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Intracellular calcium ions and intramitochondrial Ca^{2+} in the regulation of energy metabolism in mammalian tissues

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It is well-established that many hormones and other extracellular stimuli bring about their effects on key intracellular processes in mammalian cells by causing changes in the cytosolic concentration of calcium ions (e.g. Rasmussen & Barrett, 1984; Carafoli, 1987). It is also generally appreciated that all the processes stimulated by increases in the cytosolic concentration of Ca^{2+} (e.g. contraction, secretion) are energy-requiring, and that therefore the promotion of such events is also accompanied by compensatory stimulations in oxidative metabolism to produce ATP at enhanced rates (Denton & McCormack, 1985; Hansford, 1985). According to the classical theory of respiratory control, this promotion of oxidative phosphorylation would be driven by the decline in cytoplasmic phosphorylation potential (i.e. ATP/ADP × inorganic phosphate value; or a component of this) which would be brought about by the stimulated energy-requiring event(s) (e.g. Chance & Williams, 1956; Nicholls, 1984; Gibbs, 1985; Chance *et al.* 1986). However, there is now a plethora of reports which indicate that under such conditions of Ca^{2+}-mediated cell stimulation, these expected declines in phosphorylation potential are not observed, and indeed that in many such cases this key cellular variable actually increases (e.g. Siess *et al.* 1978; Titheradge & Haynes, 1980; Aprille *et al.* 1982; Balaban *et al.* 1986; From *et al.* 1986; Soboll & Scholtz, 1986; Katz *et al.* 1988, 1989). Indeed, the most notable feature of these studies is the pronounced lack of correlation between the phosphorylation potential and the varied degrees of oxidative metabolism. This obviously suggests that under such circumstances additional factors may be involved in the control of oxidative phosphorylation (Denton & McCormack, 1985; Hansford, 1985; Brand & Murphy, 1987).

An attractive alternative hypothesis for the promotion of oxidative phosphorylation in Ca^{2+}-stimulated cells during maintenance or enhancement of the phosphorylation potential, which is the main topic of the present paper, is that some degree of regulation of the respiratory rate is achieved through increases in NADH supply to the respiratory chain (Denton & McCormack, 1980, 1985; Hansford, 1985; Brand & Murphy, 1987; McMillin & Pauly, 1988; Katz *et al.* 1988, 1989). It can readily be shown that increases in the NADH:NAD^+ redox potential can promote increases in respiration and ATP production in isolated mitochondria without any changes or even increases in extramitochondrial phosphorylation potential (e.g. McCormack, 1985*a*; McCormack & Denton, 1986*a*; Koretsky & Balaban, 1987), and such a mechanism will be of obvious advantage to cells at times of increased energy utilization. There are also many reports of increased cellular or tissue NAD(P)H content under such circumstances (e.g. Sugano *et al.* 1980; Balaban & Blum, 1982; Blackmore *et al.* 1983; Katz *et al.* 1987; Koretsky *et al.* 1987).

The proposed mechanism for bringing about the increases in intramitochondrial

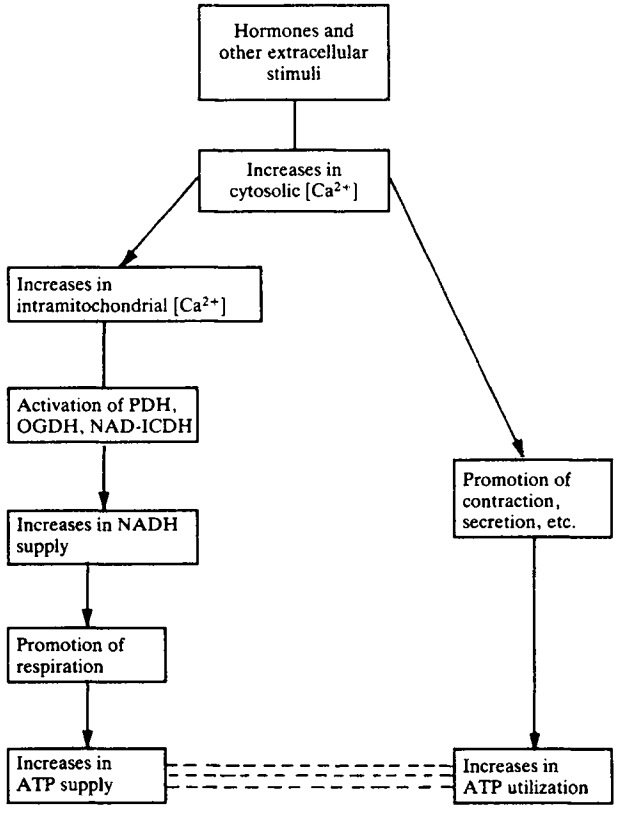

Fig. 1. Outline of the role of intramitochondrial calcium ions in providing a mechanism for increased energy production to balance the increased energy demands promoted by increases in cytosolic Ca^{2+}. PDH, pyruvate dehydrogenase complex; OGDH, oxoglutarate dehydrogenase complex; NAD-ICDH, NAD^+-linked isocitrate dehydrogenase.

NADH is also Ca^{2+}-dependent and involves the relay of the increases in cytosolic Ca^{2+} into the mitochondrial matrix. The rise in matrix Ca^{2+} then brings about the increases in NADH and, hence, ATP production by causing the activation of three key intramitochondrial dehydrogenases, namely the pyruvate (PDH), NAD^+-isocitrate (*EC* 1.1.1.41; NAD-ICDH) and 2-oxoglutarate (OGDH) dehydrogenases (see Denton & McCormack, 1985; Hansford, 1985; McMillin & Pauly, 1988). This mechanism is thus somewhat analogous to the well-accepted concept of the promotion of glycogenolysis to balance energy needs through raised cytosolic Ca^{2+} concentration increasing phosphorylase kinase (*EC* 2.7.1.38) activity (Cohen, 1978). A general outline of this mechanism, whereby the increased energy utilization promoted by raised cytosolic Ca^{2+} is balanced by increased energy production promoted by raised intramitochondrial Ca^{2+}, is shown in Fig. 1.

Another important consequence of the previously mentioned proposal is that, under normal physiological circumstances, the primary function of the mitochondrial Ca^{2+} transport system would be to regulate matrix Ca^{2+} and thus allow this to respond to

changes in cytosolic Ca^{2+} (see Denton & McCormack, 1985). This is directly in contrast to the earlier proposals that the principal function of mitochondrial Ca^{2+} transport was to act as a buffer system involved in the regulation of cytosolic Ca^{2+} concentration (see e.g. Fiskum & Lehninger, 1982; Akerman & Nicholls, 1983), and that mitochondria behaved as Ca^{2+} stores from which it could be mobilized by hormones (e.g. Exton, 1980; Williamson *et al.* 1981). These two contrasting functions are almost certainly mutually exclusive (Denton & McCormack, 1985; Hansford, 1985) and it is the former, as will be argued later, which is now thought to be the principal one under normal physiological conditions. The existing evidence in support of the previously stated concept will now be described.

THE CALCIUM-SENSITIVE INTRAMITOCHONDRIAL DEHYDROGENASES AND THEIR PROPERTIES

The enzymes of the PDH system, NAD-ICDH and OGDH are all located exclusively within the mitochondria of mammalian cells. PDH, NAD-ICDH and OGDH all catalyse irreversible oxidative decarboxylations of their appropriate substrates and are major sites of matrix NADH production which is, of course, the major substrate for the respiratory chain and subsequent ATP production. These three dehydrogenases have also been widely regarded for many years as being key regulatory sites in oxidative metabolism (e.g. Williamson *et al.* 1981) and their maximal activities are close to the maximal flux rates through the oxidative pathways.

PDH, NAD-ICDH and OGDH can each be activated severalfold by increases in Ca^{2+} concentration within the approximate range 0·05–5 μM and with the concentration of the effector required for half-maximal response ($K_{0.5}$) for Ca^{2+} of about 0·5–1 μM; however, recent findings (Rutter & Denton, 1988) suggest that NAD-ICDH may respond to a slightly higher Ca^{2+} range than the other two enzymes. Ca^{2+} activates PDH by causing increases in the amount of active, non-phosphorylated enzyme (PDH_a) through its activation of PDH phosphate phosphatase (Denton *et al.* 1972). Ca^{2+} activates NAD-ICDH and OGDH more directly by causing marked decreases in their Michaelis constant (K_m) values for their respective substrates, *threo*-D_S-isocitrate (Denton *et al.* 1978) and 2-oxoglutarate (McCormack & Denton, 1979).

All three of these key dehydrogenases can also be activated by decreases in the $NADH:NAD^+$ and ATP:ADP ratios. In addition PDH can be activated by decreases in acetyl-CoA:CoA and OGDH by decreases in succinyl CoA:CoA ratios, and both NAD-ICDH and OGDH can be activated by decreases in pH within the physiological range (see McCormack & Denton, 1979). However, the regulation of the enzymes by Ca^{2+} (as an 'extrinsic' effector) is largely independent of these other regulatory phenomena (by 'intrinsic' effectors), which is again supportive of the concept that Ca^{2+}, in its role as a second-messenger mediator for hormone effects, could override the effects of these local energy metabolites.

The Ca^{2+} sensitivity of PDH, NAD-ICDH and OGDH has been demonstrated in all vertebrate tissues so far examined, but not in invertebrates (McCormack & Denton, 1981*b*). There may, therefore, be a functional evolutionary link between the Ca^{2+} sensitivity of these enzymes and the ability of mitochondria to transport Ca^{2+} by specific pathways, as both of these phenomena may be restricted to vertebrate tissues (McCormack & Denton, 1981*b*, 1986*a*). The enzymes from insects and plants, however,

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do still exhibit regulation by nucleotide ratios and by pH (McCormack & Denton, 1981*b*). It is also of interest to point out that the concept of Ca^{2+} regulation of mitochondrial dehydrogenase activity may also still prevail in these instances, except that the dehydrogenases involved are on the outside face of the mitochondrial inner membrane, and will thus respond to changes in cytosolic, rather than intramitochondrial, Ca^{2+} concentrations. These Ca^{2+}-sensitive enzymes are the external NADH dehydrogenase (*EC* 1.6.99.3) of plant mitochondria (Moore & Akerman, 1984), and the α-glycerophosphate dehydrogenase which is found in insect mitochondria (Hansford, 1980); the latter enzyme is also present, and has been shown to exhibit Ca^{2+} sensitivity, in vertebrate tissues (Hansford, 1980).

There are two other enzymes found in the mitochondrial matrix of mammalian tissues which may be regulated by micromolar concentrations of Ca^{2+}, and which may be involved in the regulation of energy metabolism. Yamada and co-workers (Yamada *et al.* 1980; Yamada & Huzel, 1988) have described a novel mitochondrial ATPase inhibitor protein, which is distinct from the well-characterized Pullman & Munroy (1963) protein, and whose binding to the ATPase is inhibited by increases in Ca^{2+} concentration. The response to Ca^{2+} is biphasic, with optimal release and enzyme activation occurring at about 1 μM, with a return to basal levels at higher Ca^{2+} concentration. Inhibition by this protein is additive to that of the Pullman–Munroy inhibitor (Yamada & Huzel, 1988). The physiological role of the Ca^{2+}-sensitive inhibitor protein is as yet unknown, but obviously it may work in concert with the regulation of the dehydrogenases, especially if it has similar effects when the enzyme is working as the ATP synthetase rather than as an ATPase.

The other matrix enzyme which exhibits regulation by micromolar concentrations of Ca^{2+} is pyrophosphatase. Most of the pyrophosphatase activity in mammalian cells is cytosolic, but appreciable activity is also present in the mitochondrial matrix (Davidson & Halestrap, 1989), and most of the cellular pyrophosphate appears to be mitochondrial (Davidson & Halestrap, 1988). The substrate for this enzyme is magnesium pyrophosphate, but calcium pyrophosphate is a powerful competitive inhibitor, and the Ca^{2+} sensitivity of the enzyme depends on magnesium ion concentration (Davidson & Halestrap, 1989). At a probable matrix concentration of Mg^{2+} of 0·3 mM (Corkey *et al.* 1986), the $K_{0.5}$ for inhibition by Ca^{2+} is 3–4 μM (Davidson & Halestrap, 1987). It is thought that this Ca^{2+}-dependent inhibition of pyrophosphatase is responsible for the increases in tissue pyrophosphate content of the liver exposed to 'Ca-mobilizing' hormones (Davidson & Halestrap, 1988). The increases in matrix pyrophosphate concentration are then thought to bring about increases in mitochondrial matrix volume through increasing the electrophoretic uptake of potassium ions (and probably phosphate) (Davidson & Halestrap, 1987, 1988, 1989) by a mechanism that appears to involve the adenine nucleotide translocase. These increases in matrix volume can lead to the stimulation of respiration, apparently by stimulating electron flow into the ubiquinone pool by an as yet unknown mechanism. This phenomenon is clearly important and appears to occur in cells (Davidson & Halestrap, 1989), and may thus again work in concert with the activation of the dehydrogenases. The regulation and role of matrix volume is discussed further in the recent review by Halestrap (1989) to which the reader is referred for more information on this topic. The remainder of the present paper is devoted to the evidence for the physiological regulation of the previously described dehydrogenases by intramitochondrial Ca^{2+}, and also its physiological implications.

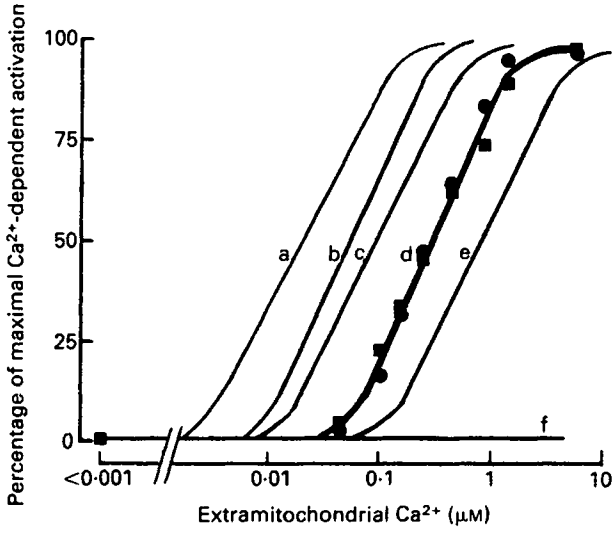

Fig. 2. The activations of the pyruvate dehydrogenase complex (●) and the oxoglutarate dehydrogenase complex (■) by calcium ions within intact rat heart mitochondria. Values are taken from Denton *et al.* (1980) from where full details can be obtained, or are similar but unpublished observations of J. G. McCormack. Mitochondria were incubated at pH 7·3 and 30° in a potassium chloride-based buffer with other appropriate additions, and in the presence of EGTA-Ca buffers to give the concentrations of extramitochondrial Ca^{2+} shown, together with (a) no further additions, (b) 10 mM-sodium chloride, (c) 0·5 mM-magnesium chloride, (d) (thick line) sodium plus magnesium, (e) 1 μM-carbonyl cyanide p-trifluoromethoxyphenylhydrozone (uncoupler; FCCP), (f) 1 μM-ruthenium red.

REGULATION OF THE DEHYDROGENASES BY CALCIUM IONS WITHIN INTACT MITOCHONDRIA

There are now available several different methods for studying the Ca^{2+} regulation of these enzymes within intact mitochondria (McCormack & Denton, 1989). Thus, studies on intact, fully coupled mitochondria from rat heart (Denton *et al.* 1980), liver (McCormack, 1985*a*,*b*), brain (Hansford & Castro, 1985), skeletal muscle (Ashour & Hansford, 1983; Fuller & Randle, 1984), kidney (McCormack *et al.* 1988), brown (McCormack & Denton, 1980) and white (Marshall *et al.* 1984) adipose tissue, and within permeabilized pig lymphocytes (Baumgarten *et al.* 1983), have now fully established that these matrix enzymes can be activated as the result of increases in the extramitochondrial concentration of Ca^{2+} within the expected physiological range, i.e. approximately 0·05–2 μM (Carafoli, 1987), and with $K_{0.5}$ values for extramitochondrial Ca^{2+} of about 0·3–0·6 μM (see Fig. 2). This is achieved in the presence of expected physiological concentrations of sodium ions and Mg^{2+} (Fig. 2). This is because the Ca^{2+} transport system of mammalian mitochondria is a cycle which consists of the electrophoretic Ca^{2+} uniporter that is driven by the membrane potential (180 mV, negative inside) set up by proton extrusion by the respiratory chain and which is inhibited by Mg^{2+} (and also artificially by ruthenium red, but activated by spermine), and probably two egress mechanisms, the principal of which is an electroneutral Na^+/Ca^{2+} exchanger that is also driven by the proton-motive gradient through subsequent Na^+/hydrogen ion exchange and which is inhibited by increases in extramitochondrial Ca^{2+} (and artificially by diltiazem and other similar drugs) (see Akerman & Nicholls, 1983; Crompton, 1985;

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Denton & McCormack, 1985); the Na^+-independent egress mechanism is poorly characterized but may involve direct $Ca^{2+}/2H^+$ exchange. Therefore, in the absence of Na^+ or Mg^{2+}, or both, there are additive diminutions in the effective Ca^{2+} concentration ranges (see Fig. 2).

The matrix Ca^{2+}-sensitive dehydrogenases exhibit sensitivity to the same ranges of Ca^{2+} in intact, but uncoupled, mitochondria as they do in mitochondrial extracts (Fig. 2) where there appears to be a free equilibrium of Ca^{2+} across the inner membrane (McCormack & Denton, 1980). This, therefore, suggests (e.g. Fig. 2) that under normal physiological conditions the gradient of Ca^{2+} ions (in:out) across the mitochondrial inner membrane is very low (of the order of 1–3). Moreover, over the ranges of Ca^{2+} where the Ca^{2+} sensitivity of these enzymes is evident, the total Ca content of the mitochondria is very low and approximately 0–4 nmol/mg mitochondrial protein (Assimacopoulos-Jeannet *et al.* 1986; Hansford & Castro, 1982). These values are in good agreement with recent estimates of total mitochondrial Ca content measured in situ using X-ray probe microanalysis, which also show very little evidence for any substantial gradients of Ca^{2+} between the matrix and the cytosol (e.g. Somlyo *et al.* 1985; Wendt-Gallitelli, 1986). This would also suggest that only about 0·1% of the mitochondrial Ca is free in the matrix (assuming a matrix space of about 1 μl/mg protein), a value of free:total Ca similar to that in the cytosol (Carafoli, 1987). It is worth pointing out that for mitochondria to behave as buffers of the extramitochondrial Ca^{2+} environment, the egress pathways for Ca^{2+} have to be saturated, and that this will not occur in the presence of Na^+ until there is in excess of some 10–15 nmol total Ca/mg mitochondrial protein (see Denton & McCormack, 1985). (There is overall approximately tenfold greater capacity for uptake than egress.) Additionally, in the presence of physiological Na^+ and Mg^{2+} concentrations, the actual set-point at which the mitochondria buffer extramitochondrial Ca^{2+} seems to be in excess of about 2–3 μM. Therefore, it would appear that under physiological circumstances, buffering behaviour would only occur when Ca^{2+}-sensitive processes in both the cytosol and the mitochondrial matrix would be saturated for Ca^{2+}. This has led to the proposal that this property of the mitochondrial Ca^{2+} transport system may be a reserved protective function for times when there is abnormal Ca^{2+} uptake into cells, as may occur during ischaemia and reperfusion of tissues (for example, see Denton & McCormack, 1985); however, the ability of mitochondria to accumulate Ca^{2+} in this way is not limitless and can lead to the deterioration of their bioenergetic functions (Denton *et al.* 1980; McCormack, 1985*a*; Crompton *et al.* 1987). The reason behind the earlier misconceptions as to mitochondria being the source of the activator Ca^{2+} released by hormones into the cytosol probably results from there being substantial contamination of crude mitochondrial fractions by inositol trisphosphate-sensitive microsomal Ca pools (see Assimacopoulos-Jeannet *et al.* 1986).

Recently it has become possible to substantially corroborate the previously mentioned estimates of matrix Ca^{2+} concentration which were effectively made by using the activity statuses of the Ca^{2+}-sensitive dehydrogenases as probes for intramitochondrial Ca^{2+} in intact mitochondria. This has followed the demonstration that the fluorescent Ca^{2+} indicators, fura-2, indo-1 and quin-2, can be successfully entrapped into the matrix of rat heart mitochondria following their incubation with the appropriate acetoxymethyl ester (Lukacs & Kapus, 1987; Davis *et al.* 1987; Lukacs *et al.* 1988; Moreno-Sanchez & Hansford, 1988; Reers *et al.* 1989; McCormack *et al.* 1989), i.e. in much the same way as the now well-developed methodology for loading such compounds into cells (Cobbold &

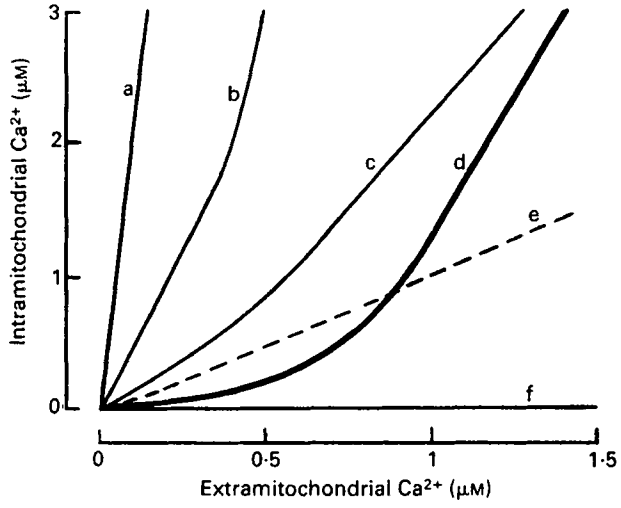

Fig. 3. Relationships between extramitochondrial free calcium ions and intramitochondrial free Ca^{2+} concentrations in fura-2-loaded rat heart mitochondria within the regulatory ranges of the matrix Ca^{2+}-sensitive dehydrogenases. Values are taken from McCormack *et al.* (1989) from where full details can be obtained. Mitochondria were loaded with fura-2 and incubated in potassium chloride-based buffer with appropriate additions and in the presence of EGTA-Ca buffers to give the extramitochondrial concentrations of Ca^{2+} shown, together with (a) no further additions, (b) 10 mM-sodium chloride, (c) 2 mM-magnesium chloride, (d) (thick line) sodium plus magnesium, (e) 1 μM-carbonyl cyanide p-trifluoromethoxyphenylhydrozone (uncoupler; FCCP), (f) 1 μM-ruthenium red.

Rink, 1987). Appropriate conditions of loading can be chosen so that the functional viability of the mitochondria do not appear to be compromised (see McCormack *et al.* 1989). Most studies have been done with fura-2; however indo-1 gives signals which are as good, whereas quin-2 has to be loaded to a higher concentration and, therefore, longer and more detrimental incubation times are required (Lukacs & Kapus, 1987; Moreno-Sanchez & Hansford, 1988). The pH indicator 2′7′-bis-2(carboxyethyl-5(6)-carboxy-fluorescein) (BCECF) has also been successfully loaded into rat heart mitochondria in a similar manner (Davis *et al.* 1987). Liver mitochondria have as yet proved more difficult to load with such indicators (Gunter *et al.* 1988), and the only other attempt with loading mitochondria to date has been with rat brain mitochondria where the mitochondria were incubated with very high Ca^{2+} concentrations (Komulainen & Bondy, 1987).

The studies with fura-2-loaded rat heart mitochondria have, therefore, allowed confirmation that at least PDH and OGDH respond to matrix Ca^{2+} over the approximate concentration range of 0·1–2 μM with $K_{0.5}$ for matrix Ca^{2+} of around 0·3–0·8 μM (Lukacs *et al.* 1988; Moreno-Sanchez & Hansford, 1988; McCormack *et al.* 1989). (NAD-ICDH is more difficult to study in these mitochondria owing to their very low activity of the tricarboxylate carrier (Chappell & Robinson, 1968).) In addition, the corresponding total mitochondrial Ca content was found to range from about 1–5 nmol/mg protein over this range of matrix free-Ca^{2+} concentrations (Davis *et al.* 1987; Lukacs *et al.* 1987). What is even more significant, however, is that these studies have revealed strong evidence to suggest that in the presence of physiological concentrations of Na^+ and Mg^{2+} there is a large degree of positive co-operativity in the transmission of the Ca^{2+} signal across the mitochondrial inner membrane and into the matrix (Moreno-Sanchez & Hansford, 1988; McCormack *et al.* 1989) (Fig. 3). Thus, at low concentrations

of extramitochondrial Ca^{2+} (e.g. about 100 nM), such as might be expected in unstimulated cells (Carafoli, 1987), there appears to be a gradient of Ca^{2+} ions across the inner membrane (in:out) of much less than unity, in fact only about 0·3. This also suggests that the matrix Ca^{2+} concentration would be below the activatory ranges for the enzymes under such conditions. As extramitochondrial Ca^{2+} is raised to around the $K_{0.5}$ for the enzymes' activations (around 0·4–0·6 μM) the gradient approaches unity (Fig. 3), whereas at higher values, e.g. about 1–2 μM as might be expected in fully stimulated cells, the gradient rises to about 2–3 (Fig. 3). Such positive co-operative behaviour in signal transmission of Ca^{2+} to the matrix had in fact been predicted in earlier computer simulations (Crompton, 1985) using the known kinetic variables of the Ca^{2+} transport system. Of particular importance in this regard are the pronounced sigmoidal characteristics of Ca^{2+} uptake in the presence of Mg^{2+}, and the inhibition of the Na^{+}-dependent Ca^{2+}-egress mechanism by increases in extramitochondrial Ca^{2+} within this range (see Crompton, 1985; McCormack *et al.* 1989).

EVIDENCE FOR THE HORMONAL REGULATION OF OXIDATIVE ENERGY METABOLISM THROUGH CHANGES IN INTRAMITOCHONDRIAL CALCIUM IONS

The most extensively studied systems to date in this regard have been the effects of positive inotropic stimulation on the rat heart, and the effects of 'Ca-mobilizing' hormones on rat liver; however, the conclusions and implications derived from the work on these two model systems are likely to be much more widespread in mammalian tissues. In each of these two circumstances, these treatments not only bring about well-characterized increases in the cytosolic concentration of Ca^{2+}, but it is also well-established that increases occur in oxygen uptake, flux through the citric acid cycle, and in the proportion of PDH existing as PDH_a (see McCormack & Denton, 1986*b* and McCormack *et al.* 1986 respectively). The evidence that these latter effects are due to the relay of the increases in cytosolic Ca^{2+} into the mitochondrial matrix and activation of the dehydrogenases is described later, and is as yet, though convincing, still rather indirect in nature. Perhaps in the not too distant future there may be actual direct evidence for changes in intramitochondrial Ca^{2+} in intact cells through the continued application of microscopic fluorescence-imaging technology, as there is already evidence that at least a proportion of compounds such as fura-2 may localize in this compartment in some cell preparations (see e.g. Davis *et al.* 1987; Steinberg *et al.* 1987).

In the heart, it is well-established that hormones such as adrenaline and other positive inotropic agents bring about their effects on contractile activity by raising the sarcoplasmic concentration of Ca^{2+}. Although this is on a beat-to-beat basis, clear increases in the 'time-averaged' concentration of cytosolic Ca^{2+} occur (e.g. Marban *et al.* 1980) which are within the ranges leading to activation of the Ca^{2+}-sensitive dehydrogenases in intact mitochondrial preparations incubated under expected physiological conditions (Fig. 2). Although these agents bring about clear increases in heart O_2 uptake, in many such cases there is no correlation at all between these and the measured (either chemically or by ^{31}P nuclear magnetic resonance spectroscopy (^{31}P-NMR)) values of components of the phosphorylation potential (e.g. Neely *et al.* 1972; Allen *et al.* 1986; Balaban *et al.* 1986; From *et al.* 1986; Katz *et al.* 1988, 1989; Unitt *et al.* 1989). Clearly, therefore, oxidation rates are not regulated entirely by the phosphorylation potential ratio or a component of this.

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Table 1. *Effects of inotropic stimulation, and of ruthenium red (RR) and pyruvate, on the active non-phosphorylated form of the pyruvate dehydrogenase complex (PDH_a) content and oxidative metabolism of the perfused rat heart*

(Hearts were perfused with 10 mM-glucose (10–20 min) and the indicated additions (for 1–2 min) as described by McCormack & England (1983) and Unitt *et al.* (1989) from where full details can be obtained)

Conditions	PDH_a (as % of total PDH)		O_2 uptake (μmol/h per g dry wt)	
	−RR	+RR(2·5 μM)	−RR	+RR
Control (1·25 mM-Ca^{2+}, 7 kPa)	10	11	1·8	1·5
5 mM-Pyruvate	34	33	nd	nd
1 μM-Adrenaline	41	13*	nd	nd
0·2 μM-Isoprenaline	46	9*	3·4	2·9
Medium Ca^{2+} raised to 5 mM	42	13*	nd	nd
Pressure increased to 16 kPa	36	13*	nd	nd

nd, not determined.
The effect of RR was significant: *$P<0·05$.

Significantly, therefore, the administration of positive inotropic agents, or raised perfusate Ca^{2+} concentration, to intact heart preparations was found to increase the amount of PDH_a by up to fourfold within about 30 s (Hiraoka *et al.* 1980; McCormack & Denton, 1981*a*) (Table 1). Moreover, these increases in PDH_a could be completely blocked by perfusing the hearts with the potent inhibitor of mitochondrial Ca^{2+} uptake, ruthenium red (McCormack & England, 1983); this was not the case for activations of PDH brought about by pyruvate or dichloroacetate which inhibit PDH_a kinase (*EC* 2.7.1.99) (see McCormack & England, 1983) (Table 1). The increases in contractile force or phosphorylase *a* activity (as indicators for cytosolic Ca^{2+}) brought about by the positive inotropes were largely unaffected by ruthenium red, although it did have some negative inotropic effects in their absence (McCormack & England, 1983). Hansford (1987) has shown that in quin-2-loaded myocytes, ruthenium red does not prevent the increases in cytosolic Ca^{2+} brought about by various treatments designed to elicit this response, but again blocked the associated increases in PDH_a. In recent studies using ^{31}P-NMR (Katz *et al.* 1988, 1989; Unitt *et al.* 1989), it has been shown that in the absence of ruthenium red the phosphorylation potential and creatine phosphate content of the hearts is remarkably stable during inotropic and oxidative stimulation, whereas in its presence there are clear decreases in phosphorylation potential and in creatine phosphate content (see Fig. 4). Unitt *et al.* (1989) also observed a slight, but significant, depression in the O_2 uptake response to isoprenaline in the presence of ruthenium red, suggesting that the transmission of the Ca^{2+} signal to the matrix may be required for the full oxidative response to occur.

The previously discussed observations strongly suggest that when the Ca^{2+}-dependent mechanism for activating oxidative metabolism via the matrix Ca^{2+}-sensitive dehydrogenases is available, it is then the preferred means for promoting the overall process of oxidative phosphorylation. When this mechanism is absent (as in the presence of ruthenium red), respiration is promoted through a decline in phosphorylation potential. The clear advantage of the Ca^{2+}-dependent mechanism is that this can allow oxidative

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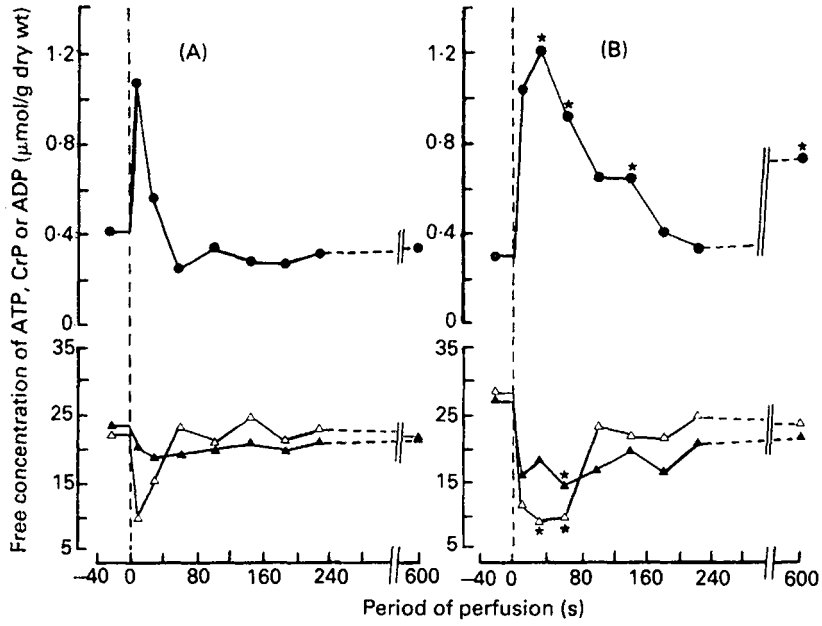

Fig. 4. Direct demonstration of the role of intramitochondrial calcium ions in the promotion of oxidative phosphorylation in rat hearts subjected to isoprenaline. Values are taken from Unitt *et al.* (1989) from where full details can be obtained. Rat hearts were perfused in either (A) the absence, or (B) the presence of 2·5 μM-ruthenium red and then subjected at zero time (vertical dashed line) to 0·1 μM-L-isoprenaline. ATP (▲) and creatine phosphate (CrP) (△) were determined by ^{31}P-NMR at the points shown, and ADP (●) calculated from the creatine kinase (*EC* 2.7.3.2) equilibrium constant, ATP × Cr/ADP × H^+ × CrP = 1·66 × 10^9/M, and the values of inorganic phosphate and pH obtained in the same experiments (Unitt *et al.* 1989). The effect of ruthenium red was significant: $^*P<0·05$.

metabolism and ATP production to be stimulated through an increase in NADH/NAD^+, whilst the cytosolic ATP/ADP value can be kept high. This would obviously be desirable when there is increased ATP demand for both increased contractile activity and increased Ca^{2+} movements. Significantly, Katz *et al.* (1987) demonstrated increases in NAD(P)H in stimulated hearts by using surface fluorescence.

Strong evidence in support of the advocated second messenger role of intramitochondrial Ca^{2+} has also come from the demonstration that the increase in PDH_a brought about by positive inotropic stimulation of the heart can persist through the preparation of mitochondria, and then during their subsequent incubation at 30° in potassium chloride-based media containing respiratory substrates and EGTA, but no Na^+ (McCormack & Denton, 1984) (Table 2). This requires rapid tissue disruption (e.g. by Polytron homogenization) into ice-cold sucrose-based media containing EGTA, where it can be shown that Ca movements across the mitochondrial inner membrane are minimal (McCormack & Denton, 1984; McCormack, 1985*b*). Moreover, it was shown that the persistent activations of PDH could be diminished by incubating the mitochondria with Na^+ to promote Ca^{2+} egress, and that diltiazem prevents these effects of Na^+ through inhibition of Na^+/Ca^{2+} exchange; or else they could be diminished by incubating the mitochondria with sufficient Ca^{2+} to lead to saturation of the Ca^{2+}-dependent activation of PDH (McCormack & Denton, 1984) (Table 2). In addition, it has been shown that OGDH activity (which can be assayed at the level of isolated mitochondria (see p. 61))

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Table 2. *Effects of hormone pretreatment on the active non-phosphorylated form of the pyruvate dehydrogenase complex (PDH_a) content, 2-oxoglutarate dehydrogenase complex (OGDH) activity (at subsaturating 2-oxoglutarate), and total calcium content, of rapidly prepared mitochondrial fractions from perfused rat heart and liver*

(Values are taken from (heart) McCormack & Denton (1984) and Crompton *et al.* (1983), and (liver) Assimacopoulos-Jeannet *et al.* (1986) from where full details can be obtained)

Tissue and treatment	Activity in isolated and incubated† mitochondria of: PDH_a (% total PDH)	OGDH (% V_{max})	Total mitochondrial Ca content (nmol/mg protein)
Perfused rat heart			
Control	8	25	1·8*
Adrenaline	20	47	4·2*
Perfused rat liver			
Control	17	6	1·2
Vasopressin	31	22	2·1
Glucagon	30	16	2·3
Glucagon plus vasopressin	44	32	4·9

V_{max}, maximum velocity of reaction.

*Crompton *et al.* (1983).

†For 3–6 min at 30° in the presence of EGTA but absence of sodium ions.

at sub-saturating concentrations of 2-oxoglutarate is also higher in mitochondria prepared from stimulated hearts when compared with controls, and that this persistent activation could also be diminished by Na^+ or saturating Ca^{2+} (McCormack & Denton, 1984) (Table 2). Positive inotropic stimulation may also result in a decrease in the 2-oxoglutarate content of intact hearts (Williamson, 1965). Additionally, Crompton *et al.* (1983) showed that mitochondria from adrenaline-treated hearts have raised total Ca content (to 2–4 nmol/mg protein) compared with controls (about 1 nmol/mg protein), and Wendt-Gallitelli (1986), using X-ray probe microanalysis, has also shown that total mitochondrial Ca content is increased in this range in situ in hearts subjected to various positive inotropic stimuli.

In the liver, it is well-established that hormones such as vasopressin, α-adrenergic agonists, angiotensin, and even glucagon bring about increases in cytosolic Ca^{2+} and also in oxidative metabolism (see e.g. Blackmore *et al.* 1983; Sistare & Haynes, 1985; Williamson & Hansen, 1987; Exton, 1988). In addition, the increases in cytosolic Ca^{2+} and cellular Ca content caused by phenylephrine or vasopressin alone are greatly enhanced when glucagon (or cyclic AMP) is also present (e.g. Mauger *et al.* 1985; Altin & Bygrave, 1986). These hormones are also known to bring about increases in cellular $NADH/NAD^+$ values in the liver (e.g. Sugano *et al.* 1980; Balaban & Blum, 1982; Haussinger & Sies, 1984; Patel & Olson, 1986), and in mitochondrial ATP/ADP values (e.g. Siess *et al.* 1978; Aprille *et al.* 1982; Soboll & Scholtz, 1986). Moreover, these changes are accompanied by decreases in the cellular contents of 2-oxoglutarate and glutamate (Siess *et al.* 1978; Haussinger & Sies, 1984; Staddon & McGivan, 1984, 1985),

and increases in the production of $^{14}CO_2$ from [1-^{14}C]-labelled glutamate or 2-oxoglutarate or octanoate (Haussinger & Sies, 1984; Taylor *et al.* 1986*a*,*b*; Rashed *et al.* 1988); these observations are clearly consistent with OGDH activation.

In the fed state, treatment of the perfused liver or isolated hepatocytes or the whole animal with these 'Ca-mobilizing' hormones caused increases in the amounts of PDH_a within a few minutes (Hems *et al.* 1978; Assimacopoulos-Jeannet *et al.* 1983, 1986). As with the stimulated heart (see p. 66), these increases in PDH_a in the liver are again persistent at the level of isolated mitochondria incubated with EGTA and in the absence of Na^+ (McCormack, 1985*b*; Assimacopoulos-Jeannet *et al.* 1986), but can be diminished by incubation with Na^+ or with sufficient Ca^{2+} to fully saturate the Ca^{2+}-dependent activations of the enzyme (Table 2). The effects of Na^+ were again found to be diltiazem-sensitive, and similar effects (to PDH) were found on OGDH activity when assayed within the mitochondria at low 2-oxoglutarate concentrations (McCormack, 1985*b*; Assimacopoulos-Jeannet *et al.* 1986) (Table 2). These observations are again consistent with the hormones causing increases in the intramitochondrial concentration of Ca^{2+} that can be largely maintained during the isolation of the mitochondria under appropriate conditions (see McCormack, 1985*b*).

In the absence of extracellular Ca all the previously mentioned intramitochondrial responses to these hormones become only transient or else barely detectable at all, whereas the addition of glucagon together with one of the other hormones greatly increases the responses (see Assimacopoulos-Jeannet *et al.* 1983, 1986; McCormack, 1985*b*) (Table 2). The responses are again accompanied by increases in mitochondrial total Ca content in the 1–4 nmol/mg protein range (Assimacopoulos-Jeannet *et al.* 1986), and values within this range are again observed in situ using X-ray probe microanalysis (see Somlyo *et al.* 1985).

There is also evidence in the liver that these hormones cause Ca^{2+}-dependent increases in mitochondrial volume through the Ca^{2+} inhibition of matrix pryophosphatase activity and, hence, increases in matrix pyrophosphate content (see p. 60) (Quinlan *et al.* 1983; Davidson & Halestrap, 1987, 1988, 1989; Halestrap, 1989), which then lead to the stimulation of the respiratory chain (Halestrap, 1989). This response may thus allow increased ATP/ADP levels to be maintained whilst the NADH/NAD^+ can then decrease in spite of there being stimulated dehydrogenase activities. This may be important for the hormonal stimulations of gluconeogenesis and urea synthesis (Taylor *et al.* 1986*a*,*b*) as high NADH/NAD^+ can be inhibitory for these processes, whereas high ATP:ADP is required (see Sistare & Haynes, 1985). The high ATP:ADP may be important for the activation of pyruvate carboxylation and citrulline synthesis (Brawand *et al.* 1980; Thienen & Davis, 1981). Indeed, there is a recent report that submicromolar concentrations of extramitochondrial Ca^{2+} may stimulate the rate of citrulline synthesis carried out by isolated rat liver mitochondria (Johnston & Brand, 1989). Decreases in the concentration of glutamate (through OGDH activation) may also play a role in the hormonal stimulation of gluconeogenesis since it is an inhibitor of pyruvate carboxylase (*EC* 6.4.1.1) (Scrutton & White, 1974). Similarly, decreases in 2-oxoglutarate may also be important in the stimulation of the exchange of glutamate for aspartate across the mitochondrial inner membrane by 'Ca-mobilizing' hormones, as it is a competitive inhibitor of aspartate formation via glutamate transamination by isolated liver mitochondria (Strzelecki *et al.* 1988).

Therefore, in rat heart and liver there is now good evidence for a key role for

intramitochondrial Ca^{2+} as a mediator for hormonal effects on oxidative (and other) metabolism. It is most likely that this will be a widespread phenomenon especially as Ca^{2+} is an important second messenger in many mammalian cell types and, in the cytosol, it generally stimulates energy-requiring events.

Already, in synaptosomal preparations isolated from rat brain and loaded with quin-2, Hansford & Castro (1985) have shown that the amount of PDH_a can be increased by a variety of treatments which cause increases in cytosolic Ca^{2+}. Moreover, these increases in PDH_a could be prevented by ruthenium red, whereas the increases in cytosolic Ca^{2+} were largely unaffected. Patel *et al.* (1988) also showed that the depolarization-induced stimulation of oxidative metabolism in synaptosomes was Ca^{2+}-dependent. Hagg *et al.* (1976) reported that ruthenium red inhibited the increases in PDH_a brought about by the stimulation of the sciatic nerve and, hence, exercise in the perfused hind-quarter of the rat; increases in PDH_a due to increased exercise have also been reported for the rat gastrocnemius muscle (Hennig *et al.* 1975).

Noradrenaline was found to cause increases in the amount of PDH_a in both white (Kilgour & Vernon, 1987) and brown (Gibbins *et al.* 1985) adipose tissue when administered to rats. In the later instance it is also known that there are large increases in citrate cycle flux and O_2 uptake under such circumstances, but no detectable changes in ATP/ADP or $NADH/NAD^+$ concentrations (e.g. Ma & Foster, 1984; Seydoux *et al.* 1984), again suggesting that increases in intramitochondrial Ca^{2+} concentration may be involved. Insulin also brings out increases in PDH_a in both white (Coore *et al.* 1971) and brown (McCormack & Denton, 1976) adipose tissue, and apparently by increasing PDH phosphate phosphatase activity (see Denton *et al.* 1984). However, it has been demonstrated that increases in intramitochondrial Ca^{2+} concentration are not involved in this instance, by using similar approaches to those which were described previously to establish the role of intramitochondrial Ca^{2+} in heart and liver (Marshall *et al.* 1984).

Acidotic conditions have been shown to cause increases in PDH_a in rat kidney (Tullson & Goldstein, 1982), and there is indirect evidence that this may be due to increases in intramitochondrial Ca^{2+}. There is also some evidence for a Ca^{2+} requirement in the activation of PDH by mitogens in pig lymphocytes (Baumgarten *et al.* 1983). However, although there is direct evidence for a transient increase in the mitochondrial Ca^{2+} pool caused by mitogens in rat thymocytes (Lakin-Thomas & Brand, 1987), this does not appear to be necessary for their stimulation of respiration in this instance (Lakin-Thomas & Brand, 1988). There is evidence from studies on permeabilized bovine adrenal corticol cells exposed to ruthenium red that intramitochondrial Ca^{2+} plays a key role in the activation of steroidogenesis (Capponi *et al.* 1988).

CONCLUSIONS AND SOME IMPLICATIONS

There is now a great deal of evidence in support of the second-messenger function of intramitochondrial Ca^{2+} in mediating hormonal effects on oxidative metabolism, as outlined in Fig. 1. It follows that the primary purpose of the mitochondrial Ca^{2+} transport system is concerned with this, and that its ability to function as a Ca^{2+} buffer for the cytosol is reserved for conditions where cytosolic Ca^{2+} is in the supraphysiological range.

In the heart it is likely that changes in matrix Ca^{2+} will be 'damped-down' with respect to those occurring on a beat-to-beat basis in the cytosol; however, there is a recent report

of small changes in PDH_a occurring within the contractile cycle of unstimulated dog hearts (Krause & Beyerdorfer, 1988). Moreover, in other cell types it is becoming apparent that hormones may initiate a train of Ca^{2+} pulses (i.e. oscillations) which increase in frequency as the concentration of agonist is raised, rather than there being sustained increases in cytosolic Ca^{2+} concentration (e.g. Woods *et al.* 1986; Grapengiesser *et al.* 1988; Monck *et al.* 1988).

There are also reports that the mitochondrial Ca^{2+} transport system itself may be subject to hormonal regulation, by as yet unknown mechanisms. For instance, α-adrenergic agonists have been shown to cause increases in the activity of the Ca^{2+}-uniporter uptake pathway in both heart (Crompton *et al.* 1983) and liver (Taylor *et al.* 1980) mitochondria, and glucagon and β-adrenergic agonists to increase the activity of the Na^+-dependent Ca^{2+}-egress pathway of liver mitochondria (Goldstone *et al.* 1983). Such mechanisms may thus allow hormones and other agents to alter the relationships for the distribution of Ca^{2+} between the cytosol and the matrix, and would, therefore, have important consequences for the hormonal control of energy metabolism.

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