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MINI-REVIEW

Dehydrogenase Activation by Ca^{2+} in Cells and Tissues

Richard G. Hansford[1]

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Abstract

The activation of intramitochondrial dehydrogenases by Ca^{2+} provides a link between the intensity of work performance by a tissue and the activity of pyruvate dehydrogenase and the tricarboxylate cycle, and hence the rate of ATP production by the mitochondria. Several aspects of this model of the control of oxidative phosphorylation are examined in this article, with particular emphasis on mitochondrial functioning in situ in cardiac myocytes and in the intact heart. Recent use of the fluorescent Ca^{2+} chelating agents indo-1 and fura-2 has allowed a more quantitative description of the dependence of dehydrogenase activity upon concentration of free intramitochondrial Ca^{2+}, in experiments with isolated mitochondria. Further, a novel technique developed by Miyata *et al.* has allowed description of free intramitochondrial Ca^{2+} within a single cardiac myocyte, and the conclusion that this parameter changes in response to electrical excitation of the cell over a range which would be expected to give substantial modulation of dehydrogenase activity.

Key Words: Pyruvate dehydrogenase; 2-oxoglutarate dehydrogenase; glycerol 3-phosphate dehydrogenase; intramitochondrial free Ca^{2+}; mitochondrial Ca^{2+} transport; adenine nucleotide phosphorylation potential.

Introduction

In mammalian cells, there are four mitochondrial dehydrogenases which are activated by micromolar, or submicromolar, concentrations of ionized Ca^{2+}. Of these, the FAD-glycerol 3-phosphate dehydrogenase is an intrinsic component of the mitochondrial inner membrane and is thought to respond directly to the free Ca^{2+} concentration of the cytosol ($[Ca^{2+}]_c$). Although first

[1] Laboratory of Cardiovascular Science, Gerontology Research Center, National Institute on Aging, Baltimore, Maryland 21224.

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described as Ca^{2+}-sensitive in insect flight muscle (Hansford and Chappell, 1967), where it is particularly abundant, this enzyme is also of importance in some mammalian tissues, and will be discussed in more detail below. The other dehydrogenases which are involved are the pyruvate dehydrogenase complex (Denton *et al.*, 1972), the NAD-isocitrate dehydrogenase (Denton *et al.*, 1978) and the 2-oxoglutarate dehydrogenase (McCormack and Denton, 1979). In each of these cases, the enzyme is present within the permeability barrier of the inner mitochondrial membrane and is sensitive to mitochondrial matrix free Ca^{2+} concentration ($[Ca^{2+}]_m$). As such, changes in $[Ca^{2+}]_c$ do not affect these enzyme systems directly, but must first be relayed into the mitochondria by the functioning of the mitochondrial Ca^{2+}-transport cycle (Crompton, 1985; Hansford, 1985; Gunter and Pfeiffer, 1990). The way in which these transport processes function to translate changes of $[Ca^{2+}]_c$, which are related to the workload experienced by the tissue, into changes in $[Ca^{2+}]_m$ thus becomes one of the themes of this article.

The mechanism of the activation by Ca^{2+} ions is allosteric in the case of NAD-isocitrate dehydrogenase (Denton *et al.*, 1978) and 2-oxoglutarate dehydrogenase (McCormack and Denton, 1979). In each case, the affinity for the oxidizable substrate is enhanced in the presence of Ca^{2+} and also by H^+ ions. By contrast, the effect of Ca^{2+} on pyruvate dehydrogenase is indirect and involves the facilitation of the activity of a phosphatase enzyme (Pettit *et al.*, 1972; Randle *et al.*, 1974; Teague *et al.*, 1982), which removes phosphoryl groups from three serine residues of the α subunit of enzyme 1, the decarboxylase portion of the complex. The phosphorylated form is catalytically inactive, and hence potentiation of phosphatase activity by Ca^{2+} ions tends to generate more of the active form of the complex (PDH_A). These relations have been well established and addressed in several recent reviews. For background on the interconversion of pyruvate dehydrogenase, the reader is referred to Wieland (1983), Reed *et al.* (1985) and Reed and Yeaman (1987). For aspects of the control of pyruvate, isocitrate and 2-oxoglutarate dehydrogenases by Ca^{2+} ions, the reader is referred to Hansford (1985), Denton and McCormack (1985), and McCormack *et al.* (1990a). Therefore, this short article will deal mainly with the evidence that dehydrogenase activation by Ca^{2+} ions occurs in living cells and tissues and will examine the consequence for cell bioenergetics of such activation.

All four Ca^{2+}-sensitive dehydrogenases share the property that they catalyze reactions which are far-displaced from equilibrium. As such, control of enzyme activity may be expected to have substantial effects on flux through the entire metabolic pathway; that is to say, these reactions may be expected to have a high control strength (see Kacser and Burns, 1973; Groen *et al.*, 1982, 1983). The pyruvate dehydrogenase reaction commits carbohydrate to either complete oxidation to carbon dioxide, or to fat synthesis, in tissues

which are capable of lipogenesis. The NAD-isocitrate dehydrogenase reaction represents a nonequilibrium step of the tricarboxylate cycle: allosteric activation of this enzyme by ADP has been clearly shown to allow the reaction to become closer to equilibrium, as flux through the tricarboxylate cycle increases (Hansford 1980), and activation by Ca^{2+} may be expected to have the same consequences. 2-Oxoglutarate dehydrogenase also catalyzes a nonequilibrium reaction of the tricarboxylate cycle, and is considered to control flux through the sector of the cycle from 2-oxoglutarate to oxaloacetate (Randle *et al.*, 1970; Smith *et al.*, 1974). This is important because of the feed-in of carbon at the level of 2-oxoglutarate, during the oxidation of glutamate, glutamine, etc. In addition, the operation of the malate/aspartate cycle for the reoxidation of glycolytically derived reducing power (Borst, 1963; Safer *et al.*, 1971) requires that mitochondria be permeable to 2-oxoglutarate, and regulate the activity of 2-oxoglutarate dehydrogenase. Aspects of the activation of the malate/aspartate shuttle by virtue of Ca^{2+}-linked activation of 2-oxoglutarate dehydrogenase will be discussed below. In tissues which possess high activities of both the mitochondrial, FAD-linked glycerol 3-phosphate dehydrogenase and the cytosolic, NAD-linked enzyme, these constitute a shuttle mechanism for the reoxidation of glycolytic NADH (Estabrook and Sacktor, 1958), and increased $[Ca^{2+}]_c$ may be expected to potentiate that process (Hansford and Chappell 1967; Wernette *et al.*, 1981; Beleznai *et al.*, 1988).

Thus, in each case, activation of these enzymes by Ca^{2+} would be expected to raise the mitochondrial $NADH/NAD^+$ ratio. This increases the ΔEh, or difference in observed reduction potentials, for the respiratory chain and therefore enhances the driving force for oxidative phosphorylation. This idea was developed in some detail in an earlier review (Hansford, 1980) which, however, emphasized allosteric activation of dehydrogenases by ADP, rather than by Ca^{2+}. This is an aspect of the control of oxidative phosphorylation within tissues which has been underemphasized, but which is beginning to receive more recognition recently (see Heineman and Balaban, 1990; McCormack *et al.*, 1990a). As noted by Van der Meer *et al.*, (1978) in a study of hepatocytes, flux through oxidative phosphorylation may be considered to be driven by an affinity term A, which has three components:

$$A = 129 + 5.9 \log [(NADH/NAD^+)_m \times (ADP \times Pi/ATP)_c^3 \times O_2^{1/2}] \text{ KJ mol}^{-1} \quad (1)$$

The dependence upon ADP × Pi/ATP has been well recognized since the pioneering studies of Lardy and Wellman (1952) and Chance and Williams (1956), using suspensions of mitochondria. Subsequent investigations have focused on the question of whether the whole phosphate potential expression is involved in control, or whether the ATP/ADP ratio, or the concentration

of ADP alone (Kunz *et al.*, 1981, 1983; Wanders *et al.*, 1984; Erecinska and Wilson, 1982; Jacobus *et al.*, 1982), is responsible for controlling respiration. Common to all of these studies was that oxidizable substrates were used which saturate dehydrogenases, e.g., 10 mM succinate or 10 mM glutamate plus malate. In addition, availability of O_2 was saturating for cytochrome *c* oxidase. Under these conditions, it is almost a matter of definition that respiration is limited by availability of ADP or Pi. By contrast there is evidence that Nature has gone to some length to ensure that dehydrogenases catalyzing nonequilibrium reactions are not saturated with substrate in non-stimulated tissue (see Hansford, 1980): conceivably, this prevents a loss of efficiency which can occur through generation of very high protonmotive forces and consequent non-ohmic conductance of protons by the mitochondrial inner membrane (Nicholls, 1974, 1977) or slippage of redox pumps (Zoratti *et al.*, 1986; Pietrobon *et al.*, 1986).

This article will examine the thesis that Ca^{2+} ions, which become more available to the mitochondria when the tissue is experiencing a heavy workload, provide a means of enhancing the degree of substrate saturation of these dehydrogenases and hence of maintaining higher $NADH/NAD^+$ ratios and fluxes through oxidative phosphorylation than would be possible in the absence of such activation. It is noted that this is not a new idea and that in fact the first paper ever on the respiratory chain (Keilin, 1925) identified an increased reduction of cytochrome *c* in the flight muscles of a wax moth when it struggled to escape. Arguably, this reflected activation of glycerol 3-phosphate dehydrogenase by Ca^{2+} and NAD-isocitrate dehydrogenase by ADP in that tissue (see Hansford, 1980).

Aspects of the interplay between control of oxidative phosphorylation in heart by Ca^{2+} and by ADP are also dealt with by Balaban in this issue.

Finally, there is evidence that Ca^{2+} ions activate oxidative phosphorylation at sites other than the dehydrogenases mentioned above. Thus Halestrap (1989) has described a potentiation of respiratory chain function linked to an increase in mitochondrial matrix volume and there is also evidence of an effect of Ca^{2+} upon an endogenous inhibitor of the mitochondrial F_1-ATP-ase (synthase) (Yamada and Huzel, 1985, 1988; Yamada *et al.*, 1980). Evidence for such an effect of Ca^{2+} in studies of intact mitochondria and cells will also be reviewed briefly in this article.

Studies on the Sensitivity of Dehydrogenases to Ca^{2+} Using Isolated Heart Mitochondria

Previous studies have involved exposing coupled, respiring mitochondria to Ca^{2+}: EGTA buffers and investigating dehydrogenase activation in the

steady-state (Hansford and Cohen, 1978; Denton *et al.*, 1980; McCormack and Denton, 1980; Hansford 1981) or using uncoupled mitochondria (Denton *et al.*, 1980; McCormack and Denton, 1980). In the latter instance, the assumption was made that no gradient of Ca^{2+} across the mitochondrial membrane remained, i.e., that $[Ca^{2+}]_m/[Ca^{2+}]_o$ was 1, where $[Ca^{2+}]_o$ is the extramitochondrial free Ca^{2+} concentration. This allowed the evaluation of the sensitivity of the dehydrogenases to Ca^{2+} in their intramitochondrial milieu. A recent advance has been made possible by the availability of the fluorescent chelating agents indo-1 and fura-2 (Grynkiewicz *et al.*, 1985), which can be introduced into mitochondria using their membrane-permeant acetoxymethyl ester forms (Davis *et al.*, 1987; Lukács and Kapus, 1987; Gunter *et al.*, 1988; Moreno-Sánchez and Hansford, 1988a; Reers *et al.*, 1989). This has allowed the measurement of $[Ca^{2+}]_m$ concomitantly with the degree of dehydrogenase activation.

When 2-oxoglutarate dehydrogenase activity was followed indirectly, by the degree of reduction of mitochondrial NAD(P) at subsaturating levels of 2-oxoglutarate (see Hansford and Castro, 1981), it was found that the $K_{0.5}$ for activation by Ca^{2+} was 300–600 nM (McCormack *et al.*, 1989), 120 nM (Wan *et al.*, 1989), or 800 nM (Lukács *et al.*, 1988). Differences in the value of the K_d for the fura-2/Ca^{2+} chelate which were used to generate these results probably do not explain the differences, in the sense that Wan *et al.* (1989) determined a K_d for intramitochondrial fura-2 of 379 nM, a value very close to that determined, also in heart mitochondria, by Reers *et al.* (1989), whereas Lukács *et al.* (1988) assumed a somewhat lower value (135 nM). Nevertheless, these direct measurements of enzyme characteristics *in situ* in the matrix tend to confirm the applicability of values previously determined with isolated enzymes (see McCormack *et al.*, 1990a for references) or toluene-permeabilized mitochondria (Rutter and Denton, 1988).

Equivalent experiments in which the content of PDH_A was measured as a function of $[Ca^{2+}]_m$ indicated a $K_{0.5}$ for activation of 300 nM (Moreno-Sánchez and Hansford, 1988a), or approximately 1 μM (Wan *et al.*, 1989). There is actually good agreement between these values, as the K_d of 95 nM for intramitochondrial indo-1 used by Moreno-Sánchez and Hansford (1988) was somewhat in error, owing to effects of light-scattering changes on the calibration experiments. Repetition of these experiments with a dual-excitation and dual-emission spectrofluorometer (P.T.I. Deltascan) has corrected the K_d value for intramitochondrial indo-1 to 244 nM (at pH 7.7), resulting in a recomputed $K_{0.5}$ for activation of PDH of 660–840 nM. Though direct, these experiments are not straightforward (*pace* McCormack *et al.*, 1989), as there are concerns about: (1) a fraction of extramitochondrial indicator (usually not a major problem with mitochondria, though extracellular indicator may be significant in experiments with cell suspensions); (2) a different

K_d in the more viscous and structured mitochondrial matrix, and at the alkaline pH characteristic of that space; (3) spectral shifts relative to the salt of these indicators studied in simple aqueous solution, such that isosbestic wavelengths may no longer be so; and (4) contribution from partially hydrolyzed, and partially Ca^{2+}-sensitive forms, of the fura-2/AM or indo-1/AM. Some of these problems were addressed by Reers *et al.* (1989) in their excellent paper. Others are addressed in the cell physiology literature (Blatter and Wier, 1990; Scalou *et al.*, 1987, Spurgeon *et al.*, 1990).

Thus, these direct experiments tended to confirm that pyruvate and 2-oxoglutarate dehydrogenases are sensitive to Ca^{2+} in the range 0.1–2 μM, when *in situ* in the mitochondrion. Comparison with experiments in which total mitochondrial calcium was measured by atomic absorption spectroscopy and related to enzyme activation (Coll *et al.*, 1982; Hansford and Castro, 1982) makes it plain that the range 0.1–2 μM for $[Ca^{2+}]_m$ corresponds to approximately 0.2–4 nmol of total calcium/mg of mitochondrial protein. A somewhat similar relation has been found by Lukács and Kapus (1987). These considerations are important in the understanding of the activation of dehydrogenases by Ca^{2+} *in vivo* (see below), as the majority of information on mitochondrial Ca^{2+} content in living tissues is from electron probe x-ray microanalysis, where results are stated in nmol total calcium/mg protein.

A startling degree of consensus emerged from these studies with fluorescent probes (Moreno-Sánchez and Hansford, 1988a; McCormack *et al.*, 1989; Reers *et al.*, 1989; Wan *et al.*, 1989) concerning the magnitude of the mitochondrial Ca^{2+} concentration gradient ($[Ca^{2+}]_m/[Ca^{2+}]_o$). When physiological concentrations of Mg^{2+} and Na^+ are present, this gradient is negative at values of less than 750 nM for $[Ca^{2+}]_o$: at higher values of $[Ca^{2+}]_o$, $[Ca^{2+}]_m$ begins to increase rapidly and the gradient becomes positive. This was shown by Reers *et al.* (1989) with fura-2-loaded and superfused mitochrondria, and by Moreno-Sánchez and Hansford (1988a) for indo-1-loaded mitochondria. These results have been amply confirmed by McCormack *et al.* (1989) and by Wan *et al.* (1989). The latter study is particularly complete in presenting the relation of $[Ca^{2+}]_m$ to $[Ca^{2+}]_o$ at various concentrations, separately, of Mg^{2+} and Na^+. Both Mg^{2+} and Na^+ ions result in lower values of the gradient $[Ca^{2+}]_m/[Ca^{2+}]_o$. In the case of Mg^{2+} (Jacobus *et al.*, 1975; Crompton *et al.*, 1976a), this effect reflects inhibition of the Ca^{2+}-uptake pathway, i.e., the uniporter (see Crompton, 1985), whereas Na^+ acts as a counter-ion for efflux of Ca^{2+} on the electroneutral $2\,Na^+/Ca^{2+}$ carrier (Crompton *et al.*, 1976b; Crompton and Heid, 1978). These relations have been reviewed previously (Akerman and Nicholls, 1983; Crompton, 1985; Gunter and Pfeiffer, 1990). In the context of the present review, it is pertinent to note that Na^+ and Mg^{2+} ions do not have any effect on the sensitivity to

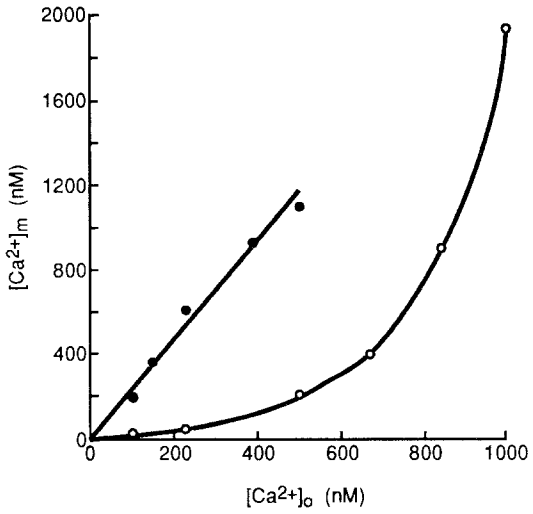

Fig. 1. The concentration gradient, $[Ca^{2+}]_m/[Ca^{2+}]_o$, as measured in a suspension of heart mitochondria. Heart mitochondria were loaded with indo-1 AM and fluorescence was measured in a PTI Deltascan. Mitochondria were respiring on glutamate plus malate, in media containing Ca^{2+} buffered with EGTA and calibrated, in separate experiments with indo-1/salt. The value of $[Ca^{2+}]_m$ was derived from the fluorescence of the mitochondrial suspension, by reference to R_{min} and R_{max} values and a K_d for indo-1/Ca^{2+} determined as 244 nM, at pH 7.7, for the intramitochondrial compound. From Hogue and Hansford (unpublished). (●) medium contained 10 mM Na^+; (○) medium contained 10 mM Na^+ plus 1 mM Mg^{2+}.

Ca^{2+} of 2-oxoglutarate dehydrogenase or pyruvate dehydrogenase interconversion within the mitochondrion (Lukács *et al.*, 1988; Moreno-Sánchez and Hansford, 1988; McCormack *et al.*, 1989): rather these ions affect sensitivity to extramitochondrial Ca^{2+} (Denton *et al.*, 1980; Hansford, 1981) by changing the magnitude of the mitochondrial Ca^{2+}-gradient.

A recent version of this experiment, using heart mitochondria loaded with indo-1/AM and studied by dual-emission spectrofluorometry, to minimize errors due to light-scattering changes, is presented as Fig. 1. The conditions chosen (10 mM Na^+, 1 mM Mg^{2+}) reflect the composition of the cytosol of cardiac myocytes (Hess *et al.*, 1982; Murphy *et al.*, 1989; Lee and Fozzard, 1975), and thus the values of the gradient $[Ca^{2+}]_m/[Ca^{2+}]_c$ are of some physiological relevance. However, such relevance is limited for this, and all previous studies, by the fact that $[Ca^{2+}]_c$ is never held constant in the range 0.2–1 μM in living cardiac muscle. Much more relevant, though more difficult, are the experiments described below.

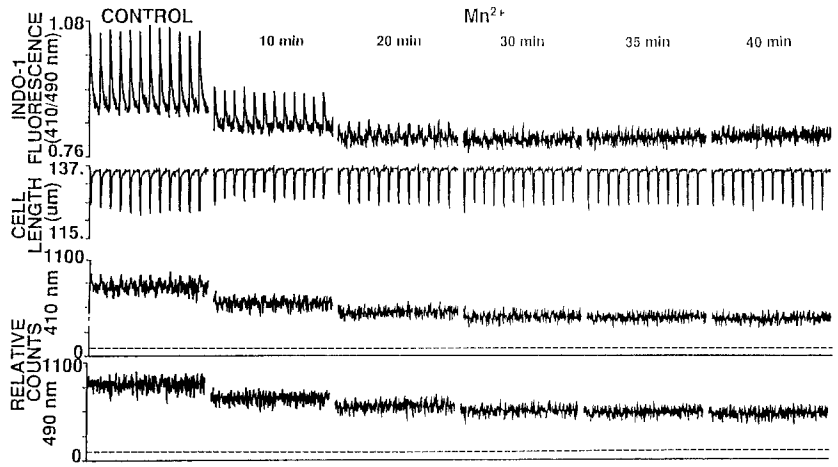

Fig. 2. Use of Mn^{2+} to quench fluorescence from cytosolic indo-1, while preserving fluorescence from mitochondrial indo-1, in a single cardiac myocyte. The upper panel gives the response of indo-1 fluorescence ratio (410/490 nm) to electrical excitation of the cell at 0.2 Hz; the panel below gives the mechanical response, i.e., shortening, of the cell. Treatment with 100 μM Mn^{2+} was for the periods of time indicated. Note that contraction is preserved, although the indo-1 fluorescence signal becomes unresponsive to electrical stimulation. For full details, see Miyata *et al.* (1991b).

Studies of $[Ca^{2+}]_m$ in Single Living Cardiac Myocytes

The fact that esterase activity within mitochondria generates some of the fluorescent chelating agents indo-1 and fura-2 within the mitochondrial matrix space when cells are exposed to the membrane-permeant esters (Davis *et al.*, 1987; Spurgeon *et al.*, 1990) has allowed us to record a signal from that compartment. The fluorescence from indo-1 within the cytosol is quenched by a graded exposure of the cells to Mn^{2+} (Miyata *et al.*, 1991a, b). Figure 2 shows that the electrically stimulated transients of indo-1 fluorescence progressively disappear as a single cardiac myocyte is superfused with Mn^{2+}: significantly, the contractions of the cell are preserved, indicating that transients of $[Ca^{2+}]_c$ are still occurring and are largely unaffected by the Mn^{2+}. The mitochondrial origin of the Mn^{2+}-resistant fluorescence is shown by the fact that it is also resistant to 5 μM, but not to 25 μM, digitonin (Miyata *et al.*, 1991b). At the lower concentration, the digitonin is shown to selectively permeabilize the plasma membrane, and leave the mitochondrial membrane intact, as based upon the release patterns of the enzymes lactate dehydrogenase and citrate synthase (see Spurgeon *et al.*, 1990). Another

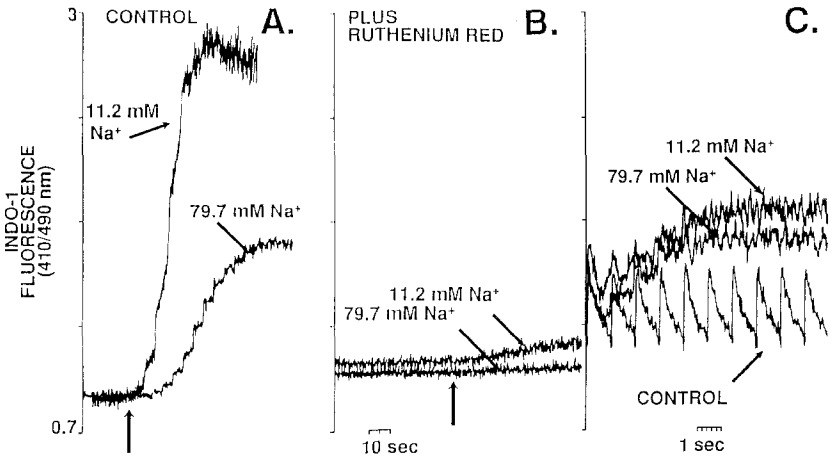

Fig. 3. Response of $[Ca^{2+}]_m$ to chronic depolarization of a single cardiac myocyte. In panels A and B, the cell had been loaded with indo-1/AM and quenched with Mn^{2+}. Partial replacement of the Na^+ in the bathing solution with K^+ causes depolarization and a rise in $[Ca^{2+}]_m$. When the cell is treated with ruthenium red in advance of the change in bathing medium (panel B), the response of $[Ca^{2+}]_m$ is largely attenuated. In panel C, a different cell was loaded with indo-1/salt, giving a fluorescence signal that derives solely from the cytosol. Extremes of Na^+ replacement (11 mM Na^+ remaining) give lower values of $[Ca^{2+}]_c$ than of $[Ca^{2+}]_m$. From Miyata *et al.* (1991b).

criterion by which the Mn^{2+}-resistant fluorescence is judged to be mitochondrial is its sensitivity to ruthenium red. Figure 3 shows that Mn^{2+}-resistant indo-1 fluorescence rises markedly when Na^+ in the cell suspension medium is replaced with K^+. This gives rise to depolarization and Ca^{2+} loading of the cells. At the lower Na^+ concentration (11 mM), $[Ca^{2+}]_m$ rises sufficiently to saturate indo-1, as determined by the 410/490 nm emission ratio. Repetition of these experiments with cells which have been pretreated with ruthenium red largely abolishes the response to Na^+-replacement (Fig. 3). Ruthenium red has previously been shown to be effective in blocking the increase in PDH_A content of the intact heart (McCormack and England, 1983; Unitt *et al.*, 1989) and of isolated cardiac myocytes (Hansford, 1987) in response to positive inotropic treatments.

When results of experiments of the type of Fig. 3 are compiled, and comparison is made with cells which have been loaded with indo-1/salt to give a read-out from the cytosol compartment, an evaluation of the gradient $[Ca^{2+}]_m/[Ca^{2+}]_c$ in living cardiac myocytes can be made. This is presented as Fig. 4. It is seen that there is remarkably good agreement with results obtained from isolated mitochondria (Fig. 1). This is the first approach to measuring the parameter $[Ca^{2+}]_m$ from mitochondria within a living cell (Miyata *et al.*, 1991a, b).

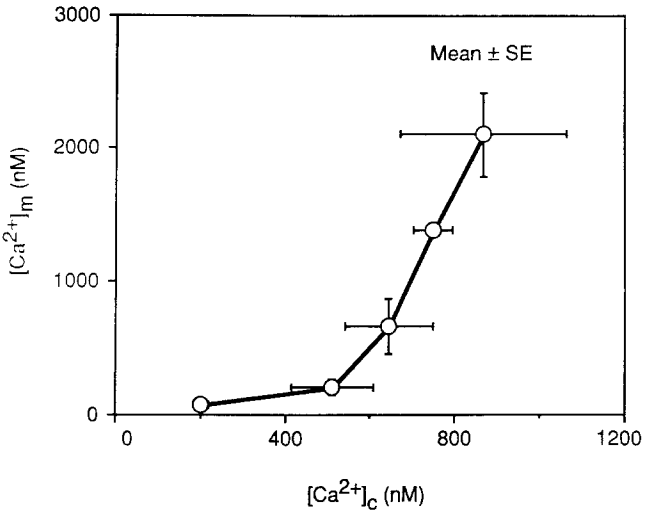

Fig. 4. The gradient $[Ca^{2+}]_m/[Ca^{2+}]_c$, as measured in single cardiac myocytes. Values of $[Ca^{2+}]_c$ were varied in a Na^+-replacement protocol (Fig. 3) and measured in cells loaded with indo-1/salt. Values of $[Ca^{2+}]_m$ were measured in indo-1/AM-loaded and Mn^{2+}-quenched cells, subjected to the same procedures. From Miyata *et al.* (1991b).

The conditions under which these experiments (Figs. 3 and 4) are performed are nonphysiological, involving a chronic Ca^{2+} overload of the cells, and changes in cytosolic Na^+. They may, however, relate to the condition under which perfusion is recommenced, following a period of cardiac ischemia (Henry *et al.*, 1977; Renlund *et al.*, 1985; Hoerter *et al.*, 1986; Allen and Orchard, 1987).

A more physiological paradigm involves the response of $[Ca^{2+}]_m$ to the frequency of electrical stimulation of a cardiac myocyte and to stimulation by catecholamines. Figure 5 shows that $[Ca^{2+}]_m$ rises from 84 nM in unstimulated cells to 240 nM as the frequency of stimulation is raised to 3 Hz. At higher frequencies (4 Hz) there is a still larger increase in $[Ca^{2+}]_m$, to 650 nM: however, β-adrenergic stimulation is required for the cell to follow this frequency, at room temperature, and the effects of stimulation frequency and β-adrenergic activation need to be further dissected apart, under these conditions. Nevertheless, the conclusion remains that $[Ca^{2+}]_m$ in a resting cell will give minimal activation of pyruvate and 2-oxoglutarate dehydrogenases (Lukács *et al.*, 1988; Moreno-Sánchez and Hansford, 1988a; McCormack *et al.*, 1989; Wan *et al.*, 1989), whereas $[Ca^{2+}]_m$ in the cell which is beating at 4 Hz, in the presence of norepinephrine, will give 50% activation, or greater (Moreno-Sánchez and Hansford, 1988a; McCormack *et al.*, 1989; Wan *et al.*, 1989). An ingredient which is still missing in these studies is the performance

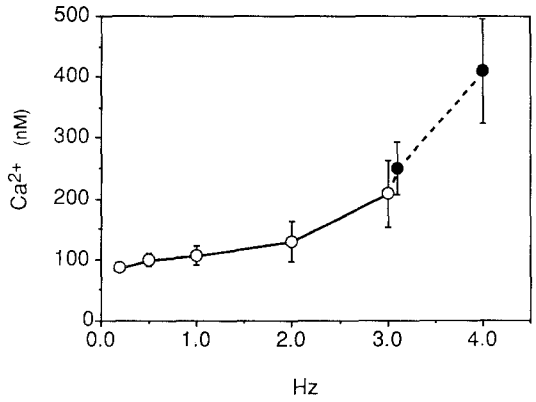

Fig. 5. Dependence of $[Ca^{2+}]_m$ upon the frequency of electrical stimulation of a cardiac myocyte. Data are from one group of four cells over the range 0.2–3 Hz, and from another group of four cells at 3 and 4 Hz in the presence of 10^{-6} M norepinephrine. From Miyata *et al.* (1991b).

of external work, in that the cells are mechanically unloaded: conceivably this could be added by making the cells contract against the resistance of a fiber optic.

The kinetics of the response of $[Ca^{2+}]_m$ to the stimulated state are shown in Fig. 6. Once the cell is fully "captured" at 4 Hz, $[Ca^{2+}]_m$ rises with a $T^{1/2}$ of approximately 30 sec, to achieve a new steady state. Cessation of stimulation then results in a biexponential fall in $[Ca^{2+}]_m$, with $T^{1/2}$ values of approximately 18 and 75 sec (Miyata *et al.*, 1991b). There is no variation of

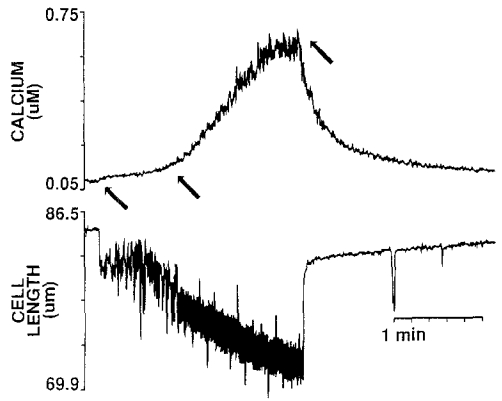

Fig. 6. Rate of response of $[Ca^{2+}]_m$ to changing the frequency of stimulation of a cardiac myocyte. At the first arrow, pacing at 4 Hz was begun: at the second arrow, the cell began to follow the pacing faithfully, as seen from the lower, length tracing. At the third arrow, pacing was discontinued. For details, see Miyata *et al.* (1991b).

$[Ca^{2+}]_m$ within a single contraction, as seen with this technique. The rate of rise and decay of fluorescence corresponding to $[Ca^{2+}]_m$ will be blunted to some extent by the presence of the intramitochondrial indo-1. However, estimates of the additional intramitochondrial Ca^{2+}-buffering power suggest that is slight.

Results from two other types of study require comparison within these findings. The application of electron probe x-ray microanalysis (EPMA) to rapidly frozen single cardiac myocytes indicates that total mitochondrial Ca rises significantly during a single electrical stimulation (Wendt-Gallitelli and Isenberg, 1991). The magnitude of the increase which these authors observe is hard to reconcile with the lack of perceptible changes in $[Ca^{2+}]_m$ (above) or with predictions made on the basis of the kinetics of Ca^{2+} influx and efflux, as studied with isolated heart mitochondria (Robertson *et al.*, 1982; Crompton, 1985). Although the EPMA technique measures total Ca, there is every reason to believe that there is a near-linear relation between total Ca and $[Ca^{2+}]_m$ over this range (Hansford and Castro, 1982; Coll *et al.*, 1982; Lukács and Kapus, 1987). The stimulation protocol used by Wendt-Gallitelli and Isenberg (1991) was also different, involving paired pulses. This clearly loaded the tissue with Ca^{2+} compared with single pulses at this low-frequency (2 Hz). However, this was done in a deliberate attempt to more clearly recreate a physiological performance of the guinea-pig cardiac myocytes studied, as this animal shows larger systolic transients of $[Ca^{2+}]_c$ at higher pacing frequencies (unlike the rat). The conditions used by Wendt-Gallitelli and Isenberg (1991) resulted in systolic transients of $[Ca^{2+}]_c$ peaking at about 800 nM Ca^{2+}, as measured with indo-1/salt. This is a reasonable value and comparable to that generated in studies with rat myocytes discussed above. Thus the reason for the discrepancy between these very different results, generated by very different techniques, awaits elucidation.

The other related study involves the use of fluorescence digital imaging microscopy applied to guinea-pig cardiac myocytes loaded with fura-2/AM (Williford *et al.*, 1990). In these images, the mitochondria show up as "hot-spots" of Ca^{2+} concentration. As the cells which were studied were unstimulated, this result is also at variance with the results of the Mn^{2+}-quench technique described above. Although individual mitochondria cannot be truly resolved by light microscopy, these studies do identify mitochondria-rich areas of the cell, which respond to mitochondrial inhibitors in a predictable way, and which show enhanced fluorescence of the mitochondria-specific probe rhodamine 1,2,3 (Jou and Sheu, 1990; Williford *et al.*, 1990). It is perfectly possible that the mitochondrial Ca^{2+} gradient is positive in resting guinea-pig cells, while negative in rat cells, as Ca^{2+} homeostasis is significantly different in other ways in myocytes from these species (see Rich *et al.*, 1988; Lewartowski *et al.*, 1990). Perhaps the aspect of these findings to

emphasize is that in neither the study by Williford *et al.* (1990) nor that cited above is the fluorescent probe close to saturation in the mitochondria of unstimulated cells. Thus, there is agreement from studies using this technique that $[Ca^{2+}]_m$ does lie within the range where the dehydrogenases show control.

Activation of Dehydrogenases, and Associated Increases in Flux, in Cardiac Mitochondria, Myocytes, and in Intact Heart

The previous sections have briefly summarized the evidence for the Ca^{2+} dependence *in situ* of pyruvate dehydrogenase, NAD-isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase and then dealt at more length with the question of the content, and free concentration, of mitochondrial Ca^{2+}. This section examines the evidence of whether dehydrogenase activation occurs in response to tissue activation and, if so, what the consequences are for flux through the pyruvate dehydrogenase reaction and the tricarboxylate cycle.

The simple model system used to address these questions has involved isolated heart mitochondria exposed to nonsaturating concentrations of pyruvate (Hansford and Cohen, 1978; Wan *et al.*, 1989) or 2-oxoglutarate (Denton *et al.*, 1980; Wan *et al.*, 1989), with Ca^{2+}-activation being imposed with Ca^{2+}: EGTA buffers. When excess ADP is present (State 3), the addition of Ca^{2+} results in raised rates of O_2 uptake, when 2-oxoglutarate is the substrate (Denton *et al.*, 1980). When ADP is absent (State 4), dehydrogenase activation is instead manifested by a greatly increased reduction of mitochondrial NAD, in the presence of nonsaturating 2-oxoglutarate as substrate (Hansford and Castro, 1981; McCormack *et al.*, 1982). A recent study of some of these relations has confirmed that increases in $[Ca^{2+}]_m$ result in increased flux through 2-oxoglutarate dehydrogenase but has found minimal effects on flux through pyruvate dehydrogenase (Wan *et al.*, 1989). Experimental conditions are critical. Wan *et al.* (1989) studied State 3 respiration generated by addition of excess ADP: ADP is an inhibitor of pyruvate dehydrogenase kinase, and thus this protocol tends to push the pyruvate dehydrogenase interconversion heavily towards PDH_A. Use of respiratory states intermediate between States 3 and 4 might be more physiological and more informative (Davis and Lumeng, 1975; Hansford, 1977). In these states, a limiting provision of ADP to the mitochondria, as maintained by exogenous ATP-ase of limiting activity, would be expected to poise the pyruvate dehydrogenase interconversion such that an effect of Ca^{2+} might be more apparent (Hansford and Cohen, 1978). Wan *et al.* (1989) also found minimal effects of Ca^{2+} upon the reduction of mitochondrial NAD by pyruvate in State 4. This is consistent with the results of an earlier study (Hansford and

Cohen, 1978), in which it was suggested that raised $NADH/NAD^+$ ratios exert a very stringent feedback control upon pyruvate dehydrogenase interconversion through a known effect at the level of the kinase (Pettit *et al.*, 1975; Cooper *et al.*, 1975). In studies in which pyruvate concentration is physiological (50 to 500 μM), ADP provision generates states between 3 and 4, and alternative respiratory substrate is available for oxidation, it is clear that Ca^{2+} activation of pyruvate dehydrogenase results in higher fluxes through this enzyme (Hansford and Cohen, 1978). As mentioned in the Introduction, activation of 2-oxoglutarate dehydrogenase is likely to be of importance in heart, as fatty acid oxidation involves the tricarboxylate cycle. Wan *et al.* (1989) make the very interesting suggestion that an important aspect of the activation of 2-oxoglutarate dehydrogenase by Ca^{2+} may be the lowering of steady-state intramitochondrial concentrations of 2-oxoglutarate, with consequently greater aspartate formation and flux through the malate/aspartate shuttle. This would be of consequence for the oxidation of glucose or lactate, but not pyruvate, by the heart.

As mentioned in the Introduction, the model of control of oxidative phosphorylation through the control of dehydrogenase activity requires that greater fluxes be associated with increases in mitochondrial $NADH/NAD^+$ ratio (Hansford, 1980; Heineman and Balaban, 1990; McCormack *et al.*, 1990a). Koretsky and Balaban (1987) investigated this in a systematic way, using suspensions of liver mitochondria. In State 3, there was found to be a linear relation between $NADH/NAD^+$ ratio and rate of O_2 uptake, when the activity of substrate dehydrogenation was varied by using a variety of NAD-linked substrates. Of special significance to the model of dehydrogenase control was the finding that when availability of ADP exerted strong control over respiration (30% State 3), an increase in $NADH/NAD^+$ caused by, say, the transition from citrate to glutamate plus malate as substrate resulted in a significant rise in the extramitochondrial $ATP/ADP \times Pi$ ratio. Thus the same flux through oxidative phosphorylation can be preserved, at different values of these two ratios, as expected from the relationship given by Akerboom *et al.* (1978) and cited in the Introduction. A somewhat similar study by Moreno-Sánchez *et al.* (1990), using heart mitochondria, varied the activity of substrate dehydrogenation by varying the concentration of pyruvate or 2-oxoglutarate and established a linear relationship between $NADH/NAD^+$ ratio and flux through oxidative phosphorylation, as measured by O_2 uptake. This was the expected result. Not so expected was the finding that increasing dehydrogenase activity by increasing $[Ca^{2+}]_o$, with nonsaturating pyruvate or 2-oxoglutarate as substrate, gave a much steeper dependence of O_2 uptake upon $NADH/NAD^+$ ratio. Over and above the activation of the dehydrogenase, the Ca^{2+} ion is clearly having an effect to activate oxidative phosphorylation. Halestrap (1987b), in a study of heart mitochondria,

showed an effect of Ca^{2+} on respiratory chain activity which is quite slow, requiring 2 min for completion, and which is linked to an expansion of the mitochondrial matrix space. Such an activation of the respiratory chain could underlie the observed steep response of O_2 uptake to changes in NADH/NAD^+, in the presence of Ca^{2+}. This mechanism will not be discussed further here, in the context of the heart, as it has been presented very recently in a major review article (McCormack *et al.*, 1990a). Other possible sites of action of Ca^{2+} which would explain these findings are the F_1-ATP-ase or the adenine nucleotide translocase (Moreno-Sánchez, 1985a, b). The dissociation of an inhibitor protein from the F_1-ATP-ase has been shown to be Ca^{2+}-dependent, in the micromolar range (Yamada *et al.*, 1980; Yamada and Huzel, 1985, 1988), and the physiological importance of this deserves further study. The possibility of an effect at the level of the adenine nucleotide translocase is suggested in the present work (Moreno-Sánchez *et al.*, 1990) by the steep dependence of flux on the NADH/NAD^+ ratio when Ca^{2+} is used to activate oxidative phosphorylation in the presence of partial inhibition of the translocase by carboxyatractyloside. Such a locus of action would also be consistent with the lack of effect of Ca^{2+} on oxidative arsenylation, a process which does not require flux through the translocase (Moreno-Sánchez, 1985b). This possibility, too, deserves further study.

Experiments with isolated cardiac myocytes from the rat (Hansford, 1987) have shown that there is a relationship between the degree of increase in $[Ca^{2+}]_c$, caused by interventions which depolarize the plasma membrane, and the increase in the content of PDH_A. Moreover, the increase in PDH_A is largely attenuated by prior treatment of the cells with ruthenium red (Table I), in keeping with prior results from McCormack and England (1983) using the intact, perfused heart (see below). The O_2 uptake of these cells is stimulated by all procedures which raise $[Ca^{2+}]_c$, with depolarization by high K^+ being relatively ineffective compared to treatment with the alkaloid veratridine, which potentiates the opening of Na^+ channels (Honenjager and Reiter, 1975; Horackova and Vassort, 1974). The enhanced respiration due to veratridine is relatively insensitive to treatment with ruthenium red, though the 15% inhibition is statistically significant (Moreno-Sánchez and Hansford, 1988b). These studies involve a chronic depolarization protocol which never occurs *in vivo*. The Na^+ overload and depolarization caused by 25 μM veratridine imposes a maximal energy demand upon the cells, as shown by a rate of O_2 uptake equal to that achieved with the uncoupling agent FCCP (Moreno-Sánchez and Hansford, 1988b), and these cells go into contracture in a matter of minutes, presumably owing to a severe drop in the ATP/ADP $\times$ Pi ratio. Under these conditions, it is not surprising that dehydrogenase activation by Ca^{2+} becomes relatively unimportant. A fall in the mitochondrial ATP/ADP ratio will lead to a rise in PDH_A content (see

Table I. Effect of Depolarization of the Plasma Membrane on the PDH_A Content of Isolated Cardiac[a] Myocytes

Condition	PDH_A, % of total
Increasing K^+	
5 mM KCl	31.7 ± 1.4
20 mM KCl	39.5 ± 0.6
40 mM KCl	49.5 ± 4.6
55 mM KCl	49.3 ± 3.6
80 mM KCl	60.6 ± 3.9
Increasing K^+ + ruthenium red (RR)	
5 mM KCl + RR	31.5 ± 2.7
40 mM KCl + RR	38.7 ± 2.3
55 mM KCl + RR	35.2 ± 3.0
80 mM KCl + RR	37.8 ± 3.3
Veratridine	
5 μM Veratridine	37.6 ± 4.5
25 μM Veratridine	51.0 ± 1.9
25 μM Veratridine + 0.2 mM ouabain	57.6 ± 3.9
25 μM Veratridine + 0.2 mM ouabain + RR	35.8 ± 2.0

[a]From Hansford (1987). Full experimental details are given in the original paper.

Hansford, 1980 for a review) as well as causing allosteric activation of NAD-isocitrate (see Hansford, 1980) and 2-oxoglutarate dehydrogenases (McCormack and Denton, 1979). When nonsaturating concentrations of veratridine (5 μM) are used, dehydrogenase activation by Ca^{2+} becomes more significant. Thus, the fall in the mitochondrial NADH/NAD^+ ratio due to stimulation of cardiac myocytes with 5 μM veratridine is amplified when the cells are pretreated with ruthenium red (Hansford *et al.*, 1988), which prevents the activation of Ca^{2+}-sensitive intramitochondrial dehydrogenases.

Of more physiological relevance than these studies with isolated myocytes are experiments in which O_2 uptake, enzyme activation, and total mitochondrial Ca content are monitored in intact hearts which are exposed to positive inotropic interventions. Early experiments showed that elevation of cardiac work output, whether by adrenergic stimulation (McCormack and Denton, 1981a; Bünger *et al.*, 1982) or by increasing left atrial influx and/or systolic pressure (Illingworth and Mullings, 1976; Pearce *et al.*, 1980; Kobayashi and Neely, 1983), resulted in an increase in PDH_A content, as well as flux through oxidative phosphorylation, as measured by O_2 uptake. Subsequently, the increase in PDH_A content caused by adrenergic stimulation, raised perfusate Ca^{2+}, or increased electrical pacing rate was found to be associated with an increase in the content of total Ca^{2+} of a preparation of mitochondria made using a procedure which minimized net influx or efflux of Ca^{2+} (McCormack and Denton, 1984; Hansford *et al.*, 1990). These results are displayed as Table II. Although these experiments measured total Ca,

Table II. Relation between Mitochondrial Calcium Content and Dehydrogenase Activation in the Heart

Preparation and conditions	PDH_A, % of total	2-OGDH, % of V_{max}	Total mitochondrial Ca (nmol/mg protein)
Perfused rat heart			
Control	8	25	1.8[a]
+ Epinephrine	20	47	4.2[a]
Perfused guinea pig heart			
Control	16		2.73 (1.37)[b]
Electrical stimulation (2 Hz)	68		4.10 (2.74)[b]

[a] From McCormack and Denton (1984).
[b] From Hansford *et al.* (1990). For the guinea pig, Ca content enclosed in parentheses was obtained by ^{45}Ca distribution: that without parentheses was obtained by atomic absorption spectroscopy.

there is every reason to believe that changes would yield proportionate changes in $[Ca^{2+}]_m$ (Hansford and Castro, 1982; McCormack *et al.*, 1989). A larger drawback to these experiments, when compared to the estimation of $[Ca^{2+}]_m$ in living cardiac myocytes (Section 3), is that they involved a one-time, destructive measurement. Despite this, they established the principle that inotropic stimulation and increased work load involve raised mitochondrial Ca^{2+} content, as well as dehydrogenase activation.

A key tenet of the model which is being presented is that activation of dehydrogenases by Ca^{2+} ions will maximize mitochrondrial NADH/NAD^+ ratios during periods of high energy-demand. According to Eq. (1), this allows high flux through the overall process of oxidative phosphorylation without the necessity of the adenine nucleotide phosphorylation potential (of which the concentration term is ATP/ADP × Pi) falling to levels which would be unacceptable for cytosolic functioning. It is not possible to predict whether mitochondrial NADH/NAD^+ will rise or fall in response to enhanced work load, only that it will fall less than would be the case if activation of dehydrogenases had not occurred. Recent work using ^{31}P nmr has suggested that the response to an imposed work load varies among different types of muscle (Chance *et al.*, 1986).

In heart, there is good evidence that only minimal decreases in ATP/ADP × Pi occur, or that there is no change at all, when the work load is raised. This is true both of the isolated glucose-perfused rat heart (Clarke and Willis, 1987; From *et al.*, 1986; Katz *et al.*, 1987, 1988) and the *in situ*, open-chested canine preparation (Balaban *et al.*, 1986; Katz *et al.*, 1989). The latter result is particularly convincing, because the heart muscle is of course perfused with blood and thus a physiological mix of substrates. These results are presented in Table III. When dehydrogenase activation is precluded by

Table III. Adenine Nucleotide Phosphorylation Potential, and Free ADP Concentration as a Function of Cardiac Work Performance[a]

A. In vivo canine heart

Protocol	Heart rate (bpm)	Rate pressure product × 10^{-3}	O_2 uptake (μmol/min/g)	[CrP]/[ATP]	[Pi]	[ADP] (μM)
Electrical pacing	121	8.6	1.8	1.9	24	56
	200	14.1	3.4	1.8	28	58
	246	15.9	4.8	1.8	22	56
Epinephrine (μg/min/kg)						
0	128	8.3	1.2	1.9	20	59
0.4	134	14.0	2.7	1.8	23	61
1.0	162	20.5	4.3	2.0	28	54
2.0	189	26.7	5.5	1.9	29	57

B. Isolated, perfused rat heart (1)

Oxidizable substrate	Heart rate (bpm)	O_2 uptake (nmol/h/g dry wt)	[CrP]/[ATP]	[Pi]
Glucose	255	1.73	0.83	166
	590	2.29	0.95	160
Pyruvate	280	1.96	1.24	54
	600	2.59	1.29	74

B. Isolated, perfused rat heart (2)

Oxidizable substrate	Time after isoproterenol (s)	10^3 × Phosphorylation potential (M^{-1})	[ATP]/[ADP]
Glucose	−10	> 20	48
	10	2.2	19
	30	3.6	29
	60	17	96
Glucose + ruthenium red	−10	> 20	84
	10	2.2	13
	30	1.7	15
	60	1.5	18

[a]Data in (A) are from Katz *et al.* (1989); (B, 1) Katz *et al.* (1988); (B, 2) Unitt *et al.* (1989). For errors and statistical significance, see the original papers. The concentration term of the phosphorylation potential is ATP/ADP × Pi. ATP/ADP can be calculated from [CrP] × [H^+] × K_{eq}/[Cr], where CrP is creatine phosphate, Cr is creatine and K_{eq} is the equilibrium constant of the creatine kinase reaction. Free ADP is calculated from the same relationship. Where [CrP]/[ATP] is presented, an increase in this parameter indicates an increase in phosphorylation potential.

prior perfusion with ruthenium red (McCormack and England, 1983), the ATP/ADP × Pi ratio does decrease with an increased pacing rate (Katz *et al.*, 1988). When these relations were studied using an improved time resolution, it was found that β-adrenergic stimulation of the isolated glucose-perfused rat heart resulted in a transient decline in ATP/ADP × Pi ratio, followed by a return to the nonstimulated steady-state (Unitt *et al.*, 1989).

Prior treatment with ruthenium red greatly extended the period over which the ATP/ADP × Pi ratio declined (Table III). Thus, these two studies are in good agreement in showing that any work-induced fall in phosphorylation potential in the glucose-perfused heart is small and transient, provided that the mechanism of activation of dehydrogenases by Ca^{2+} is functioning normally. When, by contrast, the substrate provided is a nonphysiologically high concentration of pyruvate, this overrides dehydrogenase regulation in the state of low work load, giving rise to very high values of phosphorylation potential (Katz *et al.*, 1988): increased work load then does give rise to a decline in phosphorylation potential (Table III). High concentrations of pyruvate generate high contents of PDH_A, regardless of Ca^{2+}, through inhibition of the pyruvate dehydrogenase kinase (Hucho *et al.*, 1972). In turn, high pyruvate dehydrogenase activity may tend to enhance flux through NAD-isocitrate and 2-oxoglutarate dehydrogenases, regardless of Ca^{2+}, by giving rise to higher concentrations of their substrates, isocitrate and 2-oxoglutarate.

Evidence that increased flux through oxidative phosphorylation is in fact driven by an increased mitochondrial $NADH/NAD^+$ ratio, in the response of the glucose-perfused heart to increased work load, is mixed. This reflects the difficulty of making an appropriate measurement of this parameter in heart. Thus, Hiltunen and Hassinen (1976) found that mitochondrial $NADH/NAD^+$ was higher on K^+-induced arrest, and Starnes *et al.* (1985) found no change when they raised the work load of glucose-perfused hearts. Both of these investigations used the glutamate dehydrogenase equilibrium to calculate free mitochondrial $NADH/NAD^+$. The problem here is that the enzyme may not be active enough to achieve equilibrium in the heart, and the measurement of glutamate, 2-oxoglutarate, and NH_3 in heart extracts ignores the compartmentation of these metabolites which occurs *in vivo*. By contrast, Katz *et al.* (1987) used surface fluorescence to demonstrate that an increased pacing rate (255 to 590 beats per min) is associated with a 23% increase in NAD(P)H fluorescence, and with a 32% increase in O_2 uptake, in the glucose-perfused rat heart. This probably underestimates the increase in mitochondrial NADH, as the fluorescence derives partly from NADPH, which is likely to stay highly reduced under all physiological conditions (see, e.g., Hansford and Johnson, 1975). It would seem that the evidence for an increased $NADH/NAD^+$ as a consequence of dehydrogenase activation is perhaps the weakest link in this model of the control of oxidative phosphorylation in heart.

Activation of Dehydrogenases, and Oxidative Phosphorylation, by Calcium-Mobilizing Hormones in Liver and Isolated Hepatocytes

The actions of the so-called "calcium-mobilizing" hormones on liver provide a clear example of the activation of dehydrogenases by Ca^{2+} and its effect upon oxidative phosphorylation as a whole. Vasopressin, glucagon, and α_1-adrenergic agonists are known to stimulate the release of glucose from liver and, as part of the mechanism of this process, they raise values of $[Ca^{2+}]_c$. This has been shown directly in experiments with suspensions of hepatocytes loaded with fluorescent Ca^{2+}-chelating agents (Charest *et al.*, 1983; Mauger *et al.*, 1984, 1985; Sistare *et al.*, 1985; Staddon and Hansford, 1986, 1987) or aequorin (Woods *et al.*, 1986). Associated with this increase in $[Ca^{2+}]_c$ is an increased rate of O_2 uptake (Sugano *et al.*, 1980; Balaban and Blum, 1982; Reinhart *et al.*, 1982; Blackmore *et al.*, 1983) despite an unchanged, or even increased, ATP/ADP ratio (Siess *et al.*, 1977, 1978; Soboll and Scholz, 1986; Titheradge and Haynes, 1980). The redox state of mitochondrial NAD, as indexed by fluorimetry, is found to go more reduced upon hormonal activation, and then to return partially to pre-stimulated levels (Sugano *et al.*, 1980; Balaban and Blum, 1982; Haussinger and Sies, 1984; Charest *et al.*, 1983; Sistare *et al.*, 1985; Quinlan and Halestrap, 1986; Patel and Olson, 1986; Staddon and Hansford, 1987). At the same time, the PDH_A content of the tissue or cells is found to increase (Assimacopolous-Jeannet *et al.*, 1983, 1986; Hems *et al.*, 1978; McCormack, 1985b; Staddon and Hansford, 1987) and there is evidence for an activation of 2-oxoglutarate dehydrogenase, as seen by a decrease in the steady-state concentration of 2-oxoglutarate (Haussinger and Sies, 1984; Siess *et al.*, 1977; Staddon and McGivan, 1984, 1985) and, more directly, by increased rates of $^{14}CO_2$ release from 1-^{14}C labelled glutamate, 2-oxoglutarate, or octanoate (Haussinger and Siess, 1984; Rashed *et al.*, 1988; Taylor *et al.*, 1986). These findings are consistent with a response of the mitochondrial Ca^{2+}-transport cycle to the proven increase in $[Ca^{2+}]_c$, leading to an increase in $[Ca^{2+}]_m$ and dehydrogenase activation. The consequent increase in the mitochondrial NADH/NAD^+ ratio then allows more rapid flux through oxidative phosphorylation, despite the unchanged, or enhanced, ATP/ADP $\times$ Pi ratio [see Equation (1)]. The transient nature of the increase in the NADH/NAD^+ ratio, which is particularly clear with glucagon (Staddon and Hansford, 1987), despite the sustained potentiation of O_2 uptake, could be well explained by the activation of the respiratory chain at the cytochrome b, c_1 level which occurs in response to these agonists and which is slow in onset (Halestrap, 1987a; see McCormack *et al.*, 1990a for detailed discussion). The section below scrutinizes recent (post 1985) evidence bearing upon these suggested relations.

Experiments with isolated rat liver mitochondria have confirmed that

dehydrogenase activity can be modulated by changes in $[Ca^{2+}]_o$. Thus, provided care is taken to isolate mitochondria with a low Ca content, it can be shown that pyruvate dehydrogenase activation and the reduction of mitochondrial NAD^+ by nonsaturating 2-oxoglutarate or citrate is responsive to changes in $[Ca^{2+}]_o$ in the range 50 nM to 1 μM (McCormack, 1985a). Moreover, rates of O_2 uptake with nonsaturating concentrations of pyruvate, isocitrate, and 2-oxoglutarate as substrate have now also been shown to respond to extramitochondrial $[Ca^{2+}]$, in the range 100–800 nM (Johnston and Brand, 1987). These studies are significant, in that earlier work had tended not to show modulation of respiratory activity by Ca^{2+} in liver mitochondria, in distinction to the clear effects with heart mitochondria (Hansford, 1985). Isolation of liver mitochondria with a content of total Ca close to that shown *in situ* by electron probe microanalysis, viz. around 1 nmol Ca/mg mitochondrial protein (Somlyo *et al.*, 1985; Bond *et al.*, 1987), and incubation at a physiological temperature may be prerequisites for demonstrating Ca^{2+} sensitivity (Johnston and Brand, 1987).

Experiments with suspensions of isolated hepatocytes have shown that $[Ca^{2+}]_c$ rises into the range (100–800 nM) shown above to modulate dehydrogenase activity of isolated mitochondria, when the cells are challenged with vasopressin, glucagon, and phenylephrine (Charest *et al.*, 1983; Berthon *et al.*, 1984; Thomas *et al.*, 1984; Sistare *et al.*, 1985; Staddon and Hansford, 1986, 1987). In these studies, $[Ca^{2+}]_c$ was estimated from the fluorescence of quin-2, fura-2, and indo-1. As mentioned above (Section 3), results from cells loaded with the acetoxymethyl esters of these compounds cannot simply be interpreted in terms of $[Ca^{2+}]_c$, as some loading of the mitochondrial compartment also occurs. Probably this is not so great a problem with hepatocytes as it is with cardiac myocytes, owing to the lower volume fraction occupied by mitochondria. Nevertheless, it does preclude accurate determination of $[Ca^{2+}]_c$. A much larger problem in the interpretation of these results is that studies with single hepatocytes (Woods *et al.*, 1986; Cobbold, 1989; Berridge, 1990; Kawanishi *et al.*, 1989; Rooney *et al.*, 1989; Sanchez-Bueno *et al.*, 1990; Reber *et al.*, 1990) as well as other cell types (Berridge, 1990; Berridge and Galione, 1988) have indicated that $[Ca^{2+}]_c$ does not rise monotonically to challenge by an agonist, but instead rises in a burst of oscillations. In general, higher agonist concentrations are associated with a higher oscillation frequency, and each hormone gives rise to a characteristic "signature" of oscillations (Berridge and Galione, 1988; Berridge *et al.*, 1988). Within a population, cells respond to a hormone or agonist after a variable delay, and with a pattern of $[Ca^{2+}]_c$ spikes which seems to be distinctive for each cell (see, e.g., Prentki *et al.*, 1988). Summed over a population, the result is a gradual and monotonic rise after the addition of an agonist, as seen in the studies with cell suspensions which are cited above. From the work on

hepatocytes containing the Ca^{2+} indicator aequorin (Woods *et al.*, 1986), it is clear that cytosolic Ca^{2+} "spikes" may reach 1 μM and last several seconds. It seems likely that a substantial mitochondrial Ca^{2+} uptake will occur in response to each "spike," and this could probably be followed with indo-1/AM-loaded and Mn^{2+}-quenched cells, as outlined for myocytes in Section 3. It is noted that the results of Reber *et al.* (1990), involving the simultaneous measurement of $[Ca^{2+}]_c$ and mitochondrial membrane potential ($\Delta\Psi$) in hormonally challenged hepatocytes, do not really negate such an idea. The near constancy of $\Delta\Psi$ in the face of oscillations of $[Ca^{2+}]_c$, taken by the authors as evidence that mitochondrial Ca^{2+} transport has an insignificant effect on oscillations of $[Ca^{2+}]_c$, could also be consistent with a matching of dehydrogenase activation by Ca^{2+} and the extra energy demand of Ca^{2+} transport, at both the mitochondrial and other cellular membranes.

In the absence of direct measurements of $[Ca^{2+}]_m$, conclusions based upon estimates of $[Ca^{2+}]_c$ from suspensions of hepatocytes (Charest *et al.*, 1983; Berthon *et al.*, 1984; Thomas *et al.*, 1984; Sistare *et al.*, 1985; Staddon and Hansford, 1986, 1987) and the sensitivity to $[Ca^{2+}]_o$ of liver mitochondria in steady-state experiments (McCormack, 1985a; Johnson and Brandt, 1987) remain rather tenuous. Significantly stronger are results from experiments in which dehydrogenase activity and mitochondrial Ca content are determined in tissue which has been challenged with a "calcium-mobilizing" hormone. Assimacopoulos-Jeannet *et al.* (1986) showed that treatment of isolated, perfused rat liver with either vasopressin or glucagon increased not only the PDH_A content of the tissue but also the content of total Ca of a mitochondrial fraction which was subsequently isolated. The technique of isolation is chosen to minimize redistribution of Ca^{2+} and seems to be successful in that regard (McCormack, 1985b). Some of these results are presented in Table IV, which shows that treatment with both hormones together results both in greater dehydrogenase activation and also a larger increase in mitochondrial Ca content. For comparison, Table IV also displays results from Staddon and Hansford (1987), using isolated hepatocytes, which show a sensitivity of pyruvate dehydrogenase interconversion which compares with that of the intact tissue. It is noted that there is no unanimity that the "calcium-mobilizing" hormones raise mitochondrial Ca^{2+} content. Stimulation of the liver *in vivo* with vasopressin or vasopressin plus glucagon led to no detectable increase in total mitochondrial Ca, as measured by electron probe microanalysis, in a study by Bond *et al.* (1987). However, the degree of stimulation of glycogen phosphorylase was quite modest, questioning the efficacy of the hormonal treatment. Measurement of PDH_A content of the rapidly frozen liver would have allowed a more incisive appraisal of these findings.

The increase in mitochondrial $NADH/NAD^+$ ratio which accompanies the effect of the "calcium-mobilizing" hormones seems well established

Table IV. Dehydrogenase Activation by Ca^{2+}-Mobilizing Hormones in Liver and Hepatocytes[a]

Preparation and conditions	PDH_A % of total	2-OGDH, % of V_{max}	Total mitochondrial Ca (nmol/mg protein)
Perfused rat liver			
Control	17	6	1.2[b]
+ Vasopressin	31	22	2.1[b]
+ Glucagon	30	16	2.3[b]
+ Glucagon and vasopressin	44	32	4.9[b]
Isolated hepatocytes			
Control	22		[c]
+ Phenylephrine	26		[c]
+ Vasopressin	36		[c]
+ Glucagon	49		[c]

[a]For statistical significance, see the original papers.
[b]From Assimacopoulos-Jeannet *et al.* (1986).
[c]From Staddon and Hansford (1987).

(Sugano *et al.*, 1980; Balaban and Blum, 1982; Charest *et al.*, 1983; Haussinger and Sies, 1984; Sistare *et al.*, 1985; Quinlan and Halestrap, 1986; Patel and Olson, 1986; Staddon and Hansford, 1987). The fact that the increase in $NADH/NAD^+$ ratio is not sustained, whereas the increased rate of O_2 uptake is, defines a further activation at the level of the respiratory chain. Since this has been discussed recently in some detail (Halestrap, 1989; McCormack *et al.*, 1990a), it will not be discussed further here. The elevated mitochondrial $NADH/NAD^+$ ratios seen in hepatocytes activated with "calcium-mobilizing" hormones should allow a more quantitative treatment of the model of dehydrogenase control which is being propounded here (and see Hansford, 1980; Heineman and Balaban, 1990; McCormack *et al.*, 1990a), according to the work of Akerboom *et al.* (1978) [Eq. (1)]. The advantage of this system is the presence of an active 3-hydroxybutyrate dehydrogenase in liver, allowing measurement of mitochondrial free $NADH/NAD^+$, and the sheer size of the increase in this parameter which occurs.

In terms of the physiology of the mitochondrial response to the "calcium-mobilizing" hormones, the activation of pyruvate dehydrogenase is an enigma. Increased flux through gluconeogenesis requires increased flux through pyruvate carboxylase, but not pyruvate dehydrogenase. It may be that extensive feedback inhibition of pyruvate dehydrogenase by acetyl-CoA and NADH prevents the increase in the catalytically active form, PDH_A, from being a metabolic embarrassment. In some studies of PDH_A content as a function of Ca^{2+} mobilization (see, e.g., Staddon and Hansford, 1986, 1987), pyruvate dehydrogenase interconversion was investigated as a guide to changes in $[Ca^{2+}]_m$, with the understanding that this was important for the control of

2-oxoglutarate dehydrogenase. The reason for this oblique approach is that the effect of Ca^{2+} on the pyruvate dehydrogenase interconversion is stable and can be measured in tissue extracts, whereas the effect of Ca^{2+} on 2-oxoglutarate dehydrogenase is that of an allosteric activator (McCormack and Denton, 1979; Lawlis and Roche, 1981a, b) and does not survive tissue extraction.

Activation of 2-oxoglutarate dehydrogenase by Ca^{2+} is thought to affect gluconeogenesis in two ways. The demonstrated decrease in 2-oxoglutarate concentration (Siess *et al.*, 1977; Haussinger and Sies, 1984; Staddon and McGivan, 1984, 1985) increases the export of aspartate from the mitochondria on the glutamate/aspartate exchange (Strzelecki *et al.*, 1988) by virtue of decreasing competition between 2-oxoglutarate and oxaloacetate at the level of the aspartate aminotransferase. The glutamate/aspartate exchange appears likely to have high control strength in the transport of reducing equivalents from the cytosol to the mitochondria by the malate/aspartate shuttle (see LaNoue and Schoolwerth, 1979). This mechanism is consistent with reports of increased flux through the shuttle due to "calcium-mobilizing" hormones (Leverve *et al.*, 1986; Kneer and Lardy, 1983; Taylor *et al.*, 1983). It is also noted that the α_1-adrenergic agonist phenylephrine gives rise to an oxidation of cytosolic NADH and a reduction of mitochondrial NAD^+ in isolated perfused rat liver (Taylor *et al.*, 1983), consistent with a stimulation of the malate/aspartate shuttle. The second suggested part of the mechanism of stimulation of gluconeogenesis follows from the observed fall in glutamate concentration, presumably a consequence of the decrease in 2-oxoglutarate, which would be expected to reverse the inhibition of pyruvate carboxylase (Scrutton and White, 1974).

Activation of Oxidative Metabolism by Ca^{2+} in Other Tissues

In all mammalian tissues which have been examined, the three intramitochondrial dehydrogenases discussed in this article have been found to be Ca^{2+}-sensitive (McCormack and Denton, 1981b). In addition, significant activities of the Ca^{2+}-sensitive mitochondrial glycerol 3-phosphate dehydrogenase have been found in brain (Ringler and Singer, 1959; Beleznai *et al.*, 1988), lung (Fisher *et al.*, 1973), pancreatic β-cells (MacDonald, 1981), brown adipose tissue (Bukowiecki and Lindberg, 1974; Houštek *et al.*, 1975), and a leukemia-derived ascites tumor cell (Hansford and Lehninger, unpublished: presented in Fig. 7). Since all mammalian mitochondria which have been examined possess a high-affinity Ca^{2+}-transport system (Carafoli and Lehninger, 1971), it is expected that an elevation of $[Ca^{2+}]_c$ which is profound and sustained enough to elevate $[Ca^{2+}]_m$ will lead to an activation

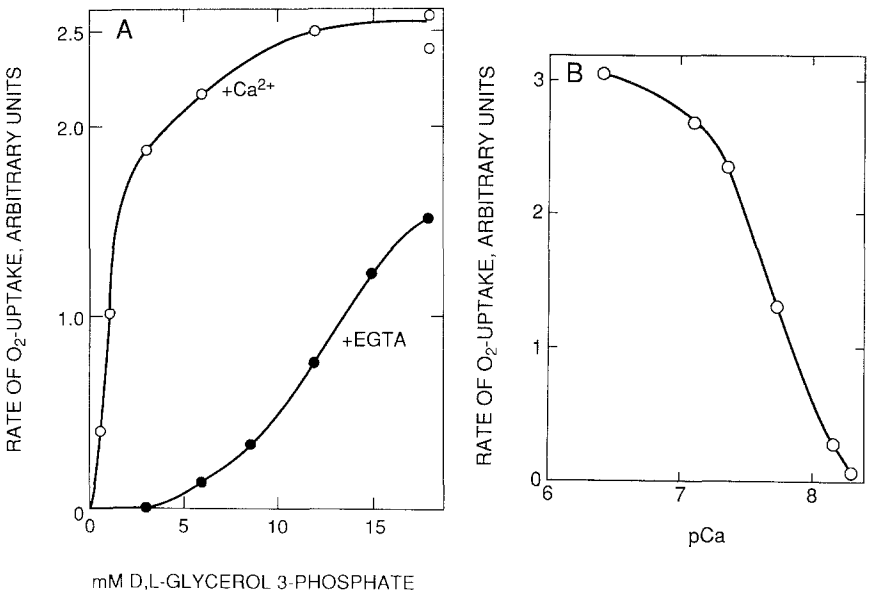

Fig. 7. The Ca^{2+} dependence of glycerol 3-phosphate oxidation by L-1210 ascites tumor cell mitochondria. Enzyme activity was followed by rates of O_2 uptake. In panel A, glycerol 3-phosphate concentration was varied, in the presence of 10^{-6}M Ca^{2+} or EGTA. In panel B, the oxidation of 3 mM D,L-glycerol 3-phosphate was stidied at different values of $[Ca^{2+}]_o$. From Hansford and Lehninger (unpublished).

of pyruvate oxidation and flux through the tricarboxylate cycle. In general, elevated $[Ca^{2+}]_c$ is associated with increased levels of work performance by the cell, and thus the stimulation of oxidative phosphorylation may be considered a mechanism by which energy supply and demand are maintained in balance (Hansford, 1980, 1985; Heineman and Balaban, 1990; McCormack *et al.*, 1990a). The rest of this section will discuss briefly some mammalian tissues, other than heart and liver, in which there is evidence for Ca^{2+}-linked activation of dehydrogenases.

In work with synaptosomes (pinched-off presynaptic terminals) from rat cerebral cortex, it was shown that depolarization with elevated K^+ concentrations (Schaffer and Olson, 1980; Hansford and Castro, 1985) or with the alkaloid veratridine (Hansford and Castro, 1985) led to an increase in the fraction of pyruvate dehydrogenase in the form PDH_A. At the same time, there was an elevation of $[Ca^{2+}]_c$, as measured with quin-2 (Hansford and Castro, 1985). Treatment with ruthenium red decreased the value of PDH_A in unstimulated synaptosomes and blunted the increase due to depolarization. The effect on resting values of PDH_A is different from the picture with isolated cardiac myocytes (Hansford, 1987), and suggests that $[Ca^{2+}]_m$ is

higher in unstimulated synaptosomes than in myocytes. This is consistent with the value of $[Ca^{2+}]_c$ of 200 nM determined for unstimulated synaptosomes using quin-2 (Hansford and Castro, 1985, but see Ashley *et al.*, 1984; Richards *et al.*, 1984). These studies (Hansford and Castro, 1985) did not establish, however, that the Ca^{2+} ion is the only signal tending to elevate PDH_A in response to depolarization. Thus, depolarization in a Ca^{2+}-free medium prevented the increase in $[Ca^{2+}]_c$ but only partially attenuated the rise in PDH_A content. Almost certainly, a decreased ATP/ADP ratio is also functioning as an effector of interconversion in these experiments which use raised K^+ concentration or veratridine to give a sustained plasma-membrane depolarization. A physiological protocol involving electrical stimulation of nervous tissue and normal action potentials would be needed to dissect apart the relative roles of Ca^{2+} and ATP/ADP ratio in dehydrogenase activation *in situ*.

There is controversy as to whether extrasynaptosomal Ca^{2+} leads to higher rates of pyruvate oxidation or not. Kauppinen and Nicholls (1986) found no requirement for Ca^{2+} when respiration was stimulated by veratridine or FCCP, conditions under which increases in mitochondrial ADP are likely to render not only pyruvate dehydrogenase interconversion but also flux through NAD-isocitrate and 2-oxoglutarate dehydrogenases less sensitive to Ca^{2+} (Denton *et al.*, 1978; McCormack and Denton, 1979). By contrast, Patel *et al.* (1988) found that flux through pyruvate dehydrogenase and the tricarboxylate cycle was only stimulated by veratridine in the presence of extrasynaptosomal Ca^{2+}. Although bioenergetically well coupled, synaptosomes are after all an experimental artefact, and the results obtained probably depend upon the degree of depletion (or repletion) of Ca^{2+} and oxidizable substrate.

In the β-cell of the pancreas, it is known that exposure to raised glucose concentrations elicits an increase in $[Ca^{2+}]_c$, and that this is involved in the signalling processes leading to release of insulin (Wollheim and Sharp, 1981; Prentki and Wollheim, 1984; Turk *et al.*, 1987). Moreover, ATP/ADP and NAD(P)H/NAP(P)$^+$ ratios are elevated (Prentki and Matschinsky, 1987; Matschinsky *et al.*, 1989), suggesting activation at the dehydrogenase level. This has been shown directly for pyruvate dehydrogenase (McCormack *et al.*, 1990b). In these studies, there was an increase in PDH_A content of rat pancreatic islets with increasing glucose concentration, in a response which mirrored insulin release. Interestingly, the PDH_A response seemed to precede that of insulin release. The β-cell of the pancreas has a very high activity of the Ca^{2+}-sensitive, mitochondrial glycerol 3-phosphate dehydrogenase (MacDonald, 1981; Meglasson and Matschinsky, 1986), and thus it seems likely that the glucose-dependent increase in $[Ca^{2+}]_c$, due to plasma membrane depolarization and the opening of voltage-dependent Ca^{2+} channels,

will result in the more active oxidation of both the pyruvate and the NADH formed by glycolysis. Stimulation of the redox shuttle which comprises mitochondrial and cytosolic glycerol 3-phosphate dehydrogenases (Estabrook and Sacktor, 1958; Klingenberg and Bücher, 1961) would oxidize cytosolic NADH and reduce mitochondrial NAD^+. Owing to the greater enhancement of mitochondrial NADH fluorescence, the expected net result would be an increase in cell fluorescence from NAD(P)H. At the moment it is not clear whether the increased $[Ca^{2+}]_c$ of pancreatic β-cells seen with exposure to elevated glucose drives the increase in O_2 uptake and ATP/ADP ratio, through this mechanism of dehydrogenase activation, or whether $[Ca^{2+}]_c$ increases secondarily as a consequence of the change in ATP/ADP ratio. The rise in pyruvate and glycerol 3-phosphate concentrations in the activated tissue (B.A. Corkey, personal communcation) provides a mechanism for increased flux through these enzymes separate from the Ca^{2+} effect.

Another system which is worth discussing in this incomplete survey of evidence for control by Ca^{2+} in intact tissues involves the mechanism of the stimulation of O_2 uptake by the invertebrate retina. Experiments by Tsacopoulos *et al.* (1983) on photoreception by slices of the compound eye of the honey bee established the intriguing finding that an increased rate of O_2 uptake precedes increases in cellular free Na^+ concentration, but not that of free Ca^{2+} concentration. A mechanism of raised O_2 uptake due to enhanced Na^+/K^+-ATP-ase activity and hence raised ADP concentrations seems precluded by these time dependences, raising the possibility that instead elevated $[Ca^{2+}]_c$ is the signal to the mitochondria in this system. If so, the locus of action is likely to be the glycerol 3-phosphate dehydrogenase, which is known to be Ca^{2+}-sensitive in insects (Hansford and Chappell, 1967), whereas the intramitochondrial trio of dehydrogenases is not (McCormack and Denton, 1981b). Related experiments from the same group have established that the light-induced increase in O_2 uptake in the ventral photoreceptor of the horseshoe crab, Limulus, does not correlate with the rise in intracellular Na^+ (Fein and Tsacopoulos, 1988a) but does correspond to the rise in $[Ca^{2+}]_c$ (Fein and Tsacopoulos 1986b). Moreover, injection of an equivalent amount of Ca^{2+} caused an increase in O_2 uptake (Fein and Tsacopoulos 1986b). As the increased respiration due to light was not blocked by injection of ruthenium red, the authors concluded that the site of action of the Ca^{2+} was the mitochondrial glycerol 3-phosphate dehydrogenase, which is also consistent with the reasoning above. This is exciting work, providing evidence for a role for the Ca^{2+}-sensitivity of this enzyme in coupling energy supply to demand in an excitable tissue.

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