NADH ratios. The kinetic constants which are altered are summarized in table 2. It seems reasonable to view the regulation by ADP/ATP and NAD^+/NADH ratios as the 'intrinsic' means whereby the rate of NADH production in mitochondria is matched to the requirements of the respiratory chain and thus ATP synthesis. The activation of these same dehydrogenases by Ca^{2+} could then be the means whereby 'extrinsic' control of intramitochondrial oxidative metabolism by such factors as hormones and neurotransmitters is superimposed on this 'intrinsic' control. It is, of course, well established that Ca^{2+} plays such a role in the regulation of a number of cytoplasmic events including muscle contraction, secretion and glycogen breakdown [28]. Nevertheless, it is always risky to ascribe regulatory importance to enzyme properties which have only been observed with separated enzymes. We therefore have sought more direct evidence to support a physiological role for the Ca^{2+}-sensitivity of these dehydrogenases by using intact mitochondria.

Table 1
Mitochondrial enzymes sensitive to activation by Ca^{2+}

Tissue	Pyruvate dehydrogenase phosphate phosphatase	NAD^+-isocitrate dehydrogenase	Oxoglutarate dehydrogenase
Muscle			
Pig heart	[13]	(a)	[20]
Rat heart	[21]	[19]	[15]
Pigeon heart	n.d.	(a)	(a)
Human heart	[22]	n.d.	n.d.
Rat skeletal	n.d.	(a)	(a)
Rat liver	[23]	(a)	(a)
Rat brain	n.d.	(a)	(a)
Rat kidney	[13]	[19]	(a)
Rat white fat	[13]	[19]	(a)
Rat brown fat	n.d.	[14,19]	[14,20]

In all cases, the $k_{0.5}$ for Ca^{2+} was 0.3–3 μM and in most the effects of Ca^{2+} have been shown to be mimicked by Sr^{2+} but with $k_{0.5}$ values about one order of magnitude greater. n.d., not determined. (a) Unpublished observations of J. G. McC. and R. M. D. and 2nd year students reading biochemistry at the University of Bristol. In other cases, reference numbers are given

Table 2
Regulation of intramitochondrial dehydrogenases

Enzyme	Effects of an increase in		
	Ca^{2+}	ADP/ATP	NAD^+/NADH
Pyruvate dehydrogenase system	Phosphatase activation Kinase inhibition	Kinase inhibition	Kinase inhibition Decrease in $K_m^{NAD^+}$
NAD^+-isocitrate dehydrogenase	Decrease in $K_m^{isocitrate}$	Decrease in $k_m^{isocitrate}$	Decrease in $K_m^{NAD^+}$
Oxoglutarate dehydrogenase	Decrease in $K_m^{oxoglutarate}$	Decrease in $K_m^{oxoglutarate}$	Decrease in $K_m^{NAD^+}$

The regulation of both NAD–ICDH and OGDH by Ca^{2+} appears to be essentially independent of their regulation by nucleotides ([19,20] and unpublished).. It should also be noted that PDH and OGDH are inhibited by their end-products, acetyl-CoA and succinyl-CoA, respectively [26,27]. For further details see [9,11–13,16,19,20,24–27]

3. Demonstration of the Ca^{2+}-sensitivity of the dehydrogenases when located within intact uncoupled mitochondria

In uncoupled mitochondria incubated in KCl-based medium there will be little or no pH gradient or membrane potential and thus the intramitochondrial $[Ca^{2+}]$ should be the same as the extramitochondrial $[Ca^{2+}]$. We have studied the sensitivity of PDHP phosphatase when located within mitochondria by incubating mitochondria in the presence of FCCP plus the calcium ionophore A23187 together with $MgATP^{2-}$ and oligomycin (to ensure adequate PDH-kinase activity) and varying $[Ca^{2+}]$ and $[Sr^{2+}]$ generated with EGTA buffers. Mitochondria were then extracted and the proportion of pyruvate dehydrogenase in its active form determined. Some results obtained with epididymal white fat cell mitochondria are shown in fig.2d. It is evident that the sensitivity to both Ca^{2+} and Sr^{2+} is very similar to that found with PDHP-phosphatase in extracts of the same mitochondria (fig.2a). Separate studies with rat heart mitochondria gave essentially the same result ([15] and table 3).

The activity of NAD–ICDH and OGDH within intact mitochondria can be determined simply by following the oxidation of isocitrate or oxoglutarate on an oxygen electrode if the respective dehydrogenases are rate-limiting. In certain mitochondria this situation occurs including brown and white adipose tissue mitochondria for both substrates and rat heart mitochondria for oxoglutarate [14,15]. In these cases, the kinetic constants (K_m, n, V_{max}) of the substrate oxidation by the uncoupled mitochondria have been determined and found to match closely those found for the appropriate dehydrogenase in mitochondrial extracts. In particular, at low concentrations of substrate, the dehydrogenases appear to be activated within the mitochondria by both Ca^{2+} and Sr^{2+} with $k_{0.5}$ close to 1 and 10 μM, respectively. This is illustrated in fig.2e and 2f for isocitrate oxidation by brown adipose tissue mitochondria and oxoglutarate oxidation by rat heart mitochondria, respectively.

These experiments clearly show that the three dehydrogenases when located within a variety of mammalian mitochondria are activated by Ca^{2+} with $k_{0.5}$ close to 1 μM. The possibility is also raised that these dehydrogenases can be used as a means of actually monitoring $[Ca^{2+}]$ within coupled mitochondria [14].

Table 3
Sensitivity of pyruvate dehydrogenase activity and oxoglutarate oxidation to changes in extramitochondrial Ca^{2+} in rat heart mitochondria

Additions	$k_{0.5}$ values (nM) for the activation of	
	Pyruvate dehydrogenase activity	Oxoglutarate oxidation
None	39	21
NaCl, 15 mM	189	82
$MgCl_2$, 0.5 mM	175	96
NaCl, 15 mM plus $MgCl_2$, 0.5 mM	464	328
FCCP, 0.5 μM	980	980

The values given refer to the extramitochondrial $[Ca^{2+}]$ required to give half-maximal response and have been calculated from the experiments illustrated in fig.3a,b [15]

4. Investigation of the effects of changes in extramitochondrial Ca^{2+} on the activities of pyruvate dehydrogenase and oxoglutarate dehydrogenase within intact coupled rat heart mitochondria

When coupled rat heart mitochondria are incubated with an oxidizable substrate (such as oxoglutarate with malate) in the presence of EGTA (i.e., Ca^{2+} $<10^{-9}$ M) only about 10% of the pyruvate dehydrogenase is found in the active form. However, on increasing the extramitochondrial $[Ca^{2+}]$ the proportion is increased to about 60% (fig.3a). In the absence of added Na^+ and Mg^{2+}, half-maximal effects of extramitochondrial Ca^{2+} are seen at about 40 nM. Since PDHP-phosphatase in both mitochondrial extracts and uncoupled mitochondria from rat heart has a $k_{0.5}$ for Ca^{2+} of about 1 μM, it follows that under these conditions the $[Ca^{2+}]$ inside the mitochondria is probably about 25-times the extramitochondrial $[Ca^{2+}]$. From the properties of the uptake and efflux components of the Ca^{2+}-transporting systems in rat heart mitochondria described by Crompton, Carafoli and their colleagues [1,2,29–33], it would be predicted that addition of either Na^+ (which stimulates the efflux pathway) or Mg^{2+} (which inhibits uptake) should reduce the gradient of Ca^{2+} across the mitochondrial inner membrane (in:out) and thus a relatively higher extramitochondrial $[Ca^{2+}]$ should be required to cause half-maximal activation of pyruvate dehydrogenase. This prediction is fulfilled (fig.3a). Moreover, the effects of Mg^{2+} and Na^+ appear to be independent of each other, as expected if Mg^{2+} and Na^+ act on the separate uptake and efflux components. More importantly, the effects of Mg^{2+} and Na^+ are largely on the $k_{0.5}$ for Ca^{2+} with little or no effects being observed on pyruvate dehydrogenase activity either in the absence of Ca^{2+} or on the extent of maximum stimulation observed with Ca^{2+}. From this it can be concluded that the effects of Na^+ and Mg^{2+} are exerted exclusively via changes in the components of the Ca^{2+}-transporting system. In the intact heart cell, the cytoplasmic $[Mg^{2+}]$ is probably about 1 mM [34] and $[Na^+]$ above 5 mM [35]. These concentrations are sufficient to bring about a maximal effect of these ions on the sensitivity to extramitochondrial Ca^{2+} of PDH activity within intact rat heart mitochondria [15]. In the presence of both Mg^{2+} and Na^+, the extramitochondrial $[Ca^{2+}]$ which gives half-maximal activation is close to 0.5 μM. In other words, under conditions which would appear to approximate to those present in the cell, the intramitochondrial Ca^{2+} is probably only about twice the extramitochondrial Ca^{2+}. Moreover, changes in extramitochondrial Ca^{2+} in the physiological range (0.1–1 μM) activate intramitochondrial pyruvate dehydrogenase activity (fig.3a).

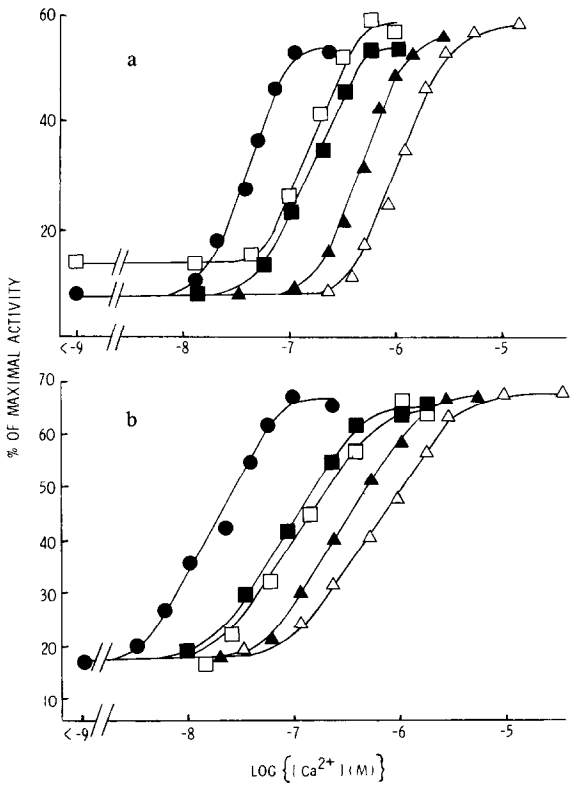

Fig.3. Sensitivity of pyruvate dehydrogenase activity and oxoglutarate oxidation in coupled rat heart mitochondria to changes in extramitochondrial Ca^{2+}. (a) Pyruvate dehydrogenase activity: Mitochondria were incubated at 30°C in 125 mM KCl, 20 mM Tris/HCl (pH 7.3), 5 mM KH_2PO_4, 15 mM oxoglutarate, 1 mM malate plus CaEGTA buffers to give required $[Ca^{2+}]$ and in the presence of: no further additions (●); 15 mM NaCl (■); 0.5 mM $MgCl_2$ (□); 15 mM NaCl plus 0.5 mM $MgCl_2$ (▲); 0.5 μM FCCP (△). (b) Oxoglutarate oxidation: Mitochondria were incubated under the same conditions except that 2 mM ADP was also added and the initial concentration of oxoglutarate was 0.5 mM. Full details may be obtained from [15].

We have been able to greatly strengthen these conclusions by studying the effects of changes in extramitochondrial Ca^{2+} on the oxidation of oxoglutarate by coupled rat heart mitochondria in the presence of malate and excess ADP (fig.3b) [15]. At low [oxoglutarate], increases in extramitochondrial Ca^{2+} enhances the rate of oxoglutarate oxidation but no effects are apparent at saturating [oxoglutarate]. The $k_{0.5}$ values

for the effects of Ca^{2+} on oxoglutarate oxidation closely match the values obtained for the elevation of pyruvate dehydrogenase activity (table 3). In particular, both Na^+ and Mg^{2+} increase the $k_{0.5}$ value by about 4-fold. In the presence of both Na^+ and Mg^{2+}, the $k_{0.5}$ is about 330 μM or about 1/3rd of the value found with both uncoupled mitochondria or with the separated rat heart oxoglutarate dehydrogenase. Thus under physiological conditions the gradient of Ca^{2+} across the mitochondrial membrane (in:out) would appear to be about 3 and changes in extramitochondrial Ca^{2+} would be expected to result in changes in OGDH activity.

We have been unable to explore the changes in activity of NAD–ICDH in heart mitochondria because of the low activity of the tricarboxylate carrier [36]. However, in coupled epididymal white fat cell mitochondria this carrier appears to be very active [37], and thus it has been possible to show in these mitochondria that (in the presence of Mg^{2+} and Na^+) PDH, NAD–ICDH and OGDH are all activated in parallel by increasing the extramitochondrial $[Ca^{2+}]$ to 0.1–1 μM (unpublished).

5. The role of Ca^{2+} within mitochondria

Overall the evidence supporting a role for Ca^{2+} in the regulation of intramitochondrial metabolism in mammalian tissues compares quite favourably with the evidence underlying the widely accepted view [3–5] that Ca^{2+} plays a key role in the regulation of a wide range of cytoplasmic events. So far three different Ca^{2+}-sensitive intramitochondrial enzymes, all dehydrogenases, have been recognised and in each case it has been possible to demonstrate similar sensitivities to Ca^{2+} not only with the separated enzymes from many different mammalian tissues but also when the enzymes are in situ within intact mitochondria. The techniques (Ca^{2+}-sensitive dyes, photoproteins and microelectrodes) which are being developed to measure the cytoplasmic $[Ca^{2+}]$ [38–40] are not (as yet!) applicable to the mitochondrial matrix. Instead, at least in isolated coupled rat heart and fat cell mitochondria, the three dehydrogenases can be used as a series of independent means of assessing the intramitochondrial $[Ca^{2+}]$. So far, this approach has indicated that within these tissues the gradient of Ca^{2+} across the inner mitochondrial membrane (in:out) is probably only about 2 or 3 and that changes in the cytoplasmic $[Ca^{2+}]$ at 0.1–1 μM are likely to be relayed into the mitochondria and alter in parallel the activity of the three dehydrogenases.

In heart and other muscles, it makes good sense that intramitochondrial oxidative metabolism is stimulated when the cytoplasmic $[Ca^{2+}]$ is increased, since this will usually occur in association with the stimulation of muscle contraction and therefore ATP utilisation. In both heart and gastroenemius muscle, increases in PDH activity have been observed under conditions of increased work load [41,42] and these may be brought about by an increase in mitochondrial Ca^{2+}. It should be noted that it may be advantageous in cells to increase the supply of reducing power to the respiratory chain under conditions of enhanced ATP utilisation without necessarily increasing the concentration ratios of ADP/ATP and/or NAD^+/NADH.

All mammalian tissues so far studied contain the three Ca^{2+}-sensitive dehydrogenases so it seems reasonable to extend the above arguments to other tissues. However, in a number of tissues including liver, kidney and smooth muscle the efflux of Ca^{2+} from mitochondria is accomplished by a Na^+-independent pathway [1,2]. Thus the relationship between intra- and extramitochondrial Ca^{2+} in these tissues may be different to that in heart and adipose tissue although the dehydrogenases show the same sensitivity to Ca^{2+}. Nevertheless, there are many examples of hormones and neurotransmitters which are thought to bring about their effects via an increase in cytoplasmic Ca^{2+} [28, 43,44]. Often these effects (such as increases in secretion or ion transport) involve increased utilisation of ATP and thus are associated with increased oxidative metabolism in the mitochondria.

In some tissues PDH and OGDH do not play a strictly catabolic role but are important control points in overall synthetic pathways. This applies to PDH in the synthesis of fatty acids from carbohydrate in liver, mammary gland and fat cells [8] and OGDH in the synthesis of glucose from glutamine, glutamate and certain other amino acids in kidney and liver [45]. The hormonal control of these processes must involve changes in the activity of PDH or OGDH and the possibility that changes in mitochondrial Ca^{2+} may be involved has to be considered. A possible role for Ca^{2+} in the increase in PDH activity in fat cells exposed to insulin [7,8,24,46] and in liver cells exposed to vasopressin [46,47] has been raised and discussed in detail elsewhere.

Nicholls has emphasised the ability of mammalian mitochondria to 'buffer' the extramitochondrial

$[Ca^{2+}]$ at about 1 μM [2,6]. Above this concentration the efflux pathway appears to be saturated and thus Ca^{2+} is taken up by the mitochondria until the extramitochondrial $[Ca^{2+}]$ returns to 1 μM and the rate of Ca^{2+} influx again balances the rate of Ca^{2+} efflux. We are doubtful about the physiological importance of this phenomenon since at an extramitochondrial $[Ca^{2+}]$ of 1 μM, the Ca^{2+}-sensitive systems in both cytoplasmic and mitochondrial compartments would be largely saturated. Moreover, uptake of Ca^{2+} under these conditions may result in sufficient intramitochondrial accumulation of calcium to cause general damage to mitochondrial function [15].

In the long term the cytoplasmic $[Ca^{2+}]$ must depend largely on the transfer of Ca^{2+} across the cell membrane. In addition, short term variations in the cytoplasmic $[Ca^{2+}]$ may be brought about by transfer across the endoplasmic reticulum in many cells and certainly in muscle. All available evidence suggests that in the resting cell, the cytoplasmic $[Ca^{2+}]$ must be considerably below 1 μM [48,49]; yet regulation of cytoplasmic $[Ca^{2+}]$ by mitochondria would appear to be restricted to situations where the cytoplasmic $[Ca^{2+}]$ is above 1 μM. It must be emphasised, in any case, that regulation of the cytoplasmic Ca^{2+} by mitochondria implies inverse changes in $[Ca^{2+}]$ in the cytoplasm and mitochondrial matrix; this is not in general compatible with a role for Ca^{2+} in the regulation of oxidative metabolism in mammalian mitochondria.

Rather than mitochondria having a role in the regulation of cytoplasmic $[Ca^{2+}]$, we view the $[Ca^{2+}]$ in the cytoplasm usually determining the intramitochondrial $[Ca^{2+}]$. Of course, the possibility also exists that certain hormones or other factors may alter the activity of one or both of the components of the mitochondrial-transport system and thus regulate intramitochondrial Ca^{2+} without necessarily changing the cytoplasmic $[Ca^{2+}]$.

If the arguments we have put forward in this article are correct then only about 0.1% of the calcium associated with mammalian mitochondria is present as free Ca^{2+} since the total mitochondrial calcium is equivalent to more than 1 mM [50], even after incubation in the presence of EGTA [51]. At first sight this ratio between bound and free is rather startling but it is probably not very different from that occurring in the extramitochondrial compartment of cells [52]. Nevertheless, very little is known about the relationship between bound and free forms of calcium in mitochondria and further study is clearly warranted.

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