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Use of toluene-permeabilized mitochondria to study the regulation of adipose tissue pyruvate dehydrogenase *in situ*

Further evidence that insulin acts through stimulation of pyruvate dehydrogenase phosphate phosphatase

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1. Rat epididymal-adipose-tissue mitochondria were made selectively permeable to small molecules without the loss of matrix enzymes by treating the mitochondria with toluene under controlled conditions. 2. With this preparation the entire pyruvate dehydrogenase system was shown to be retained within the mitochondrial matrix and to retain its normal catalytic activity. By using dilute suspensions of these permeabilized mitochondria maintained in the cuvette of a spectrophotometer, it was possible to monitor changes of pyruvate dehydrogenase activity continuously while the activities of the interconverting kinase and phosphatase could be independently manipulated. 3. Permeabilized mitochondria were prepared from control and insulin-treated adipose tissue, and the properties of both the pyruvate dehydrogenase kinase and the phosphatase were compared *in situ*. No difference in kinase activity was detected, but increases in phosphatase activity were observed in permeabilized mitochondria from insulin-treated tissue. 4. Further studies showed that the main effect of insulin treatment was a decrease in the apparent K_a of the phosphatase for Mg^{2+}, in agreement with earlier studies with mitochondria made permeable to Mg^{2+} by using the ionophore A23187 [Thomas, Diggle & Denton (1986) Biochem. J. 238, 83–91]. No effects of spermine were detected, although spermine diminishes the K_a of purified phosphatase preparations for Mg^{2+}. 5. Since effects of insulin on pyruvate dehydrogenase phosphatase activity are not evident in mitochondrial extracts, it is concluded that insulin may act by altering some high-M_r component which interacts with the pyruvate dehydrogenase system within intact or permeabilized mitochondria, but not when the mitochondrial membranes are disrupted.

INTRODUCTION

Past studies on partially purified preparations of the pyruvate dehydrogenase complex (PDH_a) plus its kinase and phosphatase have indicated that interconversion of the inactive and active forms of the complex may be regulated by a number of intracellular metabolites and metal ions (for reviews see Denton *et al.*, 1975; Wieland, 1983; Reed & Yeaman, 1986). PDH kinase can be activated by increasing ratios of [acetyl-CoA]/[CoA] and [NADH]/[NAD^+] and inhibited by ADP and pyruvate (Reed, 1974; Cooper *et al.*, 1975; Pettit *et al.*, 1975; Pratt & Roche, 1979), whereas the major regulators of PDH phosphatase are the bivalent cations Mg^{2+} and Ca^{2+} (Denton *et al.*, 1972; Siess & Wieland, 1972; Pettit *et al.*, 1972). Purified preparations of the phosphatase are completely inactive in the absence of Mg^{2+} and may be further activated by Ca^{2+} in the presence of Mg^{2+}. The effects of Ca^{2+} are both to increase $V_{max.}$ at saturating concentrations of Mg^{2+} and to diminish the K_a for Mg^{2+} (Denton *et al.*, 1972). Spermine also diminishes the K_a for Mg^{2+} (Damuni *et al.*, 1984; Thomas *et al.*, 1986), but this effect is independent of that of Ca^{2+} (Thomas *et al.*, 1986).

In such studies on purified enzymes, the concentrations of the PDH complex, kinase and phosphatase are two or three orders of magnitude less than those occurring within intact mitochondria. However, in a number of instances it has been possible to obtain evidence that potential regulators of the PDH system do act within intact mitochondria (Denton *et al.*, 1975; Wieland, 1983). For example, the PDH system is found to respond to changes in intramitochondrial concentrations of pyruvate, ADP, acetyl-CoA, CoA, NADH and NAD^+ (Martin *et al.*, 1972; Kerbey *et al.*, 1976, 1977; Hansford, 1976, 1977) and of Mg^{2+} and Ca^{2+} (Severson *et al.*, 1972; Denton *et al.*, 1972, 1980; Marshall *et al.*, 1984; Thomas *et al.*, 1986). However, one important difference has come to light recently between the properties of the isolated enzymes and those located within intact mitochondria. In intact mitochondria, the activating effect of Ca^{2+} on PDH phosphatase is only apparent at non-saturating concentrations of Mg^{2+}, whereas a marked effect of Ca^{2+} at saturating Mg^{2+} concentrations is evident with the isolated enzymes (Thomas *et al.*, 1986). This discrepancy illustrates the need to be able to explore fully the properties of the PDH system as it operates within the mitochondrial matrix.

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

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In past studies on the regulation of PDH activity within intact mitochondria, the mitochondria have been incubated under conditions designed to change the intramitochondrial concentration of the appropriate potential regulator and then the mitochondria were extracted and the proportion of the PDH complex present in the active form was determined. Such an approach has a number of drawbacks, including: (i) time courses are difficult and tedious to study; (ii) both phosphatase and kinase are usually active simultaneously, so only changes in the relative activity of the two interconverting enzymes can be observed; (iii) often the exact intramitochondrial concentrations of potential regulators are unknown, and changing the concentration of one potential regulator without changing that of other potential regulators may be impossible; (iv) the PDH complex is not catalysng the conversion of pyruvate into acetyl-CoA as *in vitro*. In this paper, we report the use of toluene-permeabilized mitochondria as a means of largely overcoming these shortcomings. As first described by Matlib *et al.* (1977), mitochondria treated under controlled conditions with toluene beome permeable to all small molecules while maintaining their structural integrity and full complement of intramitochondrial proteins. With such preparations the intramitochondrial concentrations of substrates, coenzymes and potential regulators can be easily manipulated, and so the kinetic behaviour of the PDH/system can be studied under conditions very close to those occurring in intact cells. In addition, it is possible to assay the PDH activity continuously and thus to follow accurately the time course of the effects of changing the activity of the phosphatase or kinase.

Insulin increases the amount of PDH_a in rat epididymal adipose tissue by 2–3-fold (Jungas, 1971: Denton *et al.*, 1971; Weiss *et al.*, 1971). Activation persists during the isolation and subsequent incubation of mitochondria (Denton *et al.*, 1984; Marshall *et al.*, 1984; Thomas *et al.*, 1986). This persistence has allowed evidence to be obtained which indicates that the effect of insulin may be the result of the activation of PDHP phosphatase rather than inhibition of PDH kinase (Hughes & Denton, 1976; Denton *et al.*, 1984; Thomas *et al.*, 1986). However, no persistent effect of insulin on phosphatase activity is apparent after disruption of the mitochondria (Marshall *et al.*, 1984; Thomas *et al.*, 1986). It is thus of particular interest to investigate whether the effect of insulin persists through the toluene permeabilization of adipose-tissue mitochondria.

METHODS

Chemicals and biochemicals were obtained from the sources given previously (Denton *et al.*, 1984; Thomas *et al.*, 1986). In addition, poly(ethylene glycol) (average M_r 6000) was from BDH Chemicals. Antiserum to pig heart PDH was raised as described in Hughes *et al.* (1980).

Epididymal fat-pads were from fed male Wistar rats of 170–220 g body wt. Paired fat-pads were incubated in the presence and absence of 50 nM-insulin for 30 min in bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing 5 mM-glucose. The fat-pads were briefly blotted dry and then rapidly disrupted with a Polytron PT-20 homogenizer at 0 °C. The homogenization medium was used at 2 ml/g wet wt. of tissue and was composed of 250 mM-sucrose, 20 mM-Tris/HCl, 2 mM-EGTA, 7.5 mM-GSH and 3% (w/v) essentially fatty-acid-free bovine serum albumin at pH 7.4 and 0 °C. The mitochondria from 10–20 pairs of fat-pads were isolated from the homogenate by differential centrifugation (Denton *et al.*, 1984) and then washed by resuspending the pellet in 40 ml of the homogenization medium and re-centrifugation. The final mitochondrial pellet was resuspended at about 10 mg of mitochondrial protein/ml also in the same medium. At this stage the activity of PDH in a sample of the mitochondrial preparations from control and insulin-treated tissues was determined as described by Thomas *et al.* (1986). Routinely insulin pretreatment of the tissue induced an increase of about 2-fold in the activity of PDH_a without altering total PDH activity. The yield of mitochondria was about 1 mg of mitochondrial protein/fat-pad.

The method for permeabilizing adipose-tissue mitochondria was adapted from that described by Matlib *et al.* (1977). The entire procedure was carried out in a 1.5 ml conical micro-centrifuge tube. Mitochondria were first sedimented by centrifugation for 4 min at 15000 *g* before being resuspended in a medium composed of 250 mM-sucrose, 20 mM-Tris/HCl, 2 mM-EGTA and 8.5% (w/v) poly(ethylene glycol) 6000 at pH 7.4 and 0 °C. The final concentration of mitochondria was about 20 mg of protein/ml. Permeabilization of mitochondria from control and insulin-treated tissue was carried out simultaneously. Toluene (5 μl/ml) was added to each mitochondrial suspension and the suspensions were agitated (2–3 cycles/s) for 2 min in an ice/water bath. In addition, the suspensions were gently mixed with a vortex mixer for 3 s every 30 s. The mitochondria were then rapidly separated from the medium by centrifugation at 15000 *g* for 4 min , and the toluene was carefully removed by aspiration. The residual supernatant was also removed, and the mitochondrial pellet was washed and finally resuspended in the permeabilization medium (without toluene) to give a final concentration of about 5 mg of protein/ml. The amount of mitochondrial protein was determined from the activity of glutamate dehydrogenase, assuming that rat epididymal-fat-pad mitochondria contain 0.25 unit of glutamate dehydrogenase/mg of protein (Denton *et al.*, 1984). Recovery of mitochondrial protein through the permeabilization process was about 50–70%.

All incubations of permeabilized adipose-tissue mitochondria were carried out at 30 °C in medium composed of 100 mM-sucrose, 50 mM-KCl, 50 mM-Mops, 2 mM-KH_2PO_2, 1 mM-EGTA, 1 mM-HEDTA [*N*-(2-hydroxyethyl)ethylenediaminetriacetic acid], 1 mM-dithiothreitol, 0.1 mM-$MgCl_2$, 5 μg of oligomycin/ml, 1 μg of antimycin A/ml and 1 μg of rotenone/ml at pH 7.2. To 1 ml of this buffer in a plastic cuvette was added 10 μl of mitochondrial suspension (equivalent to about 40 μg of mitochondrial protein). Enzyme reactions were followed spectrophotometrically with a Pye–Unicam SP.8-100 spectrophotometer with a kinetic attachment capable of monitoring up to four cuvettes sequentially at a sampling rate of 6 s per cuvette. The data were collected and stored by using a Hewlett–Packard 9854A computer fitted with a real-time clock which allowed plotting of specific regions of the traces, calculation of rates by linear regression through linear regions of the progress curves and differentiation of the data to facilitate analysis of non-linear regions. For glutamate dehydrogenase assays, the incubation medium was

supplemented with 0.1 mM-NADH, 10 mM-2-oxoglutarate and 2 mM-ADP. The reaction was initiated by adding 80 mM-ammonium acetate, and the utilization of NADH was monitored by the decrease in A_{340}. When PDH was assayed the basic incubation medium was supplemented with 1 mM-pyruvate, 0.5 mM-NAD^+, 0.1 mM-CoA, 1.0 mM-thiamin pyrophosphate, 10 μg of the dye *p*-(*p*-aminophenylazo)benzene-sulphonic acid (AABS)/ml and about 30 munits of arylamine acetyltransferase/ml. The production of acetyl-CoA was monitored by following the decrease in A_{460} of AABS which resulted from its acetylation catalysed by arylamine acetyltransferase (Coore *et al.*, 1971). Arylamine acetyltransferase was not appreciably inhibited in the polyethylene glycol-containing incubation medium.

The traces shown in this paper are all representative of observations made with at least three separate preparations of mitochondria. Free Ca^{2+} and Mg^{2+} concentrations were calculated as described previously (Thomas *et al.*, 1986). One unit of enzyme activity is the amount catalysing the utilization of 1 μmol of substrate/min at 30 °C.

RESULTS AND DISCUSSION

Characterization and use of permeabilized adipose-tissue mitochondria

Optimal conditions for solvent-induced permeabilization of rat liver and heart mitochondria have been thoroughly investigated by Matlib *et al.* (1977) and confirmed by Lof *et al.* (1983). Adipose-tissue mitochondria permeabilized under our conditions (see the Methods section) behaved in a broadly similar manner to that described previously by these two groups. The main requirements for successful permeabilization in our hands were that the mitochondra should be suspended at a concentration of not less than 15 mg of mitochondrial protein/ml, and that the exposure to toluene should be even but not excessive. The presence of poly(ethylene glycol) 6000 at about 8.5% (w/v) was found essential to prevent disruption of the mitochondria.

Permeabilized adipose-tissue mitochondria could be sedimented by centrifugation without any appreciable loss to the medium of matrix marker enzymes such as glutamate dehydrogenase, citrate synthase, malate dehydrogenase and PDH. The ratio of the activities of these enzymes to the activity of the inner-membrane marker enzyme succinate dehydrogenase was constant for both non-permeabilized and permeabilized mitochondria before and after centrifugation (results not shown). The key criterion used to show that the mitochondrial membranes were permeable to small molecules was the ability to measure the activity of enzymes within the mitochondrial matrix without further disruption. Fig. 1 shows an experiment where glutamate dehydrogenase was measured directly. For mitochondria taken through the full permeabilization procedure but without adding any toluene, the detectable activity of glutamate dehydrogenase was very low (Fig. 1*a*), owing to the permeability barrier to NADH and NAD^+ imposed by the still intact mitochondrial membranes. When the detergent Triton X-100 was added, the intramitochondrial glutamate dehydrogenase was released into the medium and all of the activity could be detected (Fig. 1*a*). With

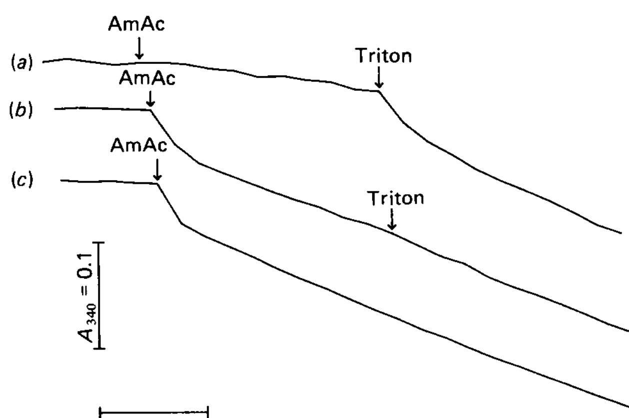

Fig. 1. Accessibility of glutamate dehydrogenase within toluene-permeabilized mitochondria

Adipose-tissue mitochondria were permeabilized with toluene by the procedure described in the Methods section (*b*, *c*) or taken through the same procedure but without adding the toluene (*a*). The mitochondria were then suspended at about 25 μg of protein/ml in the poly(ethylene glycol)-based incubation medium supplemented with 0.1 mM-NADH, 10 mM-2-oxoglutarate and 2 mM-ADP. The changes in the A_{340} were followed. After a steady baseline had been attained, the glutamate dehydrogenase reaction was initiated by adding 80 mM-ammonium acetate (AmAc). For traces (*a*) and (*b*) 0.2% Triton X-100 was added where indicated, and the sample used in trace (*c*) was treated with 0.2% Triton X-100 before initiating the reaction.

toluene-permeabilized mitochondria the full activity of the intramitochondrial glutamate dehydrogenase was detectable without the need for any further disruption of the mitochondrial membranes (Fig. 1*b*).

Similar results were obtained when the activities of other matrix enzymes, including citrate synthase and malate dehydrogenase, were assayed in permeabilized and non-permeabilized mitochondria. One partial exception to this general behaviour was PDH itself. Although PDH was accessible to its many substrates, cofactors and effectors in permeabilized mitochondria (see below), the addition of Triton X-100 to toluene-permeabilized mitochondria always resulted in an increase in PDH activity of about 50%. The reason for this has not been established, but one possibility is that the large size of the PDH complex ensures that a proportion of its catalytic sites are blocked by the close proximity of the mitochondrial inner membrane in permeabilized mitochondria.

PDH activity in permeabilized mitochondria was generally monitored by measuring acetyl-CoA production because, although it was possible to monitor NADH production over short periods, linear rates were not maintained once the NADH accumulated to appreciable concentrations. This was probably partly due to rotenone-insensitive NADH oxidase activity and contaminating lactate dehydrogenase and partly due to increasing ratios of [NADH]/[NAD^+] and [acetyl-CoA]/[CoA], which feedback-inhibit PDH. Formation of acetyl-CoA was measured by including arylamine acetyltransferase, which uses acetyl-CoA as a substrate for acetylation of the dye AABS, and monitoring the

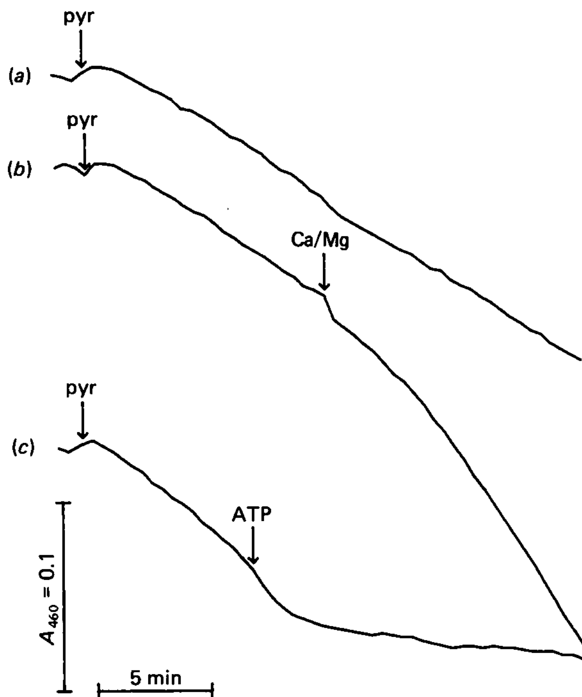

Fig. 2. Measurement of PDH activity and activation of PDH phosphatase and PDH kinase in permeabilized mitochondria

Permeabilized adipose-tissue mitochondria were suspended at about 50 μg of protein/ml in the basic incubation medium (see the methods section) supplemented with 0.5 mM-NAD$^+$, 0.1 mM-CoA and 1.0 mM-thiamin pyrophosphate. In addition 0.15 mM of the dye AABS and 30 munits of arylamine acetyltransferase/ml were also included so that acetyl-CoA production could be monitored spectrophotometrically by the decrease in A_{460} of the AABS. At the point indicated, the PDH reaction was initiated by adding 1 mM-pyruvate (pyr). In trace (*a*), no further additions were made. In trace (*b*), 1.9 mM-$CaCl_2$ plus 1.5 mM-$MgCl_2$ (Ca/Mg) was added to give free concentrations of 100 μM-Ca^{2+} and 0.8 mM-Mg^{2+}. In trace (*c*), 0.2 mM-ATP plus 50 μM-$MgCl_2$ (ATP) was added, giving final concentrations of 14 μM-MgATP with little change in the free Mg^{2+} concentration from its starting value of 7 μM.

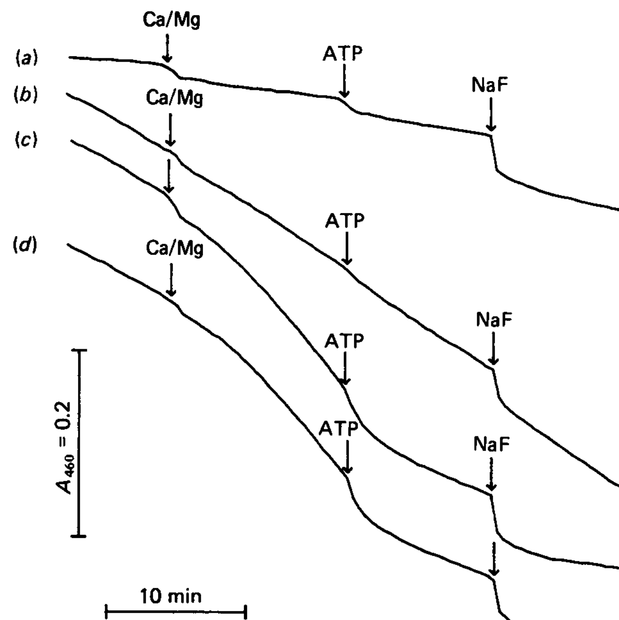

Fig. 3. Exclusion of PDH antibody by toluene-permeabilized mitochondria

Toluene-permeabilized mitochondria were incubated with substrates for the assay of PDH as described in the Methods section and the legend to Fig. 2. The traces show the decrease in A_{460} after addition of substrates in the absence (*c*, *d*) or presence (*a*, *b*) of 0.2% Triton X-100 and either 20 μl of PDH antiserum/ml (*a*,*c*) or 20 μl of control serum/ml (*b*, *d*). Sequential additions of 1.9 mM-$CaCl_2$ plus 1.5 mM-$MgCl_2$ (Ca/Mg), 0.2 mM-ATP plus 50 μM-$MgCl_2$ (ATP) and 25 mM-NaF were also made at the points indicated.

decrease in A_{460}. At this wavelength there was no interference by NADH. The activity of added arylamine acetyltransferase was always at least 20-fold greater than the maximal activity of PDH in the cuvette. Taking the K_m of the acetyltransferase for acetyl-CoA as 7 μM (Andres *et al.*, 1983), it may be calculated that even under non-steady-state conditions the acetylation of AABS should never lag by more than 10 s behind the production of acetyl-CoA. Indeed, addition of twice the normal activity of arylamine acetyltransferase did not have any appreciable effect on time courses similar to those in Figs. 2(*b*) and 2(*c*).

In Fig. 2, traces from three parallel experiments are shown in which PDH activity was monitored under different conditions with permeabilized mitochondria. Fig. 2(*a*) shows that the reaction was linear for prolonged periods under conditions when PDH kinase was inactive because of the absence of ATP, and PDH phosphatase was inactive because Ca^{2+} was absent and the concentration of Mg^{2+} (7 μM) was well below the K_m value. Addition of Ca^{2+} plus Mg^{2+} caused a time-dependent increase in the activity of the PDH (Fig. 2*b*), which could be blocked by 25 mM-NaF and represents activation of endogenous PDH phosphatase with a consequent increase in PDH_a. Subsequent extraction of the mitochondria and direct assay of initial and total activity of PDH in the extract (as described by Denton *et al.*, 1984) showed that treatment with Ca^{2+} plus Mg^{2+} was capable of causing essentially complete conversion of all of the PDH into the active PDH_a form. It was also possible to follow the inactivation of PDH within the permeabilized mitochondria by adding ATP plus $MgCl_2$ to give 14 μM-MgATP (Fig. 2*c*). Under these conditions, PDH kinase was able to diminish PDH_a activity to less than 3% of the maximum value achieved after addition of Ca^{2+} plus Mg^{2+}.

In order to examine further the protein permeability of toluene-permeabilized mitochondria, an antiserum to PDH was used which completely inhibits PDH activity on binding to the enzyme. In Fig. 3(*a*), toluene-permeabilized mitochondria were further disrupted with Triton X-100, and PDH antiserum was added. Under these conditions the antiserum caused almost complete inhibition of the initial PDH_a rate. In contrast, there was no effect of PDH antiserum on the initial PDH_a rate in toluene-permeabilized mitochondria in the absence of Triton X-100 (Fig. 3*c*), which was identical with the

equivalent rate with control serum (Fig. 3*d*). Thus the PDH antibody was unable to gain access to PDH within the matrix of toluene-permeabilized mitochondria.

In addition to showing that toluene-permeabilized adipose tissue mitochondria excluded PDH antibody, the data of Fig. 3 also show that PDH phosphatase and PDH kinase were ineffective once the mitochondrial membranes had been disrupted by adding Triton X-100. PDH phosphatase was activated by adding Ca^{2+} and Mg^{2+}, which led to a time-dependent increase in PDH_a in the absence of Triton X-100 (Figs. 3*c* and *d*), but had no effect in its presence (Fig. 3*b*). ATP was then added to activate PDH kinase, and the activity of the PDH_a was diminished to a lower steady-state value set by the competing kinase and phosphatase acting on PDH within the matrix of the permeabilized mitochondria. Subsequent addition of NaF to inhibit the phosphatase, leaving PDH kinase to act alone, led to complete inactivation of the PDH (Figs. 3*c* and 3*d*; the initial rapid decrease in absorbance after NaF addition is a dilution artefact). As with PDH phosphatase, PDH kinase was unable to act on PDH once the mitochondrial membranes were disrupted with toluene (Fig. 3*b*). The simplest explanation of these observations is that PDH phosphatase and PDH kinase are unable to act on the PDH once the mitochondrial membranes have been disrupted by Triton X-100 because of the large dilution that occurs. Even in extracts of mitochondria 100 times more concentrated than the suspensions of peremabilized mitochondria used here we have found that both the kinase and phosphatase act much more slowly than in the permeabilized mitochondria (A. P. Thomas & R. M. Denton, unpublished work). Taking a mitochondrial matrix volume of 1 µl/ml (see Quinlan *et al.*, 1983), the 25 µg of mitochondrial protein used in these assays is equivalent to 0.025 µl, which is thus diluted some 40000-fold once the protein contents are released into the 1 ml incubation medium after addition of Triton X-100. Kerbey & Randle (1982) were the first to report the apparent dissociation of kinase activity from the PDH complex after extensive dilution.

The observations presented above demonstrate that the toluene-permeabilized mitochondria offer a system in which PDH and its interconverting kinase and phosphatase remain active within their native intramitochondrial environment but are freely accessible to small-M_r substrates, cofactors and ions. The main advantage of the permeabilized mitochondria is the combination of having the PDH complex at its normal very high intramitochondrial concentration, while substrates and effectors can easily be manipulated and measured in the surrounding bulk medium. It thus becomes possible to investigate the regulation of PDH kinase and PDH phosphatase while PDH is catalysing its normal reaction under condtions where changes in PDH activity can be monitored continuously.

Effect of insulin pretreatment of adipose tissue on subsequently prepared permeabilized mitochondria

In Fig. 4 two pairs of traces are shown for PDH assays with permeabilized mitochondria from control (Figs. 4*a* and 4*c*) or insulin-treated (Figs. 4*b* and 4*d*) adipose tissue. At the start of the traces neither kinase nor phosphatase is active, and the activity of PDH_a is essentially the same as that in the source tissue, with a clearly increased activity in the mitochondria from insulin-pretreated adipose tissue. In the experiment shown in Fig. 4, the mean initial PDH_a activities were 101 and 171 munits/ml of mitochondrial preparation from control and insulin-treated adipose tissue respectively. Addition of $CaCl_2$ and $MgCl_2$ to give final calculated concentrations of 0.2 mM-Mg^{2+} and 0.9 µM-Ca^{2+} (Figs. 4*c* and 4*d*) resulted in activation of PDH phosphatase and conversion of all of the PDH into PDH_a, so that the insulin effect was no longer apparent. The total PDH activities calculated from the maximum rates after addition of Ca^{2+} and Mg^{2+} were 185 munits/ml for the control and 194 munits/ml for the mitochondrial preparation from insulin-treated tissue. Addition of ATP to the permeabilized mitochondria after treatment with Ca^{2+} and Mg^{2+} allowed both the kinase and the phosphatase to act simultaneously, with the result that a new intermediate steady-state activity of PDH_a was achieved (Figs. 4*c* and 4*d*). Under these conditions, the insulin-induced elevation in the activity of PDH_a was once more apparent.

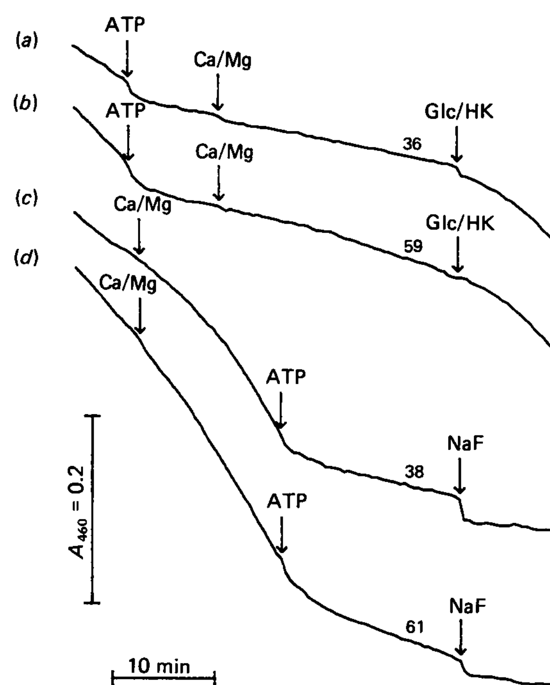

Fig. 4. Persistent elevation of PDH_a activity in permeabilized mitochondria prepared from insulin-treated adipose tissue

The activity of PDH in permeabilized mitochondria prepared from control (*a*, *c*) and insulin-treated (*b*, *d*) adipose tissue was monitored as described in the Methods section and the legend to Fig. 2. After a linear rate had been achieved, the following additions were made as indicated on the traces: 0.2 mM-ATP plus 50 µM-$MgCl_2$ (ATP); 1.0 mM-$CaCl_2$ plus 1.0 mM-$MgCl_2$, giving 0.9 µM-Ca^{2+} plus 0.2 mM-Mg^{2+} (Ca/Mg); 10 mM-glucose plus 1.4 unit of hexokinase/µl (Glc/HK); 25 mM-NaF (NaF). The numbers on the traces represent the rate of the PDH reaction in munits/ml of stock mitochondrial suspensions, calculated by linear regression through the local linear region of the record.

Similar results were obtained when the sequence of additions was reversed, with kinase activation before initiation of the phosphatase reaction (Figs. 4*a* and 4*b*). With both of the protocols shown in Fig. 4, the effect of

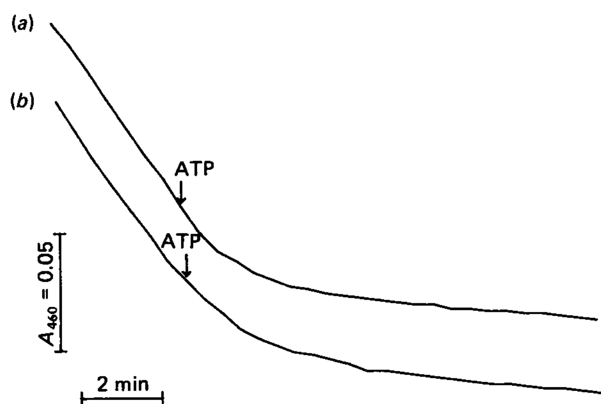

Fig. 5. Time course for the inhibition of PDH resulting from phosphorylation by PDH kinase in permeabilized mitochondria

Permeabilized mitochondria prepared from (*a*) control or (*b*) insulin-treated tissue were preincubated with 1.5 mM-$MgCl_2$ for 15 min to activate the PDH maximally, and then phosphatase activity was blocked by adding 25 mM-NaF. Substrates and cofactors for the PDH reaction were then added to the incubation cuvettes, and the production of acetyl-CoA was monitored as described in the Methods section and the legend to Fig. 2. After a linear rate was obtained, 0.2 mM-ATP plus 50 μM-$MgCl_2$ (maintaining the free Mg^{2+} concentration at 7 μM) was added (ATP), and the resulting inactivation of PDH was followed as the decline in the rate of acetyl-CoA generation.

insulin was lost once either the phosphatase or the kinase was allowed to act alone, resulting in total activation or near-complete inactivation of PDH respectively. In Figs. 4(*a*) and 4(*b*), kinase activity was blocked by removing the ATP with glucose and hexokinase, whereas in Figs. 4(*c*) and 4(*d*) the phosphatase was inhibited by adding NaF. The conditions where PDH kinase and PDH phosphatase are operating simultaneously to maintain a steady-state intermediate activity of PDH_a are closely analogous to the conditions that pertain during incubation of intact mitochondria. The mechanism responsible for the insulin-induced elevation of this steady-state PDH_a activity in both systems must involve either activation of PDH phosphatase or inhibition of PDH kinase.

In order to examine whether PDH kinase activity is altered in permeabilized mitochondria from insulin-treated adipose tissue, it was first necessary to eliminate any differences in the starting activity of PDH_a. This was achieved by preincubating the permeabilized mitochondria with 1.5 mM-$MgCl_2$ to activate PDH phosphatase and convert all of the endogenous PDH into the dephosphorylated PDH_a. After this, the phosphatase was inhibited by adding NaF and then the kinetics of the PDH kinase reaction were monitored directly by following the inactivation of PDH after ATP addition (Fig. 5). When ATP was added, the PDH kinase reaction was initiated and PDH was inhibited in a time-dependent manner. There was no significant difference in the kinetics of the kinase reaction between the two preparations of mitochondria, with a 50% decrease in PDH_a occurring after 90 s and almost complete inactivation of the PDH after 4 min. Similar observations were made in several other experiments and are in line with earlier conclusions that PDH kinase may not be the main site at which insulin acts to elevate steady-state PDH_a activities (Hughes & Denton, 1976; Denton *et al.*, 1984).

The effect of insulin on PDH phosphatase was also examined by a similar approach. For these experiments, PDH was completely inactivated by incubating the permeabilized mitochondria with ATP for 10 min (Fig. 6). The ATP was then removed by adding glucose and hexokinase, and after a further 5 min the phosphatase reaction was initiated by adding $MgCl_2$ to give a free Mg^{2+} concentration of 0.3 mM. This concentration is below that necessary to obtain a maximum rate of dephosphorylation (see below), and thus a fairly slow re-activation of PDH was observed, until all of the PDH was in the active dephosphorylated form. Under these conditions it was apparent that the re-activation of PDH by the phosphatase was enhanced in permeabilized mitochondria from insulin-treated adipose tissue (Fig. 6*b*) compared with controls (Fig. 6*a*). In order to quantify this difference, the amount of PDH_a was 'frozen' at fixed times after Mg^{2+} addition by adding NaF. For the traces shown in Figs. 6(*a*) and 6(*b*) (continuous lines), two parallel incubations were also carried out in which the mitochondria were treated identically until 6 min after Mg^{2+} addition, when 25 mM-NaF was added to stop the PDH phosphatase reaction completely and essentially instantaneously. The parts of the traces after NaF addition are shown by the broken lines in Figs. 6(*a*) and 6(*b*). These traces thus show a linear rate of PDH activity which was equivalent to the amount of PDH_a produced by the phosphatase during the time up to NaF addition, and are therefore effectively a tangent to the kinetic curve of phosphatase activity. At the time point selected for NaF addition in Fig. 6, the amount of PDH_a that had been generated in the permeabilized mitochondria from insulin-treated adipose tissue was some 60% greater than that in the controls. In four separate experiments carried out in this manner the mean increase ($\pm$S.E.M.) was 55 ± 6.1%. Similar results were obtained when NaF was added at between 2 and 8 min after the addition of Mg^{2+}. No changes in PDH activity were apparent if NaF was added with or before the Mg^{2+} or when activation was complete (20 min after addition of Mg^{2+}).

In order to monitor the initial re-activation of PDH more directly, NADH production was measured by following the increase in A_{340}. As discussed above, it is impractical to measure NADH generation for very long, because of the presence of NADH oxidase activities which prevent NADH from accumulating. The initial production of NADH upon phosphatase activation in an experiment carried out under essentially the same conditions as those of Figs. 6(*a*) and 6(*b*) are shown in the insert to Fig. 6. Within about 6 min of Mg^{2+} addition the rate of NADH production ceases to increase, and shortly after this the rate may actually decline. However, although quantification is not possible in this system, since more NADH is presumably being produced than detected spectrophotometrically, it is still apparent that the mitochondria from insulin-treated tissue (Fig. 6*d*) show a more rapid acceleration in the rate of NADH production than the controls (Fig. 6*c*) over the initial period after Mg^{2+} addition. Overall, all three of the different approaches illustrated in Fig. 6 have provided

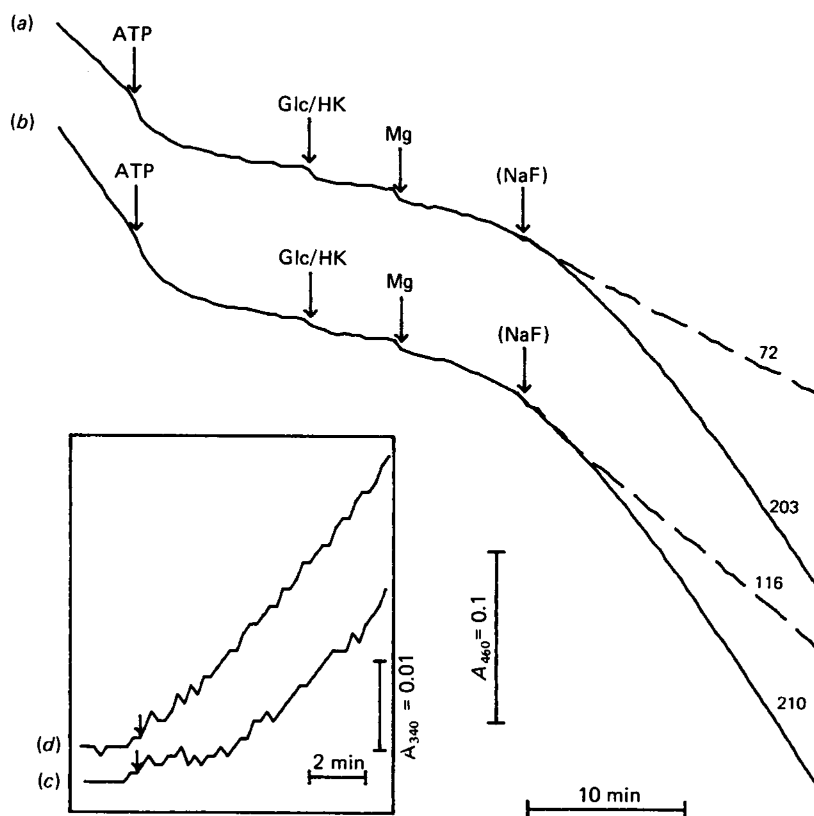

Fig. 6. Activation of PDH resulting from dephosphorylation caused by PDH phosphatase in permeabilized mitochondria from control and insulin-treated adipose tissue

The PDH reaction was monitored as described in the Methods section, either by using AABS to trap and monitor acetyl-CoA (*a*, *b*) or by direct measurement of NADH production at 340 nm (*c*, *d*). The permeabilized mitochondria were prepared from either control (*a*, *c*) or insulin-treated (*b*, *d*) adipose tissue. In traces (*a*) and (*b*), PDH was initially fully phosphorylated and inactivated by adding 0.2 mM-ATP plus 50 μM-$MgCl_2$ (ATP). Subsequently 10 mM-glucose plus 1.4 unit of hexokinase/ml (Glc/HK) was added to remove the ATP, and then the phosphatase reaction was initiated by adding 1.3 mM-$MgCl_2$ to give 0.3 mM free Mg^{2+} (Mg). A parallel pair of cuvettes was treated exactly as for traces (*a*) and (*b*), but 6 min after $MgCl_2$ addition the phosphatase reaction was stopped by adding 25 mM-NaF. The traces after NaF addition are shown by the broken lines; the traces drawn with continuous lines show the continuing increase in PDH_a in the absence of NaF. Inset: permeabilized mitochondria from control (*c*) and insulin-treated (*d*) tissue were treated exactly as for traces (*a*) and (*b*), except that arylamine acetyltransferase and AABS were absent and the PDH substrates were not added until 2 min before initiation of the phosphatase reaction with Mg^{2+}. Numbers on the traces represent rates of PDH reaction as in Fig. 4.

evidence that insulin brings about increases in PDH activity through a stimulation of PDH phosphatase.

Mg^{2+}-sensitivity of PDH phosphatase in toluene-permeabilized mitochondria

We have previously proposed that insulin may activate PDH phosphatase through an increase in the sensitivity of this enzyme to Mg^{2+} (Thomas *et al.*, 1986). In experiments of the type shown in Figs. 4 and 6 it was also found that the effect of insulin was most apparent at low Mg^{2+} concentrations. In the experiment shown in Fig. 7, permeabilized mitochondria from control and insulin-treated adipose tissue were first treated with ATP to inactivate the PDH. Subsequently $MgCl_2$ additions were made to give the free concentrations of Mg^{2+} indicated. After each Mg^{2+} addition the mitochondria were allowed to establish a new steady-state activity of PDH_a, proportional to the activity of PDH phosphatase in competition with the essentially unaltered PDH kinase activity. Although there was an effect of insulin over the lower range of Mg^{2+} concentrations, the effect became proportionally smaller as the Mg^{2+} concentration was increased. In this experiment the calculated apparent $K_{0.5}$ value for Mg^{2+} was 0.7 mM in the control mitochondria, and this was decreased to 0.3 mM in the mitochondria from the insulin-treated tissue without any appreciable difference in the apparent maximal effect.

A more accurate measurement of Mg^{2+}-sensitivity of PDH phosphatase in permeabilized mitochondria is shown in Fig. 8, which presents data combined from several experiments where the steady-state activities of PDH_a at different Mg^{2+} concentrations were determined. For these experiments only one Mg^{2+} concentration was tested in each incubation. The curves drawn in Fig. 8 represent the best fit of the data by non-linear regression to a co-operative binding equation (see the legend). The calculated parameter values (means $\pm$ S.E.M.) for mitochondria from control and insulin-treated tissue were respectively: 0.36 ± 0.02 and 0.16 ± 0.02 mM for the K_a for Mg^{2+}: 49.7 ± 1.5 and $45.1 \pm 2.3\%$ of total for the maximal PDH_a (V_{max}): 1.03 ± 0.07 and 1.15 ± 0.13 for the Hill coefficient (*h*). The effect of insulin to decrease

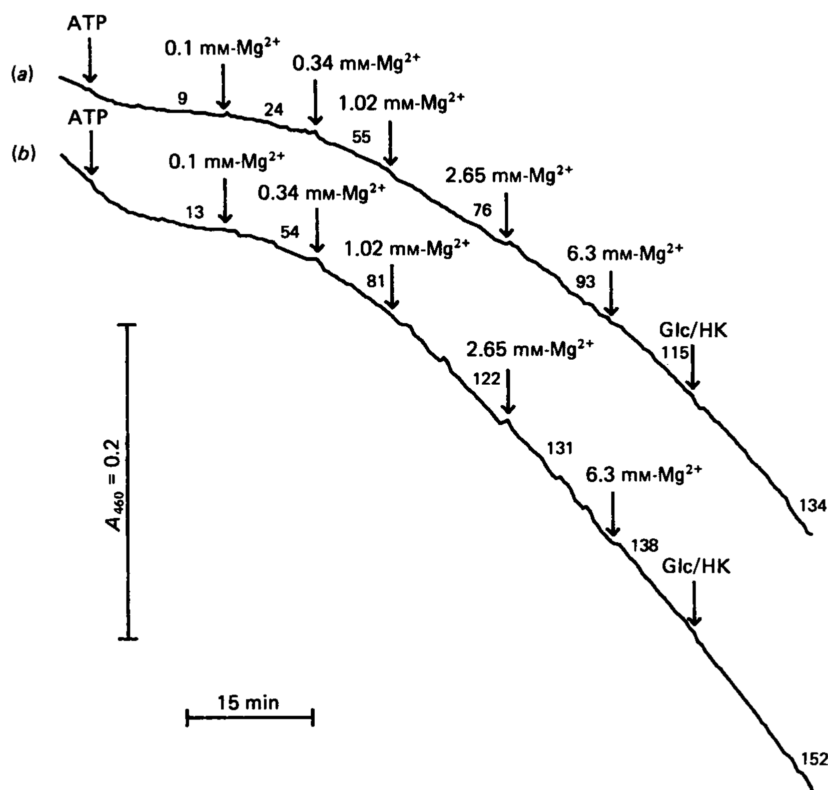

Fig. 7. Effect of varying the Mg^{2+} concentration on steady-state PDH_a activities in permeabilized mitochondria

Permeabilized mitochondria from control (*a*) or insulin-treated (*b*) adipose tissue were incubated as described in the Methods section for assay of PDH by measuring acetyl-CoA production. The PDH was first inactivated by adding 0.2 mM-ATP plus 50 μM-$MgCl_2$ (ATP) and then $MgCl_2$ was added to give the indicated free concentrations of Mg^{2+}. The numbers above different portions of the traces represent the rate of PDH_a over the local linear region calculated by non-linear regression. At the end of the trace 10 mM-glucose plus 1.4 unit of hexokinase/ml (Glc/HK) was added to block kinase activity.

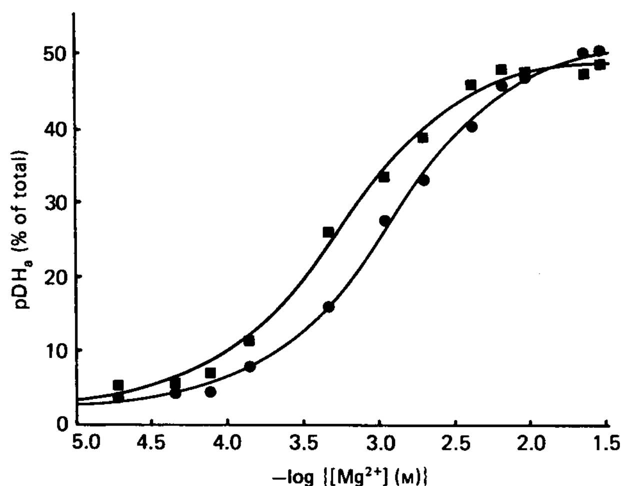

Fig. 8. Mg^{2+}-sensitivity of PDH phosphatase in permeabilized mitochondria from control and insulin-treated adipose tissue

A series of experiments were carried out similar to that in Fig. 7, except that only one $MgCl_2$ addition was made to each sample, so that a more prolonged linear steady-state activity of PDH could be obtained. PDH_a activities are expressed as a percentage of the total PDH activity in each sample. The data shown here are combined from experiments using three separate preparations of permeabilized mitochondria from control (●) and from insulin-treated (■) adipose tissue. Each point represents the mean value obtained from two or three experiments. The curves were fitted to the data by non-linear least-squares regression to the equation:

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

where $V_{min.}$ and $V_{max.}$ are the calculated values of PDH_a as a percentage of total activity in the absence of Mg^{2+} and in the presence of saturating concentrations of Mg^{2+} respectively.

the K_a for Mg^{2+} was significant at $P < 0.05$ by Student's *t* test. The insulin-induced decrease in the K_a for Mg^{2+} is similar to that which we have observed when the Mg^{2+} content of intact mitochondria was varied by using the ionophore A32187 (Thomas *et al.*, 1986).

We have shown that the polyamine spermine increases the sensitivity of purified PDH phosphatase to Mg^{2+} in a manner very similar to the action of insulin in the intact tissue (Thomas *et al.*, 1986). This raises the possibility that insulin might act by increasing the concentration of spermine or a related polybasic compound (see Thomas *et al.*, 1986, for further discussion of this point). However, the results with toluene-permeabilized mito-

chondria are not consistent with this possibility, for two reasons. Firstly, if such a polyamine was present in the mitochondria, it seems probable that it would diffuse away after the permeabilization treatment, with the loss of the insulin effect. Although the insulin effect does gradually deteriorate over a period of several hours when the mitochondria are stored at 0 °C, this loss seems to be extremly slow. Secondly, attempts to activate PDH phosphatase by adding spermine to toluene-permeabilized mitochondria have so far proved negative. It seems most unlikely that there is any barrier to the entry of spermine into permeabilized mitochondria, in view of the free permeation of cations such as Ca^{2+} and Mg^{2+} and metabolites of the size of NAD^{+}, acetyl-CoA and ATP. An alternative possibility is that the spermine-sensitive site is occupied by some higher-M_r component within the mitochondria, perhaps even associated with the mitochondrial inner membrane, and that this is the component which is altered by insulin. Clearly the toluene-permeabilized mitochondria offer an ideal system for the further elucidation of the mechanism by which insulin activates PDH.

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