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Effects of Ca^{2+} and Mg^{2+} on the activity of pyruvate dehydrogenase phosphate phosphatase within toluene-permeabilized mitochondria

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1. Mitochondria from rat epididymal white adipose tissue were made permeable to small molecules by toluene treatment and were used to investigate the effects of Mg^{2+} and Ca^{2+} on the re-activation of pyruvate dehydrogenase phosphate by endogenous phosphatase. 2. Re-activation of fully phosphorylated enzyme after addition of 0.18 mM-Mg^{2+} showed a marked lag of 5–10 min before a maximum rate of reactivation was achieved. Increasing the Mg^{2+} concentration to 1.8 mM (near saturating) or the addition of 100 μM-Ca^{2+} resulted in loss of the lag phase, which was also greatly diminished if pyruvate dehydrogenase was not fully phosphorylated. It is concluded that, within intact mitochondria, phosphatase activity is highly sensitive to the degree of phosphorylation of pyruvate dehydrogenase and that the major effect of Ca^{2+} may be to overcome the inhibitory effects of sites 2 and 3 on the dephosphorylation of site 1. 3. Apparent $K_{0.5}$ values for Mg^{2+} and Ca^{2+} were determined from the increases in pyruvate dehydrogenase activity observed after 5 min. The $K_{0.5}$ for Mg^{2+} was diminished from 0.60 mM at $<$ 1 nM-Ca^{2+} to 0.32 mM at 100 μM-Ca^{2+}; at 0.18 mM-Mg^{2+}, the $K_{0.5}$ for Ca^{2+} was 0.40 μM. Ca^{2+} had little or no effect at saturating Mg^{2+} concentrations. Since effects of Ca^{2+} are readily observed in intact coupled mitochondria, it follows that Mg^{2+} concentrations within mitochondria are sub-saturating for pyruvate dehydrogenase phosphate phosphatase and hence less than 0.5 mM.

INTRODUCTION

The pyruvate dehydrogenase (PDH) complex of higher animals is an intramitochondrial enzyme regulated by a phosphorylation/dephosphorylation cycle, with the phosphorylated form being virtually inactive. Cycling is achieved by the opposed actions of a specific kinase, which appears to be tightly associated with the complex, and a more loosely bound phosphatase (for reviews see Denton *et al.*, 1975; Randle *et al.*, 1981; Wieland, 1983; Reed & Yeaman, 1986). Phosphorylation occurs *in vitro* and *in vivo* on the α-subunits of the pyruvate decarboxylase component (E1) of the complex, on three serine residues, designated sites 1, 2 and 3 (Yeaman *et al.*, 1978; Sugden *et al.*, 1979; Hughes *et al.*, 1980; Sale & Randle, 1981*a*,*b*). Extensive studies on isolated enzymes, intact mitochondria and *in vivo* indicate that the major site responsible for inactivation may be site 1 (Yeaman *et al.*, 1978; Sale & Randle, 1981*a*,*b*, 1982*a*,*b*). Phosphorylation of site 2 alone may also cause inactivation, but rarely occurs in the absence of phosphorylation of site 1; phosphorylation of site 3 is non-inactivating (Hughes *et al.*, 1980; Sale & Randle, 1981*b*, 1982*a*,*b*). Randle and colleagues have argued, on the basis of extensive studies on purified pig heart enzyme and intact rat heart mitochondria, that the role of phosphorylation of sites 2 and 3 may be to inhibit re-activation by diminishing the rate of dephosphorylation at site 1 by PDHP phosphatase (Sugden *et al.*, 1978; Kerbey & Randle, 1979; Sugden & Simister, 1980; Sale & Randle, 1982*b*).

Studies on partially purified and purified preparations of PDH complex, phosphatase and kinase have identified a number of potential regulators of the extent of phosphorylation *in vivo*. The kinase may be activated by increasing the [acetyl-CoA]/[CoA] and [NADH]/[NAD+] ratios and inhibited by increases in the concentrations of ADP and pyruvate (Reed, 1974; Cooper *et al.*, 1975; Pettit *et al.*, 1975; Pratt & Roche, 1979). The isolated phosphatase requires Mg^{2+} and is activated by Ca^{2+} (Denton *et al.*, 1972; Pettit *et al.*, 1972; Siess & Wieland, 1972). The action of Ca^{2+} seems to be to decrease both the apparent K_m for PDHP at saturating Mg^{2+} concentrations (Pettit *et al.*, 1972; Randle *et al.*, 1974) and the apparent K_m for Mg^{2+} (Denton *et al.*, 1972; Thomas *et al.*, 1986). Studies using intact mitochondria have, in general, confirmed that the kinase and phosphatase are sensitive to changes in the intramitochondrial concentrations of these potential regulators (Martin *et al.*, 1972; Kerbey *et al.*, 1976; Hansford, 1976, 1977; Denton *et al.*, 1980; Marshall *et al.*, 1984; Thomas *et al.*, 1986). One important difference is that the major effect of Ca^{2+} on PDHP phosphatase within these mitochondria appears to be to decrease the apparent K_m for Mg^{2+}, with little or no effect of Ca^{2+} at saturating concentrations of Mg^{2+} (Thomas *et al.*, 1986). This discrepancy between results from intact mitochondria and isolated enzymes may be due to the low concentrations of PDHP employed in studies on purified preparations of PDHP phosphatase (Thomas *et al.*, 1986), and illustrates the need to examine the control of PDH activity while the PDH system is located within the mitochondrial matrix.

A major problem in the investigation of the regulation

Abbreviations used: PDH, pyruvate dehydrogenase; PDH_a, PDHP, active (phosphorylated) and inactive (non-phosphorylated) forms of pyruvate dehydrogenase respectively; AABS, *p*-(*p*-aminophenylazo)benzenesulphonic acid; HEDTA, *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid. Throughout this paper Mg^{2+} and Ca^{2+} refer to the unbound species of these bivalent ions.

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of PDH activity in intact mitochondria has been that, until recently, it was necessary to extract the complex from previously incubated mitochondria in order to measure the extent of activation or changes in phosphorylation. This is inconvenient and potentially inaccurate (see Thomas *et al.*, 1986). We have now developed a technique, based on the toluene-permeabilization procedure of Matlib *et al.* (1977), which allows the continuous assay of PDH activity while all the components of the PDH system remain within the mitochondrial matrix (Thomas & Denton, 1986). The characteristics and advantages of this technique have been described in some detail (Thomas & Denton, 1986). In the present paper, we describe further refinements which allow an accurate time course of the re-activation of PDH by endogenous PDHP phosphatase to be determined in the absence of any PDH kinase activity. We also report the effects of Mg^{2+} and Ca^{2+} on the activity of PDHP phosphatase within these permeabilized mitochondria. The role of Ca^{2+} in the regulation of intramitochondrial oxidative metabolism has been reviewed (Hansford, 1985; Denton & McCormack, 1985; McCormack & Denton, 1986).

METHODS

Materials

Chemicals and biochemicals were obtained from sources given previously (Thomas & Denton, 1986). In addition, plasmocorinth B [3-(5-chloro-2-hydroxyphenylazo)-4,5-dihydroxynaphthalene-2,7-disulphonic acid] was from Sigma Chemical Co., Poole, Dorset, U.K. Epididymal fat-pads of male Wistar rats (170–220 g) were incubated in the absence of hormones, and mitochondria were prepared as in Thomas & Denton (1986). The mitochondria were then permeabilized with toluene by the modification of the procedure of Matlib *et al.* (1977) described by Thomas & Denton (1986).

Assay of PDH activity

The permeabilized mitochondria (about 40 μg of mitochondrial protein/ml) were incubated at 30 °C in a medium composed of 100 mM-sucrose, 50 mM-KCl, 50 mM-Mops, 35 mM-Tris, 8.5% (w/v) polyethylene glycol 6000, 2 mM-KH_2PO_4, 1 mM-EGTA, 1 mM-HEDTA, 1 mM-dithiothreitol, 0.1 mM-$MgCl_2$, 5 μg of oligomycin/ml, 1 μg of antimycin A/ml, 1 μg of rotenone/ml, pH 7.2. PDH activity was measured after addition of 1 mM-pyruvate, 0.5 mM-NAD^+, 0.1 mM-CoA and 1 mM-thiamin pyrophosphate together with the dye AABS (10 μg/ml) and arylamine acetyltransferase (about 30 munits/ ml, where 1 unit of activity converts 1 μmol of substrate into products in 1 min). Acetyl-CoA produced from pyruvate caused a loss of absorbance at 460 nm owing to acetylation of the dye AABS, which was measured with a Pye–Unicam SP.8-100 spectrophotometer. Other additions were made as given in Figure legends.

In experiments where the activity of PDH was continually changing (Figs. 1–3), absorbance measurements were collected and manipulated with a Hewlett–Packard 9854A computer connected directly to the SP.8-100 analogue output via an analogue-to-digital converter. Data points were collected every 3s, each being the average of 132 separate measurements made throughout that period. PDH activity was calculated at each data point by fitting a quadratic polynomial to 25 sequential data points and hence calculating a gradient at the central data point. For data points 2–13 fewer points were fitted, but an equal number of points on either side of the calculated point was always used. This method was based on the least-squares fitting procedure of Savitzky & Golay (1964). In these studies, signal noise was minimized by (i) carrying out incubations in a total volume of 2 ml in a standard cuvette (1 cm × 1 cm × 4 cm) placed in a cuvette holder immediately in front of the photomultiplier to diminish light scattering, and (ii) computer-averaging sets of data points from up to three runs carried out under identical conditions but with separate preparations of permeabilized mitochondria.

In experiments where PDH activity was allowed to attain a steady-state value (Figs. 4–6), assays were carried out in a total volume of 1 ml in micro-cuvettes (0.4 cm path width × 1 cm × 4 cm), four at a time, by using an automatic cell changer. In this case, rates were measured directly from the SP.8-100 chart recorder.

Calculation of free concentrations of Mg^{2+} and Ca^{2+}

These were calculated as given in Thomas *et al.* (1986) on the basis that, at pH 7.2, the dissociation constants for Mg–EGTA, Ca–EGTA, Mg–HEDTA and Ca–HEDTA were 14.1 mM, 0.83 μM, 43 μM and 1.94 μM respectively. The published values for the stability constant for Mg–HEDTA vary from 5.2 to 7.1 (Sillén & Martell, 1964; Martell & Smith, 1974; Durham, 1983), which correspond to a range of calculated dissociation constants at pH 7.2 of 2550 to 34 μM. We therefore determined the apparent dissociation constant at pH 7.2 by using changes in the absorbance of the dye plasmocorinth B to monitor changes in Mg^{2+} (Scarpa, 1979). By following changes in the value of $A_{554-592}$ on addition of various concentrations of Mg^{2+} and HEDTA at a constant pH to a solution containing 50 μM-plasmocorinth B, a dissociation constant at pH 7.2 of 43 ± 2 μM was determined. This corresponds to a stability constant of 7.0, in agreement with the values of 7.0 given by Martell & Smith (1974) and 7.1 by Durham (1983). Calculations were therefore based on a stability constant of 7.0.

In the presence of added Ca^{2+}, calculated adjustments were made to the added $MgCl_2$ to ensure that the free Mg^{2+} concentrations were the same as in the absence of Ca^{2+}. The dye plasmocorinth B was used to check that the adjustments were correct. Close attention was also paid to keeping the pH constant at 7.2 throughout the studies, because of the great sensitivity of the dissociation constants of both EGTA and HEDTA to changes in pH.

RESULTS

Time course of re-activation of PDHP by endogenous PDHP phosphatase within toluene-permeabilized mitochondria

In the experiment shown in Fig. 1, toluene-permeabilized mitochondria were first incubated with

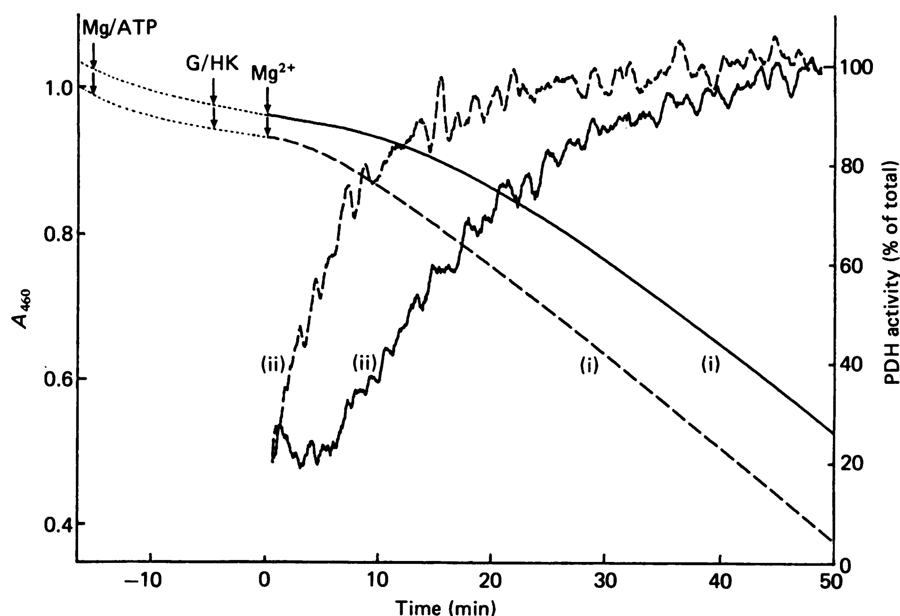

Fig. 1. Time course of the re-activation of PDHP by endogenous phosphatase in toluene-permeabilized mitochondria

Permeabilized mitochondria were incubated with 0.2 mM-ATP and 0.15 mM-$MgCl_2$ for 10 min, resulting in phosphorylation of PDH and hence near-complete loss of activity (see Thomas & Denton, 1986); 10 mM-glucose and hexokinase (1.4 units/ml; G/HK) were then added to remove excess ATP. After 5 min, re-activation of PDHP was initiated by addition of further $MgCl_2$ to give calculated concentrations of 0.18 mM-Mg^{2+} and 1.8 mM-Mg^{2+}. The Figure shows: (i) the time course of the loss of A_{460} corresponding to the acetylation of the dye AABS in the presence of 0.18 mM-Mg^{2+} (——) or 1.8 mM-Mg^{2+} (----); (ii) time courses of the increases in PDH obtained by differentiation of curves in (i). Data are from a single preparation of mitochondria.

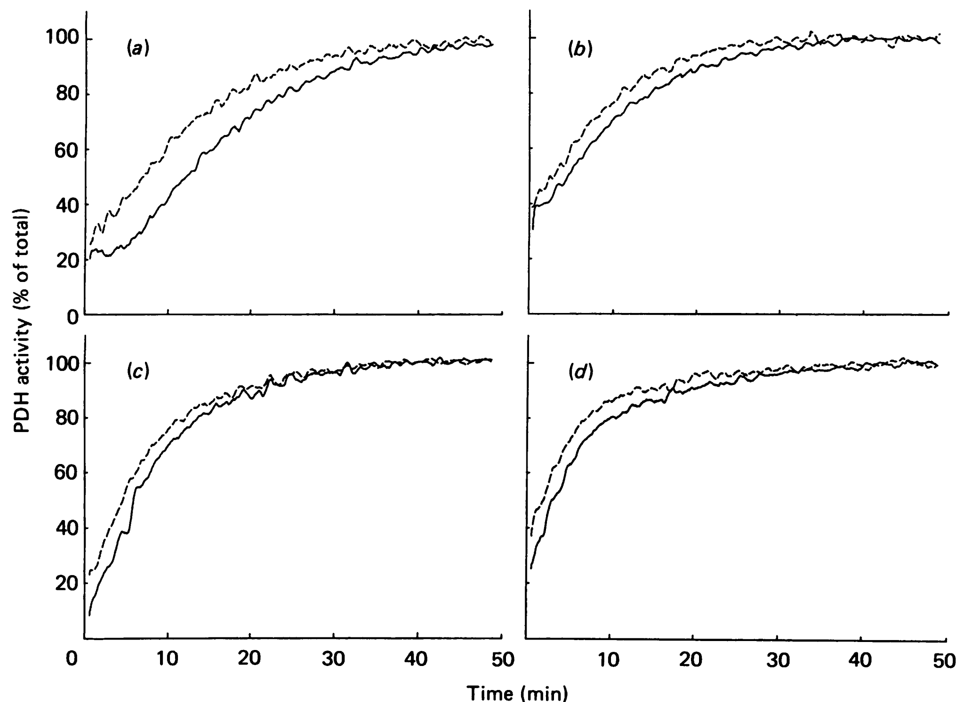

Fig. 2. Effects of Ca^{2+}, Mg^{2+} and extent of phosphorylation on rates of re-activation of PDHP by endogenous phosphatase in toluene-permeabilized mitochondria

In (*a*) and (*c*), PDH was fully phosphorylated before re-activation, as described in Fig. 1. In (*b*) and (*d*), PDHP was re-activated without prior treatment with MgATP. Re-activation was initiated by adding amounts of $MgCl_2$ and $CaCl_2$ to give either 0.18 mM-Mg^{2+} (*a* and *b*) or 1.8 mM-Mg^2 (*c* and *d*) together with either < 1 nM-Ca^{2+} (——) or 0.1 mM-Ca^{2+} (----). Each curve is the differential of computer-averaged data from three separate mitochondrial preparations. Initial rates of re-activation (as % of total activity/min, ±S.D.) were calculated by fitting the time courses to a first-order rate equation. Values obtained in the absence of added $CaCl_2$ were as follows: (*a*) not determined; (*b*) 6.2±0.1; (*c*) 11.2±0.2; (*d*) 10.7±0.3. The corresponding values in the presence of Ca^{2+} were: (*a*) 6.2±0.1; (*b*) 7.6±0.1; (*c*) 10.7±0.2; (*d*) 10.3±0.3.

MgATP for 10 min at 30 °C. This resulted in maximal inactivation of PDH. Glucose and hexokinase were added to convert all the ATP into ADP, and incubation was continued for 5 min. Substrates of the pyruvate dehydrogenase reaction, the dye AABS and arylamine acetyltransferase were then added, and the time courses of re-activation were observed after addition of $MgCl_2$ to give either 0.18 mM-Mg^{2+} or 1.8 mM-Mg^{2+} [Fig. 1, trace (i)], and the differentials were computed [Fig. 1, trace (ii)].

At the lower, sub-saturating, concentration of Mg^{2+} there was a marked lag period before the maximum rate of re-activation was attained. In ten separate experiments this period was always in the range of 5–10 min. In contrast, at the higher, near-saturating, Mg^{2+} concentration, there was no appreciable lag period.

The noise on the differential traces was mainly a result of the light-scattering by the mitochondrial preparations. However, the time courses with different mitochondrial preparations were highly reproducible, allowing computer averaging, with the elimination of much of this noise. This procedure was carried out in Fig. 2. At 0.18 mM-Mg^{2+}, the mean differential trace, in the absence of added Ca^{2+}, showed a lag of about 10 min, as before (Fig. 2*a*). In contrast, in the presence of 100 μM-$CaCl_2$, there was a lag of less than 1 min. Ca^{2+} had little effect on the maximum rate of re-activation in the presence of either 0.18 mM- or 1.8 mM-Mg^{2+} (Figs. 2*a* and 2*c*).

When permeabilized mitochondria were first prepared, the activity of PDH was about 30% of the total activity. From the extensive studies of Sale & Randle (1982*a*,*b*) on the relationship between rat heart PDH activity and site occupancy both *in vivo* and in isolated mitochondria, it is probable that under these conditions the phosphorylation of sites 2 and 3 will be considerably less than that of site 1. Addition of 0.18 mM-Mg^{2+} in the absence of Ca^{2+} to such mitochondria caused re-activation, with little or no lag period (Fig. 2*b*). Time courses of re-activation were in fact very similar to those observed after pre-treatment with MgATP (Fig. 2*a*) after about 30% re-activation had occurred: only a modest effect of Ca^{2+} was evident. As with mitochondria pre-treated with MgATP, there was little or no effect of Ca^{2+} evident in the presence of 1.8 mM-Mg^{2+} (Fig. 2*d*).

All the time courses of activation shown in Fig. 2 closely fitted a first-order rate equation, except that in Fig. 2(*a*) obtained in the absence of added Ca^{2+}. The legend to Fig. 2 gives the calculated initial rates of re-activation, obtained by multiplying the first-order rate constants by the initial amount of inactive phosphorylated complex.

Fig. 3 further demonstrates the effects of the time of pre-treatment of the permeabilized mitochondria with MgATP on the time course of re-activation with a sub-saturating concentration of Mg^{2+} (0.18 mM) in the absence of added Ca^{2+}. Some lag is apparent after 1 min pre-treatment, whereas the lag after 3 min pre-treatment approaches that after 10 min pre-treatment. In all cases the maximum rates of re-activation were apparent when the PDH activity was 20–40% of the maximum rate, and these rates were essentially unaffected by the time of pre-treatment with MgATP.

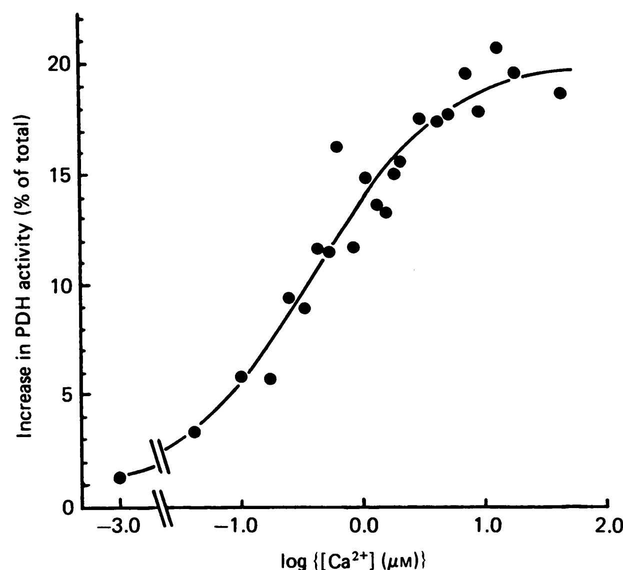

Fig. 4. Sensitivity of re-activation of PDHP by endogenous phosphatase to Ca^{2+} within toluene-permeabilized mitochondria determined by the NaF-stopped assay technique

PDH within permeabilized mitochondria was fully phosphorylated and ATP was removed as described in Fig. 1. Re-activation was initiated by addition of $MgCl_2$ (to give 0.18 mM-$MgCl_2$) and $CaCl_2$ to give different concentrations of Ca^{2+}. After 5 min, PDHP phosphatase activity was blocked by addition of 30 mM-NaF, and the then constant PDH activity was determined. Averaged data from three separate preparations of mitochondria are shown and have been fitted by non-linear least-squares regression to the equation:

$$v - V_{min.} = V_{max.}/\{1+(K_{0.5}/[Ca^{2+}])^h\}$$

where $V_{min.}$ is the PDH activity in the absence of added Ca^{2+}, $V_{max.}$ is the maximal increase in PDH activity with Ca^{2+}, $K_{0.5}$ is the concentration of Ca^{2+} giving half-maximal stimulation and h is the Hill coefficient.

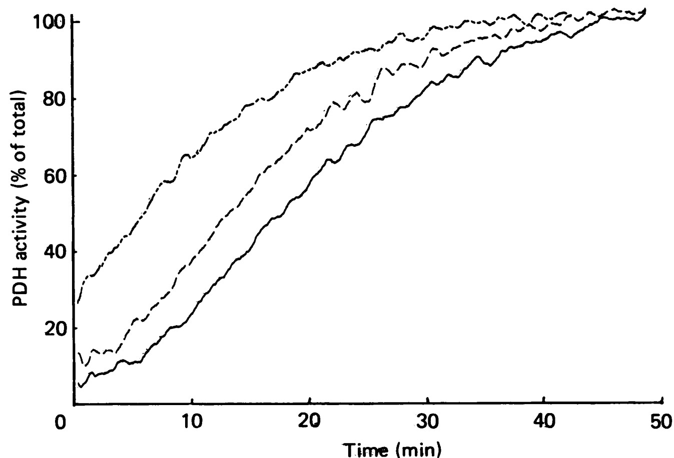

Fig. 3. Relationship between extent of phosphorylation and time course of PDHP re-activation

Permeabilized mitochondria were incubated with 0.2 mM-ADP and 0.15 mM-$MgCl_2$ for 0.5 min (– – – –) or with 0.2 mM-ATP and 0.15 mM-$MgCl_2$ for 1 min (– – – –) or 3 min (——), followed by 5 min incubation with 10 mM-glucose and hexokinase (1.4 units/ml). Re-activation of PDHP was initiated by addition of further $MgCl_2$ to give 0.18 mM-Mg^{2+}. Each curve is the differential of data from a single preparation of mitochondria.

Sensitivity of re-activation of PDHP within toluene-permeabilized mitochondria to changes in the concentration of Ca^{2+} and Mg^{2+}

The time courses of re-activation shown in Figs. 1–3 indicated that the maximum effects of Ca^{2+} and Mg^{2+} were apparent after about 5 min. To study the sensitivity of re-activation of fully phosphorylated PDHP to Ca^{2+} and Mg^{2+} concentrations, re-activation was allowed to proceed for 5 min in the presence of various concentrations of these ions. After this period, re-activation was stopped by addition of 30 mM-NaF to inhibit further PDHP phosphatase activity (Thomas & Denton, 1986). Constant activities of PDH were then observed, which could be accurately determined.

Fig. 4 shows the effects of various concentrations of Ca^{2+} at 0.18 mM-Mg^{2+}. In the absence of added Ca^{2+} (< 1 nM), the increase in PDH activity after 5 min was about 2% of the total activity of PDH. In the presence of 10–100 μM-Ca^{2+}, the increase in PDH activity was some 10 times greater and approached 20%. Under these conditions the apparent $K_{0.5}$ for Ca^{2+} was 0.4 ± 0.1 μM and the Hill coefficient, h, 0.7 ± 0.15 (parameter values given ± S.D. for 19 degrees of freedom).

Fig. 5 shows the sensitivity found to changes in Mg^{2+} concentration in the presence and absence of 100 μM-Ca^{2+}. At saturating concentrations of Mg^{2+}, little or no effect of Ca^{2+} was found, and approximately half the PDHP was re-activated. The major effect of Ca^{2+} was to diminish the $K_{0.5}$ for Mg^{2+} from 0.60 ± 0.07 mM to 0.32 ± 0.03 mM (values given ± S.D. for 8 degrees of freedom; $P < 0.05$). The corresponding calculated values (± S.D.) of h and $V_{max.}$ were 2.1 ± 0.5 and 46 ± 4% in the absence of Ca^{2+} and 1.8 ± 0.3 and 48 ± 2% in its presence. Similar results were obtained in a separate series of experiments carried out in the presence and absence of 25 μM-Ca^{2+} (results not shown).

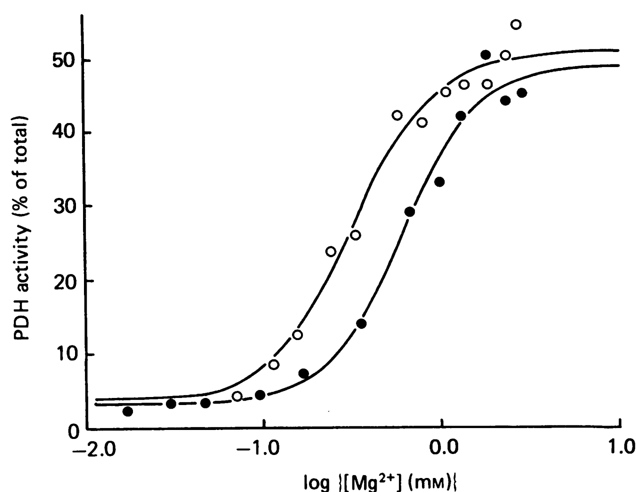

Fig. 5. Sensitivity of re-activation of PDHP by endogenous phosphatase to Mg^{2+} within toluene-permeabilized mitochondria determined by the NaF-stopped assay technique

Details were as for Fig. 4, except that re-activation was initiated with additions of various amounts of $MgCl_2$ wither in the absence of added $CaCl_2$ (●; [Ca^{2+}] < 1 nM) or in the presence of sufficient added $CaCl_2$ to give 100 μM-Ca^{2+} (○). Curve fitting to meaned data from three separate preparations of mitochondria was carried out as described in Fig. 4, except that [Ca^{2+}] in the equation was replaced by [Mg^{2+}].

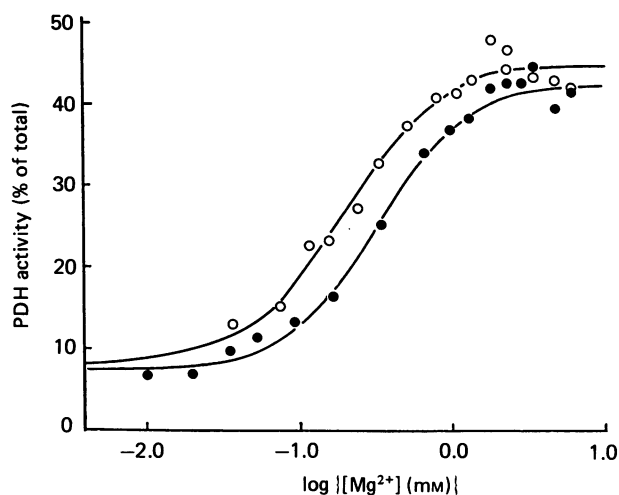

Fig. 6. Effect of Ca^{2+} on the Mg^{2+}-sensitivity of changes in the steady-state activity of PDH in toluene-permeabilized mitochondria incubated in the presence of MgATP

Steady-state activities of PDH were determined in toluene-permeabilized mitochondria incubated in medium containing 0.2 mM-ATP, and with various additions of $MgCl_2$ plus either no added $CaCl_2$ (●; Ca^{2+} < 1 nM) or added $CaCl_2$ to give a calculated free concentration of 100 μM-Ca^{2+} (○). Curve fitting of mean data of three separate experiments was carried out as in Fig. 5.

The effects of Ca^{2+} on Mg^{2+}-sensitivity were also examined by measuring the steady-state activity of PDH obtained in the presence of MgATP to produce a constant activity of PDH kinase, together with varying the concentrations of $MgCl_2$ and $CaCl_2$ (Fig. 6). In this system the sensitivity to Mg^{2+} appeared to be somewhat greater, but again the major effect of Ca^{2+} was to diminish the $K_{0.5}$ for Mg^{2+}. Values calculated for $K_{0.5}$ for Mg^{2+} from the data shown in Fig. 6 were 0.33 ± 0.03 mM and 0.20 ± 0.02 mM in the absence and presence of 100 μM-Ca^{2+} respectively (values given ± S.D. for 12 degrees of freedom; $P < 0.05$). The corresponding values of h and $V_{max.}$ were 1.5 ± 0.2 and 36 ± 2% in the absence of Ca^{2+} and 1.3 ± 0.2 and 37 ± 1% in the presence of Ca^{2+}.

DISCUSSION

The present studies involved further refinements in the use of toluene-permeabilized mitochondria. This allowed direct measurement of the effects of Mg^{2+} and Ca^{2+} on the time course of PDHP re-activation by endogenous phosphatase in the absence of PDH kinase activity within intact mitochondria. Some interesting observations were made. The most striking was the very marked lag in the time course of re-activation of PDHP under certain conditions. The lag was only evident after the permeabilized mitochondria had been pre-treated with MgATP to phosphorylate the complex fully, and then re-activation was carried out at sub-saturating concentrations of Mg^{2+} and Ca^{2+}. The most likely explanation for this is that, under these conditions, phosphorylation of sites 2 and/or 3 inhibits the dephosphorylation of site 1 and hence re-activation.

Randle and his colleagues have obtained evidence with the purified pig heart complex that the rate of re-activation of the complex in which all three sites are phosphorylated may be markedly diminished compared with the complex only phosphorylated in site 1 (Sugden *et al.*, 1978; Kerbey & Randle, 1979; Kerbey *et al.*, 1981). However, these studies were carried out at saturating concentrations of Ca^{2+} and Mg^{2+} and much lower concentrations of PDH complex and phosphatase than those occurring within mitochondria. The present results suggest that this phenomenon can occur under conditions likely to pertain within mitochondria, i.e. at high concentrations of complex and phosphatase and limiting concentrations of Mg^{2+} and Ca^{2+}. The presence of Ca^{2+} may overcome the inhibitory effects of phosphorylation of sites 2 and 3, perhaps by specifically stimulating their dephosphorylation. Direct studies on the extents of phosphorylation of sites 1, 2 and 3 within toluene-permeabilized mitochondria are required to confirm these suggestions.

In the presence of high concentrations of Mg^{2+} with or without Ca^{2+}, there was very little lag in the onset of re-activation and the time courses could be fitted closely to a first-order rate equation (see legend to Fig. 2). Attempts were also made to estimate a value for the apparent K_m for PDHP under these conditions by using the integrated form of the Michaelis–Menten equation and plotting $\ln[(P_{total}-P_0)/(P_{total}-P_t)]/t$ against $(P_t-P_0)/t$, where P_0, P_t and P_{total} were the activity of PDH at time 0, time t and time infinity. However, values for the apparent $K_{0.5}$ were of the order of twice the initial PDHP and therefore could not be determined with any precision (hence the close fit to a first-order rate equation). The total PDH in rat epididymal adipose-tissue mitochondria is about 100 munits/mg of mitochondrial protein (Denton *et al.*, 1984), which corresponds to about 100 units/ml of matrix volume. A minimum estimate of the apparent $K_{0.5}$ for PDHP in the permeabilized mitochondria incubated with saturating Ca^{2+} and Mg^{2+} was thus about 200 units of PDHP/ml (where 1 unit of PDHP yields 1 unit of PDH on activation). This is much greater than the value of about 2 units/ml obtained with purified pig heart PDH and PDHP phosphatase under apparently comparable conditions (Randle *et al.*, 1974). The reasons for this marked discrepancy between the behaviour of the purified PDH system and the system when located within toluene-permeabilized mitochondria remain to be established, but may reflect interactions of the PDH system with other intramitochondrial components, including the mitochondrial inner membrane, which are lost on purification. However, it can be concluded from the present studies that the rate of re-activation by the phosphatase within mitochondria will be highly sensitive to changes in the ratio of PDH_a to PDHP under all conditions.

Finally, the present studies have shown directly and in the absence of PDH kinase activity that the major effect of Ca^{2+} on PDHP phosphatase activity within mitochondria is to diminish the apparent $K_{0.5}$ for Mg^{2+} and that there is little or no effect of Ca^{2+} at saturating concentrations of Mg^{2+}. This confirms the conclusions of previous studies using mitochondria made permeable to bivalent metal ions by the ionophore A23187, in which PDH kinase activity was maintained at an assumed constant value by the addition of MgATP. It should be noted that the calculated apparent $K_{0.5}$ values for Mg^{2+} found in the present studies are lower than those found for the isolated enzyme and with A23187-permeabilized mitochondria, whereas the $K_{0.5}$ values for Ca^{2+} were very similar (McCormick & Denton, 1980; Thomas *et al.*, 1986). The basis for these differences requires further study. Nevertheless, effects of Ca^{2+} are readily observed with intact coupled mitochondria (Hansford & Cohen, 1978; Denton *et al.*, 1980, 1984; McCormack, 1985), indicating that Mg^{2+} concentrations within mitochondria are sub-saturating for PDHP-phosphatase. The estimates of Mg^{2+} in liver mitochondria by a null-point titration support this conclusion (Corkey *et al.*, 1986).

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