Sensitivity of pyruvate dehydrogenase phosphate phosphatase to magnesium ions

Similar effects of spermine and insulin

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1. The effects of Mg^{2+} on the activity of pyruvate dehydrogenase phosphate phosphatase within intact mitochondria prepared from control and insulin-treated rat epididymal adipose tissue was explored by incubating the mitochondria in medium containing the ionophore A23187. The apparent K_a for Mg^{2+} was approximately halved in the mitochondria derived from insulin-treated tissue in both the absence and the presence of Ca^{2+} . In this system, the major effect of Ca^{2+} was also to decrease the apparent K_a for Mg^{2+} , rather than to change the V_{max} of the phosphatase. 2. Damuni, Humphreys & Reed [(1984) *Biochem. Biophys. Res. Commun.* **124**, 95–99] have reported that spermine activates ox kidney pyruvate dehydrogenase phosphate phosphatase. Studies were carried out on phosphatase from pig heart and rat epididymal adipose tissue which confirm and extend this observation. The major effect of spermine is shown to be a decrease in the K_a for Mg^{2+} , which is apparent in both the presence and the absence of Ca^{2+} . Spermine did not affect the sensitivity of the phosphatase to Ca^{2+} at saturating concentrations of Mg^{2+} . Other polyamines tested were not as effective as spermine. 3. No alteration in the maximum activity or Mg^{2+} -sensitivity of pyruvate dehydrogenase phosphate phosphatase was apparent in extracts of mitochondria from insulin-treated tissue. 4. The close similarity of the effects of spermine and the changes in kinetic properties of pyruvate dehydrogenase phosphate phosphatase within mitochondria from insulin-treated adipose tissue suggests that insulin may activate pyruvate dehydrogenase by increasing the concentration of spermine within the mitochondria. However, it is concluded that insulin is more likely to alter the interaction of the pyruvate dehydrogenase system with some other polybasic intramitochondrial component whose action can be mimicked by spermine.

INTRODUCTION

The reaction catalysed by pyruvate dehydrogenase (PDH) represents the point at which carbohydrate is irreversibly lost to the pathways of the citrate cycle, fatty acid synthesis and other non-carbohydrate products. The enzyme, which is exclusively mitochondrial, is highly regulated by a range of metabolic and hormonal factors in all mammalian tissues. Much of the acute regulation of PDH is achieved through alterations in the phosphorylation state of the enzyme (for reviews see Denton *et al.*, 1975; Wieland, 1983; Reed & Yeaman, 1986). Phosphorylation of the α -subunits of the pyruvate decarboxylase components of the PDH complex by a tightly associated kinase results in almost total inactivation of the enzyme. The inactive PDHP can be re-activated by a specific phosphatase, which may be only loosely associated with the PDH complex. Within the intact mitochondria it is the balance between kinase and phosphatase activities which determines the steady-state amount of the active dephosphorylated form of the complex (PDH_a).

PDH kinase represents the site at which PDH_a activity may be modulated by the metabolic and energy demands of the cell, since the kinase is activated with increasing

ratios of [acetyl-CoA]/[CoA] and [NADH]/[NAD⁺] and the enzyme is inhibited by ADP and pyruvate (Cooper *et al.*, 1974, 1975; Reed, 1981; Wieland, 1983; Reed & Yeaman, 1986). In contrast, the major regulators of PDHP phosphatase are the bivalent cations Mg^{2+} and Ca^{2+} . The enzyme is completely inactive in the absence of Mg^{2+} and may be activated 10–20-fold by Ca^{2+} in the presence of Mg^{2+} (Denton *et al.*, 1972; Siess & Wieland, 1972; Hucho *et al.*, 1972). PDHP phosphatase appears to be the site at which several hormones act to alter PDH_a activity. For example, the activation of PDH by catecholamines in heart and liver results from an increased mitochondrial Ca^{2+} content, with consequent activation of PDHP phosphatase (Denton & McCormack, 1980, 1985; McCormack & Denton, 1984; McCormack, 1985a,b). Evidence has also been obtained to suggest that activation of PDHP phosphatase is the means whereby insulin-induced PDH activation occurs in adipose tissue (Hughes & Denton, 1976; Denton *et al.*, 1984; Marshall *et al.*, 1984). Mitochondria prepared from insulin-treated adipose tissue and incubated under conditions where both phosphatase and kinase are active maintain a higher steady-state PDH_a activity than do mitochondria from untreated tissue. This persistent effect of insulin is not reversed by depletion of mitochondrial Ca^{2+} , and this

Abbreviations used: PDH, pyruvate dehydrogenase; PDH_a , PDHP, active non-phosphorylated and inactive phosphorylated forms of pyruvate dehydrogenase respectively. Throughout this paper Mg^{2+} and Ca^{2+} refer to the free unbound species of these bivalent cations.

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observation, together with several other lines of evidence, has led to the conclusion that insulin does not activate PDHP phosphatase through changes in mitochondrial $[Ca^{2+}]$ (Marshall *et al.*, 1984).

Damuni *et al.* (1984) have reported on a new potential regulator of PDHP phosphatase, spermine. This polyamine was shown to activate PDHP phosphatase at low Mg^{2+} concentrations, although having lesser effects at saturating Mg^{2+} concentrations. In the present paper the effects of polyamines on PDHP phosphatase are characterized further and compared with the persistent effect of insulin on this enzyme. It is shown that polyamines decrease the K_a of PDHP phosphatase for Mg^{2+} in a Ca^{2+} -independent manner. The persistent activation of PDHP phosphatase in mitochondria from insulin-treated adipose tissue is also shown to result from an apparent increased sensitivity to Mg^{2+} , and the possible role of polyamines in this effect of insulin is discussed.

METHODS

Materials

Chemicals and biochemicals were obtained from the sources given previously (Denton *et al.*, 1984). All polyamines were from Sigma (London) Chemical Co., Poole, Dorset, U.K. Pig heart PDH was purified by the method of Cooper *et al.* (1974). PDHP phosphatase was partially purified from a crude preparation of pig heart mitochondria as described by Severson *et al.* (1974). The preparations were free of PDHP and PDH_a and were capable of generating 2–5 units of PDH_a from added pig heart PDHP/min per mg of protein. $[^{32}P]$ PDHP was prepared from pig heart PDH_a essentially as given by Denton *et al.* (1972). The resulting $[^{32}P]$ PDHP contained about 1 nmol of ^{32}P /unit of original PDH_a activity [this corresponds to about 3 mol/mol of the pyruvate dehydrogenase component ($\alpha_2\beta_2$) (Randle *et al.*, 1981)]. All phosphorylation was found to be associated with the α -subunit of the pyruvate decarboxylase component when the preparation was analysed by SDS/polyacrylamide-gel electrophoresis and could be completely released by incubation with PDHP phosphatase.

Preparation and incubation of mitochondria: assay of PDH activity

Adipose-tissue mitochondria were prepared from epididymal fat-pads obtained from male rats (170–220 g) as described previously (Denton *et al.*, 1984). For the determination of steady-state PDH_a activities, the mitochondria were incubated in a standard medium composed of 120 mM-KCl, 30 mM-Mops, 2 mM-potassium phosphate, 1 mM-EGTA, 1 mM-HEDTA [*N*-(2-hydroxy-ethyl)ethylenediaminetriacetic acid], pH 7.2, with other additions as required, for 5 min at 30 °C. The incubations were terminated by centrifugation for 40 s at 15000 *g* in a mini-centrifuge, and the resulting mitochondrial pellets were frozen in liquid N₂ for subsequent assay of PDH_a and total PDH.

The frozen samples were extracted in 100 μ l of buffer composed of 100 mM-potassium phosphate, 5 mM-EDTA and 2 mM-dithiothreitol, pH 7.2, with 0.2% Triton X-100 and 5% (v/v) rat serum added. Initial and total PDH activity were then assayed as described by Stansbie *et al.* (1976).

Assay of PDHP phosphatase

PDHP phosphatase was assayed with purified $[^{32}P]$ PDHP as substrate and by measuring the release of $[^{32}P]P_i$ as described by Denton *et al.* (1972), except for the following changes. The assay medium consisted of 50 mM-Mops, 1 mM-EGTA, 1 mM-HEDTA and 2 mM-dithiothreitol, with the pH adjusted to 7.2 with Tris. For most experiments, the proteinase inhibitors pepstatin, antipain and leupeptin were also added at 1 μ g/ml each, and the assay volume was 35 μ l. Free concentrations of Mg^{2+} and Ca^{2+} were calculated by an iterative computer program from the absolute stability constants of the relevant ligands corrected to the working pH of 7.2 (Denton *et al.*, 1978). The combination of EGTA and HEDTA as a Ca^{2+}/Mg^{2+} buffering system gave excellent control of the free concentrations of both ions over the physiological range of 0.1–10 μ M for Ca^{2+} and 0.1–10 mM for Mg^{2+} . The calculated dissociation constants used at pH 7.2 for EGTA were 0.83 μ M with Ca^{2+} and 14.1 mM with Mg^{2+} , and for HEDTA they were 1.94 μ M with Ca^{2+} and 43 μ M with Mg^{2+} (Martell & Smith, 1974). Phosphatase assays were initiated by adding $[^{32}P]$ PDHP (corresponding to 10–20 munits of original PDH) to give 10000–20000 c.p.m. per assay tube, and the reactions were stopped by adding 700 μ l of 10% (v/v) trichloroacetic acid. If less than 30% of the $[^{32}P]$ PDHP substrate was dephosphorylated, the assays were linear with time.

Where phosphatase activity was measured in mitochondrial extracts, the mitochondria were suspended in the phosphatase assay medium described above and frozen and thawed three times to ensure complete disruption of the mitochondrial membranes. Mitochondrial extracts prepared in this manner were used in the standard $[^{32}P]$ PDHP phosphatase assay at a final concentration of about 2 mg of mitochondrial protein/ml. In these assays, added $[^{32}P]$ PDHP was in about 4-fold excess over endogenous PDHP.

Analysis and expression of results

Kinetic constants for the effects of Mg^{2+} and Ca^{2+} on PDHP phosphatase have been calculated by fitting the relevant data by non-linear least-squares regression to either a simple hyperbolic equation or, where indicated, allowing for co-operative kinetics by using a computer program written by Dr. P. J. England (present address Smith, Kline & French Research, Welwyn, Herts, U.K.). One unit of PDH activity is the amount catalysing the utilization of 1 μ mol of substrate/min at 30 °C.

RESULTS

Increased sensitivity of PDHP phosphatase to Mg^{2+} in mitochondria from insulin-treated adipose tissue

In order to examine the Mg^{2+} -sensitivity of PDHP phosphatase in intact rat epididymal-adipose-tissue mitochondria, the mitochondrial inner membrane was made permeable to bivalent cations by using the ionophore A23187. In addition, to ensure equilibration of Mg^{2+} ions by the ionophore, the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone ('FCCP') was added to dissipate any transmembrane electrical or pH gradients that might build up, and oligomycin together with ATP and an ATP-regenerating system was added to provide a constant concentration of ATP for PDH kinase. In this way a measure of PDHP phosphatase

Fig. 1. Sensitivity of PDHP phosphatase to Mg^{2+} in mitochondria from control and insulin-treated adipose tissue

Rat epididymal fat-pads were incubated for 30 min in the absence (●) or presence (■) of 50 nM-insulin before isolation of the mitochondria. The mitochondria were incubated in the basic incubation medium described in the Methods section with 10 μ M-ionophore A23187, 0.5 μ M-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide, 5 μ g of oligomycin/ml, 2 mM-ATP, 10 mM-phosphocreatine, 10 units of creatine kinase/ml plus $MgCl_2$ and $CaCl_2$ to give the required free concentrations. After 5 min the incubations were terminated by rapid centrifugation and the mitochondrial pellets subsequently assayed for PDH_a . The total amount of PDH activity was unaffected by insulin treatment or by the variations in Mg^{2+} and Ca^{2+} concentrations. In (a) the sensitivity to Mg^{2+} is shown in the presence of approx. 100 μ M- Ca^{2+} , whereas in (b) no Ca^{2+} was added ($Ca^{2+} < 1$ nM). The curves represent the best fit to the data calculated as described in the legend to Table 2. Each point is the mean of duplicate measurements obtained from a single experiment. The mean calculated K_a values for data combined from a series of three such experiments are included in Table 2.

activity could be obtained from the steady-state activity of PDH_a under conditions where kinase activity was held constant (Fig. 1). A similar approach has been used previously to explore the Ca^{2+} -sensitivity of PDHP-phosphatase (McCormack & Denton, 1980; Marshall *et al.*, 1984).

In Fig. 1, the activity of PDHP phosphatase has been

manipulated within the A23187-treated mitochondria by varying the extramitochondrial Mg^{2+} concentration in the presence and absence of Ca^{2+} . For these experiments the concentration of MgATP was always well in excess of the K_m of PDH kinase. The apparent K_a for Mg^{2+} of the PDHP phosphatase in control mitochondria calculated by non-linear regression was 2.7 ± 0.6 mM in the absence of Ca^{2+} and 1.1 ± 0.2 mM in the presence of about 100 μ M- Ca^{2+} (this concentration is sufficient to result in full Ca^{2+} -activation of the phosphatase). A similar effect of Ca^{2+} on the Mg^{2+} -sensitivity of purified PDHP phosphatase preparations is also found (Denton *et al.*, 1972; see also Fig. 3). This is probably the major means whereby Ca^{2+} activates PDHP phosphatase within mitochondria, since little effect of Ca^{2+} is evident at a saturating concentration of Mg^{2+} (Fig. 1). This is discussed more fully below.

When the apparent K_a for Mg^{2+} was calculated in mitochondria prepared from insulin-treated tissue, a significant shift to lower concentrations of Mg^{2+} was found in both the absence and the presence of Ca^{2+} (to 1.8 ± 0.2 mM and 0.6 ± 0.1 mM respectively; see Table 2). There was no appreciable difference between the mitochondria from control and insulin-treated tissue in terms of the maximal activity of PDH_a achieved at saturating concentrations of Mg^{2+} . Thus insulin treatment appears to cause a persistent increase in the sensitivity of PDHP phosphatase to Mg^{2+} in a manner which is essentially independent of the increase observed with Ca^{2+} and does not apparently involve an increase in the V_{max} of the enzyme.

Effects of spermine on the sensitivity of PDHP phosphatase to Ca^{2+} and Mg^{2+}

The effect of insulin to increase the apparent sensitivity to Mg^{2+} of PDHP phosphatase within the intact mitochondria has some similarity to the activation of purified ox kidney PDHP phosphatase by spermine at low Mg^{2+} concentrations reported by Damuni *et al.* (1984). It was therefore decided to examine the effect of spermine directly on the Mg^{2+} -sensitivity of PDHP phosphatase under defined Ca^{2+} concentrations. For these experiments, PDHP phosphatase partially purified from crude pig heart mitochondria was used initially and the substrate was PDH purified from the same source and phosphorylated with [32 P]ATP. In agreement with the results of Damuni *et al.* (1984), we have found that the most effective concentration of spermine was 0.5 mM (Fig. 2). Although Ca^{2+} activated the phosphatase by more than 10-fold, both the sensitivity to spermine and the percentage stimulation were similar in the absence and presence of Ca^{2+} .

In Fig. 3, data combined from a series of experiments to examine the effects of Ca^{2+} and spermine on the sensitivity of pig heart PDHP phosphatase to Mg^{2+} are shown. Each of the four Mg^{2+} -titration curves represents the best fit to the data calculated by non-linear regression. In the absence of Ca^{2+} (Fig. 3b) the K_a for Mg^{2+} was 3.11 mM (see Table 2 for a full list of calculated kinetic parameters with standard errors and significance of changes). When 0.5 mM-spermine was added, the PDHP phosphatase was activated by more than 3-fold at low concentrations of Mg^{2+} , and the calculated K_a for Mg^{2+} decreased to 1.06 mM.

The effect of spermine on the Mg^{2+} -sensitivity of PDHP phosphatase was independent of the other

Fig. 2. Sensitivity of PDHP phosphatase to spermine

Purified pig heart PDHP phosphatase was assayed by measuring the release of [32 P] P_i from purified [32 P]PDHP as described in the Methods section. Spermine concentrations were varied as indicated in the presence of 1.0 mM- Mg^{2+} in the absence of added Ca^{2+} (●) or in the presence of 25 μ M- Ca^{2+} (■). Each point represents the mean of values obtained from two separate experiments.

well-characterized phosphatase activator, Ca^{2+} . In the presence of saturating concentrations of Ca^{2+} (approx. 100 μ M), phosphatase activity was greatly stimulated at all concentrations of Mg^{2+} (Fig. 3a). Even when Mg^{2+} was present at a maximal concentration, PDHP phosphatase could still be activated by about 15-fold with Ca^{2+} . In addition to its effect on the V_{max} of PDHP phosphatase, Ca^{2+} also caused a decrease in the K_a for Mg^{2+} similar in magnitude to that observed with spermine. However, when spermine was added together with the maximal concentration of Ca^{2+} , there was a further decrease in the K_a for Mg^{2+} , indicating that the effects of Ca^{2+} and spermine were essentially independent. In the presence of approx. 100 μ M- Ca^{2+} , the K_a for Mg^{2+} was decreased from 1.01 to 0.41 mM when spermine was added. The Mg^{2+} titrations shown in Fig. 3 were obtained by using a combination of EGTA and HEDTA to buffer the bivalent-cation concentrations. However, similar values were obtained for the Mg^{2+} -sensitivity when the metal chelators were omitted (apart from a minimal amount of EGTA to remove the endogenous Ca^{2+} for the Ca^{2+} -depleted conditions).

In order to differentiate further between the activation of PDHP phosphatase caused by spermine and by Ca^{2+} , the effects of spermine and Mg^{2+} on the Ca^{2+} -sensitivity of the phosphatase were examined. In the experiment of Fig. 4, the free [Ca^{2+}] was varied over the range from about 1 nM (no added Ca^{2+}) to 150 μ M at subsaturating and saturating Mg^{2+} concentrations. In the presence of 10 mM- Mg^{2+} (Fig. 4a), the calculated K_a for Ca^{2+} was 0.87 ± 0.12 μ M. This is similar to previous values obtained in this (Denton *et al.*, 1972; McCormack & Denton, 1980; Denton & McCormack, 1980) and other laboratories (Wieland, 1983; Roche & Cate, 1977). However, although a K_a for Ca^{2+} of about 1 μ M was typical of several experiments, it is interesting to note that one of the preparations of pig heart PDHP phosphatase used in

Fig. 3. Effects of Ca^{2+} and spermine on the sensitivity of purified PDHP phosphatase to Mg^{2+}

PDHP phosphatase was assayed in the presence of approx. 100 μ M- Ca^{2+} (a) or in the absence of added Ca^{2+} (b), and the Mg^{2+} concentration was varied as indicated. The sensitivity to Mg^{2+} is shown in the absence (●) and presence (■) of 0.5 mM-spermine. Each point represents the mean of values from seven separate experiments. The plotted curves represent the best fit calculated by non-linear regression (see Table 2).

this study had a K_a for Ca^{2+} of the order of 5 μ M. The reason for this difference is unknown, but no other appreciable variations in kinetic properties of the phosphatase were obtained between the preparations. There have been reports of high values of K_a with other purified preparations of PDHP phosphatase (Kerbey & Randle, 1979; Teague *et al.*, 1982).

As shown in Fig. 4(a), there was no effect of spermine at any concentration of Ca^{2+} tested when the Mg^{2+} was present at a saturating concentration. The data of Fig. 4(b) show that at subsaturating Mg^{2+} (0.7 mM) spermine caused a substantial activation of PDHP phosphatase at all Ca^{2+} concentrations. These data are consistent with those shown in Fig. 3 in showing that spermine acts in a manner which is distinct from the mode of action of Ca^{2+} and can only be observed at limiting concentrations of Mg^{2+} . The K_a for Ca^{2+} was about 10 μ M at the low Mg^{2+} concentration and was diminished by spermine to about 2 μ M.

Table 1 compares the effects of a variety of

Fig. 4. Effects of Mg^{2+} and spermine on the sensitivity of purified PDHP phosphatase to Ca^{2+}

PDHP phosphatase was assayed in the absence (●) or presence (■) of 0.5 mM-spermine with the free Ca^{2+} concentration varied as indicated. The Mg^{2+} concentration was held at 10 mM in (a) and at 0.7 mM in (b). Data combined from two separate experiments using a single preparation of pig heart PDHP phosphatase are shown.

commercially available polyamines on partially purified pig heart PDHP phosphatase activity measured at limiting $[Mg^{2+}]$. The effects of each polyamine at 100 μM and 400 μM were tested, as these concentrations gave about half-maximal and maximal activation with spermine. Spermine was considerably more effective as an activator of PDHP phosphatase than any of the other polyamines tested. Spermidine, N^1 -acetylspermine and N^1 -acetylspermidine were weak activators, but N^8 -acetylspermidine, putrescine and N^1 -acetylated putrescine were all without effect.

PDHP phosphatase and kinase assays using endogenous PDH_a and PDHP in extracts of adipose-tissue mitochondria

Fig. 5 summarizes results of a series of experiments where PDHP phosphatase and PDH kinase were assayed after disruption of mitochondria, prepared from control and insulin-treated rat epididymal adipose tissue. For the phosphatase assays (Fig. 5a) the mitochondria were preincubated with 2-oxoglutarate and malate to decrease PDH_a activity to a minimum (Denton *et al.*, 1984). The

Table 1. Effects of different polyamines on pig heart PDHP phosphatase

PDHP phosphatase was assayed in the presence of 0.5 mM- Mg^{2+} and 100 μM - Ca^{2+} together with polyamine concentrations of 100 μM or 400 μM . For each condition the data from four to ten separate experiments were combined, and the values given are expressed as a percentage of the maximal rate in the absence of any polyamine (this mean control rate was 17.5% of $[^{32}P]$ PDHP dephosphorylated/5 min). Significance of polyamine effects compared with controls, calculated by Student's *t* test: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

Polyamine	Phosphatase (% of control rate) in the presence of polyamine concentration of:	
	100 μM	400 μM
Spermine	154.4 $\pm$ 8.69***	195.19 $\pm$ 19.16***
N^1 -Acetylspermine	114.15 $\pm$ 2.23***	134.17 $\pm$ 3.46***
Spermidine	108.9 $\pm$ 1.86*	137.13 $\pm$ 6.87***
N^1 -Acetylspermidine	121.72 $\pm$ 4.46**	116.01 $\pm$ 5.83*
N^8 -Acetylspermidine	85.93 $\pm$ 8.80	103.04 $\pm$ 9.63
Putrescine	84.47 $\pm$ 8.86	111.68 $\pm$ 16.73
N^1 -Acetylputrescine	107.75 $\pm$ 5.78	99.05 $\pm$ 4.38

phosphatase reaction was initiated by adding Mg^{2+} (sub-saturating) and Ca^{2+} (saturating), and the dephosphorylation of endogenous PDHP was followed by measuring the change of PDH_a with time. Although the initial activity of PDH_a was higher in the mitochondrial extract from insulin-treated tissue, there was no significant difference in the kinetics of PDH activation between the extracts from control and insulin-treated adipose tissue. For the assay of PDH kinase in extracts of adipose-tissue mitochondria with the endogenous PDH_a as substrate (Figs. 5b and 5c), the intact mitochondria were first incubated with Ca^{2+} and ADP to convert the PDHP into PDH_a as completely as possible. The mitochondria were then disrupted, and the kinase reaction initiated by $MgATP$ was followed by monitoring the decrease of PDH_a . As with the phosphatase assay, there was no appreciable difference in the kinetics of the kinase reaction between the mitochondrial extract from control adipose tissue and that from the insulin-treated tissue (Fig. 5b). Furthermore, when both kinase and phosphatase were allowed to act on the endogenous preactivated substrate simultaneously (Fig. 5c), there was still no trace of any insulin effect, even though a partially elevated steady-state activity of PDH_a was attained, similar to that which occurs in intact mitochondria.

Effects of spermine on PDHP phosphatase in extracts of adipose-tissue mitochondria

One possible explanation for the loss of the insulin-induced activation of PDHP phosphatase subsequent to mitochondrial disruption is that there is a dissociable effector present in the mitochondria which becomes diluted once the mitochondrial membranes are broken (the matrix is diluted by at least 100-fold after mitochondrial disruption in a minimal volume). An obvious candidate for this was one of the polyamines, and we therefore investigated whether PDHP phosphatase from white-adipose-tissue mitochondria was activated by spermine in a manner similar to the pig heart PDHP

Fig. 5. PDHP phosphatase and PDH kinase in mitochondrial extracts assayed with endogenous PDH_a and PDHP as substrate

Mitochondria were prepared from control (●) or insulin-treated (■) adipose tissue as described in the Methods section. In (a) the mitochondria were preincubated with 5 mM-2-oxoglutarate and 0.5 mM-malate before being separated from the medium by centrifugation. Subsequently the mitochondria were disrupted by freezing and thawing as described in the Methods section. The PDHP phosphatase reaction was initiated by adding 100 μ M-Ca²⁺ and 2 mM-Mg²⁺, and the dephosphorylation of endogenous PDHP was followed by measuring the increase in PDH_a with time. In (b) the mitochondria were preincubated with 1 μ M-Ca²⁺ and 2 mM-ADP before disruption. The assay of PDH kinase activity was initiated by adding 1 mM-MgATP, and the decrease of PDH_a was followed with time. In (c) both the PDHP phosphatase and PDH kinase were allowed to act on the Ca²⁺- and ADP-preactivated endogenous PDH_a. For this experiment the reaction was initiated by adding 1 mM-ATP plus MgCl₂ and CaCl₂ to give 2 mM-Mg²⁺ and 100 μ M-Ca²⁺ respectively.

phosphatase. We were able to show spermine-induced stimulation of PDH phosphatase by following the activation of endogenous PDHP in extracts of adipose-tissue mitochondria by the method described above. However, to facilitate measurements of the adipose-tissue phosphatase activity, exogenously added pig heart [³²P]PDHP was generally used as a substrate.

In agreement with the observations obtained with partially purified pig heart PDHP phosphatase, the K_a for Mg²⁺ of the adipose-tissue enzyme was significantly decreased by the addition of 0.5 mM-spermine, in both the presence and the absence of Ca²⁺ (Fig. 6; Table 2). As found previously (Denton *et al.*, 1972; McCormack & Denton, 1980), Ca²⁺ increased PDHP phosphatase activity in extracts of epididymal rat-adipose-tissue mitochondria at saturating Mg²⁺ concentrations 6–10-fold, as well as decreasing the K_a for Mg²⁺ (Table 2). This is in marked contrast with the situation in intact adipose-tissue mitochondria, where only the effect of Ca²⁺ on the K_a for Mg²⁺ is evident (Fig. 1). As found with the partially purified pig heart PDHP phosphatase (see Fig. 4b), at a submaximal Mg²⁺ concentration (1 mM), 0.5 mM-spermine was found to activate rat adipose tissue PDHP phosphatase 1.5–8-fold at all Ca²⁺ concentrations from less than 1 nM to 100 μ M (results not shown).

K_a for Mg²⁺ of PDHP phosphatase in mitochondrial extracts from control and insulin-treated tissue was also compared (Fig. 6; Table 2). No alterations in Mg²⁺-sensitivity or the effect of spermine were evident. Insulin pre-treatment was also without effect on the sensitivity of extracted PDHP phosphatase to Ca²⁺, as reported previously (Marshall *et al.*, 1984).

DISCUSSION

Two important characteristics of the insulin-induced elevation of PDH_a activity in adipose tissue are that the effect persists through subsequent isolation and incubation

Fig. 6. Effect of spermine on the Mg²⁺ sensitivity of PDHP phosphatase in mitochondrial extracts from control and insulin-treated adipose tissue

PDHP phosphatase was assayed in extracts of disrupted mitochondria by measuring the [³²P]P_i released from exogenously added pig heart [³²P]PDHP. The assays were carried out at about 100 μ M-Ca²⁺ with the indicated Mg²⁺ concentrations in the absence (○, ●) or presence (□, ■) of 0.5 mM-spermine. Mitochondrial extracts prepared from control (●, ■) and insulin-treated (○, □) adipose tissue are compared. There was no significant effect of insulin, and therefore only the mean curve is shown for the two treatments with (—) and without (—) spermine. Phosphatase activity is expressed as a percentage of the maximum rate at saturating Mg²⁺ in the absence of spermine.

of the mitochondria and that no differences in PDH kinase or PDHP phosphatase resulting from insulin pretreatment of the tissue can be detected once the

Table 2. Calculated K_a values for the activation of PDHP phosphatase by Mg^{2+}

The concentrations of Mg^{2+} required for half-maximal activation (K_a) of PDHP-phosphatase under the various conditions used in the present study were calculated by fitting the relevant data to the equation:

$$v = \frac{V_{\max.}}{\{1 + (K_a/[Mg^{2+}])^h\}}$$

where v is the measured phosphatase activity (or level of PDH_a), $[Mg^{2+}]$ is the free Mg^{2+} concentration, h is the Hill coefficient and $V_{\max.}$ is the phosphatase activity in the presence of saturating Mg^{2+} . For each condition the data from at least three separate experiments were combined, and K_a values are given as means $\pm$ S.E.M. as calculated from the best fit to the above equation by non-linear least-squares regression. Values calculated for h in these experiments were within the range 1.5–2.5, and none of the conditions tested caused any significant alteration in this parameter. $V_{\max.}$ values varied with the type of phosphatase preparation and assay system, but, when compared with the relevant control, none of the treatments altered the $V_{\max.}$ except for Ca^{2+} , which increased it by 10–20-fold (see the text). The following differences in the calculated K_a values for Mg^{2+} were significant at $P < 0.05$ by Student's t test: ^aeffect of spermine compared with relevant control; ^beffect of Ca^{2+} compared with equivalent condition with no added Ca^{2+} ; ^ceffect of insulin treatment of tissue before mitochondrial preparation.

PDHP phosphatase	K_a of PDHP phosphatase for Mg^{2+} (mM)	
	No Ca^{2+}	25 μ M- Ca^{2+}
Pig heart		
– spermine	3.11 $\pm$ 1.06	1.10 $\pm$ 0.21 ^b
+ spermine	1.06 $\pm$ 0.21 ^a	0.41 $\pm$ 0.13 ^{ab}
In extracts of adipose-tissue mitochondria		
Control tissue		
– spermine	3.23 $\pm$ 0.50	1.38 $\pm$ 0.06 ^b
+ spermine	2.32 $\pm$ 0.35 ^a	0.67 $\pm$ 0.13 ^{ab}
Insulin-treated tissue		
– spermine	3.63 $\pm$ 0.30	1.49 $\pm$ 0.10 ^b
+ spermine	—	1.02 $\pm$ 0.13 ^{ab}
In intact A23187-treated adipose-tissue mitochondria		
From control tissue	2.64 $\pm$ 0.59	1.05 $\pm$ 0.21 ^b
From insulin-treated tissue	1.64 $\pm$ 0.21 ^c	0.59 $\pm$ 0.11 ^{bc}

mitochondria have been disrupted. The persistence of the insulin effect through isolation of the mitochondria has allowed the demonstration that insulin action on PDH probably results from an increase in PDHP-phosphatase activity (Hughes & Denton, 1976; Denton *et al.*, 1984), a conclusion which we have recently confirmed using toluene-permeabilized adipose-tissue mitochondria (Thomas & Denton, 1986). Experiments with isolated mitochondria have also largely ruled out a role for either Ca^{2+} or any of the other known metabolic effectors of the PDH system in the insulin effect (Denton *et al.*, 1984; Marshall *et al.*, 1984). These observations suggest that insulin acts to alter the intramitochondrial content of an as yet unidentified effector of PDHP phosphatase, which does not readily leave the mitochondria but dissociates and becomes lost or diluted once the mitochondrial membranes are disrupted.

A further characteristic of the activation of PDH by insulin that emerged during the present study was that the sensitivity of PDHP phosphatase to Mg^{2+} within the mitochondrial matrix was apparently increased when mitochondria from insulin-treated adipose tissue were compared with control mitochondria. For these experiments, the mitochondria had to be treated with the ionophore A23187 to allow manipulation of intramitochondrial Mg^{2+} concentrations, and phosphatase activity was estimated from changes in the steady-state activity of PDH_a in the presence of a constant PDH kinase activity. However, despite these limitations it is clear that insulin caused a small but statistically significant decrease

in the apparent K_a for Mg^{2+} of PDHP phosphatase, both in the absence of added Ca^{2+} and in the presence of saturating Ca^{2+} . We have also observed a similar insulin-induced decrease in the apparent K_a of PDHP phosphatase for Mg^{2+} , using mitochondria made permeable with toluene (Thomas & Denton, 1986). It should be noted that insulin does not appear to alter the total mitochondrial Mg^{2+} content in adipose tissue (Denton *et al.*, 1984); however, activation of PDHP phosphatase would occur provided that the matrix free Mg^{2+} concentration was not already saturating. A value for mitochondrial-matrix free $[Mg^{2+}]$ of about 0.4 mM has been determined by a null-point titration technique (Corkey *et al.*, 1986), and with Mg^{2+} concentrations of this order the insulin-induced decrease in the K_a for Mg^{2+} would cause a marked stimulation of PDHP phosphatase.

Comparison of the data in Figs. 1, 3 and 6 clearly shows the similarity between the elevation of PDH_a activity by insulin and the stimulation of PDHP phosphatase by spermine. Both effects result from a shift in Mg^{2+} -sensitivity without altering the $V_{\max.}$ of the phosphatase, and in both cases the effects can be considered to be essentially independent of the effects of Ca^{2+} . Table 2 summarizes the K_a values for Mg^{2+} calculated for a partially purified preparation of pig heart PDHP phosphatase, PDHP phosphatase in A23187-treated mitochondria from control and insulin-treated adipose tissue, and PDHP phosphatase in extracts of mitochondria. In all cases the K_a for Mg^{2+} was lower in the presence

of Ca^{2+} than in its absence and, essentially independently of this effect, spermine also caused a decrease in the K_a of PDHP phosphatase for Mg^{2+} . Spermine had no effect on the K_a for Mg^{2+} when added to A23187-treated mitochondria (results not shown), but it is probable that spermine would not enter the mitochondrial matrix under these conditions. Some evidence has been presented for a phosphate-linked energy-dependent spermine transport system in rat liver mitochondria (Toninello *et al.*, 1985), but this appears to be rather slow and unlikely to operate with the de-energized mitochondria used in the studies reported here.

A further but quite distinct effect of spermine which may also lead to the activation of PDHP phosphatase is the activation of Ca^{2+} uptake into mitochondria (Nicchitta & Williamson, 1984). The addition of spermine (in the range 0.1–1.0 mM) to intact mitochondria derived from rat heart, liver or epididymal adipose tissue and incubated in medium containing appropriate limiting concentrations of Ca^{2+} has been shown to result in increase in PDH_a because of the Ca^{2+} -activation of PDHP phosphatase (J. G. McCormack & R. M. Denton, unpublished work). The importance of this action of spermine in intact tissue preparations is unknown, but it should be emphasized that it has no direct bearing on the observations made in the present study.

Apart from its ability to activate PDHP phosphatase in a manner similar to insulin, spermine has several other effects which also mimic the actions of insulin. Early studies with isolated adipocytes showed that spermine added to the extracellular medium caused increased glucose uptake and lipogenesis (Lockwood & East, 1974; Czech *et al.*, 1974). However, subsequent studies have indicated that this is probably a secondary effect of increased H_2O_2 formation (Livingston *et al.*, 1977; Cascieri *et al.*, 1979). Another effect of insulin which can be mimicked by direct addition of spermine to intact cells is the dephosphorylation of phenylalanine hydroxylase in hepatocytes (Fisher *et al.*, 1986). Spermine has also been shown to activate purified protein phosphatase IIA, and this activation appears to be particularly directed towards site 3a, 3b and 3c of muscle glycogen synthase (Tung *et al.*, 1985), the same sites whose phosphorylation is decreased by insulin in the intact tissue (Cohen *et al.*, 1985). The activation of phosphatase IIA by spermine differs from its effect on PDHP phosphatase in that it is not Mg^{2+} -dependent. However, not all of the reported effects of spermine on protein phosphorylation are consistent with a role for this polyamine in insulin action. For instance, in addition to activating phosphatase IIA, spermine has also been reported to cause a substrate-directed activation of cyclic AMP-dependent protein kinase against phosphorylase kinase (Singh & Huang, 1985). Furthermore, it has been reported that, although spermine addition to liver cells results in decreased phosphorylation of several proteins, two of these proteins fall into the class whose phosphorylation state is increased by insulin, and this action of insulin is consequently antagonized by spermine (Auberger *et al.*, 1984).

Spermine is generally present in mammalian tissues in the range of 1–2 $\mu\text{mol/g}$ wet wt. of tissue (Tabor & Tabor, 1976, 1984; Pegg & McCann, 1982). Polyamine contents can change under some conditions, such as tissue regeneration, diabetes and growth (Grillo & Bedino, 1977; Grillo *et al.*, 1978a,b; Grillo & Pezzali, 1984; Corti

et al., 1985), but, apart from increases in putrescine, insulin does not appear to cause very marked changes in the cellular contents of the major polyamines (Grillo *et al.*, 1978a,b; Brosnan & Hu, 1983). In contrast with this, two key enzymes involved in polyamine biosynthesis, ornithine decarboxylase and *S*-adenosylmethionine decarboxylase, can be induced by several-fold subsequent to insulin treatment. However, although both of these enzymes have half-lives in the order of 10–20 min, several hours are required before maximal activities are achieved (Grillo *et al.*, 1978a,b; Grillo & Pezzali, 1984; Rinehart & Canellakis, 1985). Thus at this stage it must be concluded that, although spermine can mimic the activation by insulin of PDHP phosphatase, as well as several other insulin effects, it seems an unlikely candidate to act as an intracellular messenger for insulin. It is possible, however, that a more minor polybasic compound, structurally related to spermine, might play such a role; but other polyamines that we have studied were less effective than spermine (Table 1). Damuni *et al.* (1984) have pointed out the possibility that polyamines might be related to the putative mediator proposed to be involved in the insulin-induced activation of PDH and glycogen synthase, but it should be noted that this mediator has generally been stated as having a net negative charge at neutral pH (Cheng *et al.*, 1985; Seals, 1985).

Even if polyamines are not directly involved in the activation by insulin of PDHP phosphatase, they still represent an important new class of regulators for this enzyme. It is clear that spermine does not work at the same site as Ca^{2+} , since it is apparently equally effective in the presence and absence of this ion. It also seems unlikely that spermine simply substitutes for Mg^{2+} , since in the absence of added Mg^{2+} spermine was unable to cause any activation of PDHP phosphatase. Presumably polyamines act at a distinct site to bring about an increased affinity for Mg^{2+} . The physiological ligand for this site may be a polyamine, but it is also possible that a macromolecular or even a membrane component might play this role.

Finally, it should be noted that, in addition to increasing the maximal activity of PDHP phosphatase preparations, Ca^{2+} also increases the sensitivity of the enzyme to Mg^{2+} . Studies with purified PDHP phosphatase have shown that the major mechanism by which Ca^{2+} acts on the separated enzyme is through a decrease in the K_m of the phosphatase for its PDH substrate (Pettit *et al.*, 1972; Randle *et al.*, 1974). However, within the mitochondrial matrix, the concentration of PDHP is very high (at least 50 μM with respect to protein-bound phosphate, compared with a K_m value of around 30 μM in the absence of Ca^{2+}), and thus it might be expected that the phosphatase is near saturation with its substrate, such that Ca^{2+} should have little further effect (Severson *et al.*, 1974). Despite this prediction, Ca^{2+} is an effective activator of PDHP phosphatase in intact mitochondria (Severson *et al.*, 1974; Denton & McCormack, 1980, 1985; Hansford, 1985). The most likely explanation for this is that Ca^{2+} acts mainly within intact mitochondria through its effect on the Mg^{2+} -sensitivity of PDHP phosphatase. Our data with A23187-treated adipose-tissue mitochondria (Fig. 1) support this hypothesis. In this system, Ca^{2+} effectively decreased the K_a for Mg^{2+} , thus activating PDH at sub-saturating Mg^{2+} concentrations, but had little effect at high Mg^{2+} concentrations.

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