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Calcium and Magnesium Ions as Effectors of Adipose-Tissue Pyruvate Dehydrogenase Phosphate Phosphatase

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The metal-ion requirement of extracted and partially purified pyruvate dehydrogenase phosphate phosphatase from rat epididymal fat-pads was investigated with pig heart pyruvate dehydrogenase [^{32}P]phosphate as substrate. The enzyme required Mg^{2+} (K_m 0.5mM) and was activated additionally by Ca^{2+} (K_m 1μM) or Sr^{2+} and inhibited by Ni^{2+}. Isolated fat-cell mitochondria, like liver mitochondria, possess a respiration- or ATP-linked Ca^{2+}-uptake system which is inhibited by Ruthenium Red, by uncouplers when linked to respiration, and by oligomycin when linked to ATP. Depletion of fat-cell mitochondria of 75% of their total magnesium content and of 94% of their total calcium content by incubation with the bivalent-metal ionophore A23187 leads to complete loss of pyruvate dehydrogenase phosphate phosphatase activity. Restoration of full activity required addition of both $MgCl_2$ and $CaCl_2$. $SrCl_2$ could replace $CaCl_2$ (but not $MgCl_2$) and $NiCl_2$ was inhibitory. The metal-ion requirement of the phosphatase within mitochondria was thus equivalent to that of the extracted enzyme. Insulin activation of pyruvate dehydrogenase in rat epididymal fat-pads was not accompanied by any measurable increase in the activity of the phosphatase in extracts of the tissue when either endogenous substrate or ^{32}P-labelled pig heart substrate was used for assay. The activation of pyruvate dehydrogenase in fat-pads by insulin was inhibited by Ruthenium Red (which may inhibit cell and mitochondrial uptake of Ca^{2+}) and by $MnCl_2$ and $NiCl_2$ (which may inhibit cell uptake of Ca^{2+}). It is concluded that Mg^{2+} and Ca^{2+} are cofactors for pyruvate dehydrogenase phosphate phosphatase and that an increased mitochondrial uptake of Ca^{2+} might contribute to the activation of pyruvate dehydrogenase by insulin.

Insulin activates pyruvate dehydrogenase (EC 1.2.4.1) in rat epididymal adipose tissue by effecting the conversion of the inactive (phosphorylated) form of the enzyme into an active (dephosphorylated) form (Jungas, 1971; Coore *et al.*, 1971; Weiss *et al.*, 1971; Randle & Denton, 1973). Interconversion of these two forms of pyruvate dehydrogenase are catalysed by pyruvate dehydrogenase kinase (utilizing $MgATP^{2-}$) and pyruvate dehydrogenase phosphate phosphatase (Linn *et al.*, 1969*a*,*b*). The action of insulin is thus assumed to involve inhibition of the kinase or activation of the phosphatase, or both.

Pyruvate dehydrogenase kinase is inhibited by ADP (competitive with ATP) and by pyruvate [non-competitive with ATP (Linn *et al.*, 1969*b*) or uncompetitive with ATP (R. H. Cooper, P. J. Randle & R. M. Denton, unpublished work)]. Although evidence has been obtained that increased concentrations of ADP and of pyruvate may activate pyruvate dehydrogenase in fat-cells or fat-cell mitochondria, there is no convincing evidence that insulin action is mediated by an elevated intracellular ADP/ATP concentration ratio, nor does it appear that insulin action is mediated solely by elevated intracellular concentrations of pyruvate (see Martin *et al.*, 1972).

Pyruvate dehydrogenase phosphate phosphatase requires Mg^{2+} for activity (Linn *et al.*, 1969*a*,*b*). Studies with EGTA [ethanedioxybis(ethylamine)-tetra-acetate] and with CaEGTA buffers suggest that the phosphatase from rat adipose tissue, pig heart and pig kidney cortex mitochondria is activated by Ca^{2+} (in the μM concentration range) in the presence of a saturating concentration of Mg^{2+} (Denton *et al.*, 1972). More detailed studies with the phosphatase from pig heart mitochondria have confirmed this effect of Ca^{2+} by use of EGTA and CaEGTA buffers and have demonstrated effects of Ca^{2+} in the absence of EGTA by using reagents depleted of Ca^{2+} with Chelex-100 resin. It was found, however, that higher concentrations of Ca^{2+} may be required in the absence of EGTA. These studies also showed that Sr^{2+} can substitute for Ca^{2+}, that Ni^{2+} is inhibitory and that

the effect of Ca^{2+} (in the presence of EGTA) is to lower the K_m of the phosphatase for pyruvate dehydrogenase phosphate from 30μM-protein-bound phosphate to 1.6μM (Randle *et al.*, 1974). The concentration of pyruvate dehydrogenase phosphate in fat-cell or rat heart mitochondria may be of the order of 30μM, and at this high substrate concentration pig heart phosphatase is rather insensitive to Ca^{2+}. It has seemed important therefore to attempt to show the dual metal-ion requirement (for Ca^{2+} and Mg^{2+}) of the phosphatase within fat-cell mitochondria and to study the effects of added $SrCl_2$ and $NiCl_2$. This has become possible with the availability of the bivalent-cation ionophore A23187 and the results of such experiments are described here.

Insulin might activate pyruvate dehydrogenase in adipose tissue through activation of pyruvate dehydrogenase phosphate phosphatase. We have therefore assayed the phosphatase activity in extracts prepared from epididymal fat-pads previously incubated with and without insulin. It seemed possible that uptake of Ca^{2+} by mitochondria might regulate the activity of pyruvate dehydrogenase through an effect on the phosphatase and that insulin may affect this Ca^{2+} uptake. This has led to an investigation of the uptake of Ca^{2+} by fat-cell mitochondria, and the effect on insulin activation of fat-cell pyruvate dehydrogenase of agents such as Ruthenium Red, $NiCl_2$ and $MnCl_2$, which can impair the uptake of Ca^{2+} by cells and/or mitochondria.

Experimental

Materials

Rats. Epididymal fat-pads were obtained from male albino Wistar rats (150–220g) with free access to stock laboratory diet (modified 41B; Oxoid Ltd., London S.E.1, U.K.). The animals were killed by decapitation and in any one experiment were closely matched for age and weight.

Chemicals. Biochemicals and enzymes were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K., with the following exceptions. Crystalline insulin was a gift from Boots Pure Drug Co. Ltd., Nottingham, U.K.; a stock solution of 10 units/ml was prepared in 3mM-HCl and 0.2ml portions were kept frozen at −20°C. Bovine albumin (fraction V), defatted by the method of Chen (1966), and collagenase (type I, *Clostridium histolyticum*) were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Ruthenium Red was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. A23187, a gift from Dr. R. L. Hamill, Eli Lilly and Co., Indianapolis, Ind., U.S.A., was diluted from a stock solution of 1mg/ml in ethanol. [γ-^{32}P]-ATP and ^{45}Ca were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Highly purified $MgSO_4$ and $CaCl_2$ (spectrographically standardized) were obtained from Johnson Matthey Chemicals Ltd., London E.C.4, U.K. These were only used when specifically indicated; otherwise analytical-grade chemicals were used.

Pyruvate dehydrogenase phosphate and pyruvate dehydrogenase phosphate phosphatase. Pyruvate dehydrogenase [^{32}P]phosphate was prepared from pig heart pyruvate dehydrogenase (15–30 units/ml) (see Randle *et al.*, 1974). The specific radioactivity was 0.5–2μCi/unit of pyruvate dehydrogenase (equivalent to 0.3–1.2μCi/nmol of protein-bound phosphate). Pig heart pyruvate dehydrogenase phosphate phosphatase was prepared from an extract of pig heart mitochondria obtained by freezing and thawing, by $(NH_4)_2SO_4$ fractionation and precipitation at pH6.1 (as described below for preparation of fat-pad enzyme). Pyruvate dehydrogenase was removed as a pellet by centrifugation at 150000*g* for 100min at 4°C. The preparations used in this study contained 3munits of phosphatase/ml (i.e. 1ml hydrolyses 3nmol of protein-bound phosphate/min). In practical terms, complete hydrolysis of pyruvate dehydrogenase phosphate in tissue extracts was complete in 5min with equal volumes of extract and phosphatase.

Rat epididymal fat-pad pyruvate dehydrogenase phosphate phosphatase was concentrated and partially purified (fivefold) by the following procedure. Fat-pads (45g) were frozen, powdered under liquid N_2 and extracted with 135ml of 30mM-potassium phosphate buffer (pH7.0) containing 2mM-$MgCl_2$ and 1mM-2-mercaptoethanol at 0°C with a Polytron PT10 tissue homogenizer. The extract was centrifuged at 35000*g* for 40min at 4°C, and the infranatant aspirated from below the fat layer was treated with solid $(NH_4)_2SO_4$ (208g/litre). The precipitate was redissolved in 5ml of 20mM-potassium phosphate buffer (pH7.0) containing 5mM-2-mercaptoethanol and dialysed overnight against 100vol. of the same buffer. The solution was adjusted to pH6.1 with 10% (v/v) acetic acid and the precipitate discarded. The supernatant after adjustment to pH7.0 and centrifugation for 90min at 150000*g* at 4°C contained approx. 20–25% of the pyruvate dehydrogenase phosphate phosphatase activity contained in the original extract and was free of pyruvate dehydrogenase activity.

Media. Fat-pads were incubated in bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing half the recommended $CaCl_2$ concentration and gassed with O_2+CO_2 (95:5).

Isolated fat-cells and fat-cell mitochondria. Isolated fat-cells were prepared by the method of Rodbell (1964) by shaking rat epididymal fat-pads in bicarbonate-buffered medium containing 2% defatted bovine albumin and collagenase (0.5mg/ml). Medium containing albumin was dialysed before use for 16h

against the medium buffer. Mitochondria were prepared from these cells as described by Martin & Denton (1970).

Methods

Assay of enzyme activities in extracts of fat-pads and fat-cell mitochondria. Frozen fat-pads were powdered with a pestle under liquid N_2 and extracted at 0°C with 100mM-potassium phosphate–2mM-EDTA, pH7.0 in a Polytron PT-10 tissue homogenizer, for 30s at position 6. Mitochondria were extracted by triple freezing and thawing in the same medium.

Pyruvate dehydrogenase and glutamate dehydrogenase were assayed in extracts by methods given in Martin *et al.* (1972).

Pyruvate dehydrogenase phosphate phosphatase activity was assayed by the release of [^{32}P]phosphate from pyruvate dehydrogenase [^{32}P]phosphate. Assays in extracts of fat-pads were performed in quadruplicate in a total volume of 25μl containing 10μl of extract and approx. 30pmol of protein-bound phosphate. The other components were 52mM-potassium phosphate, 3mM-2-mercaptoethanol, 25mM-$MgCl_2$, 0.4mM-EDTA, 8mM-EGTA, ±7.8mM-$CaCl_2$, pH7.0. The reaction was initiated by addition of substrate and terminated after 5min at 30°C with 100μl of 10% (w/v) trichloroacetic acid and 100μl of 1% (w/v) albumin. [^{32}P]Phosphate was assayed in the supernatant after the addition of scintillator [toluene (600ml), methoxyethanol (400ml), naphthalene (80g) and 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (6g)] in a Nuclear–Chicago mark I liquid-scintillation spectrometer. Results from incubations without extract allowed corrections to be applied for small amounts of [^{32}P]phosphate and phosphatase present in the substrate.

The same assay was used for experiments with partially purified phosphatase except that the incubation volume was 20μl and the incubation mixture contained 20mM-potassium phosphate–5mM-mercaptoethanol, pH7, and other additions as given in the text or tables. In all assays the quantities of extract or phosphatase were such that no more than 20% of substrate was hydrolysed. Hydrolysis was linear with time and the rate was proportional to enzyme or extract concentration under these conditions.

Glycerol output of fat-pads. Glycerol was measured spectrophotometrically in neutralized $HClO_4$ extracts of incubation media as described by Garland & Randle (1962).

O_2 uptake and ATP content of fat-cell mitochondria. Rates of respiration of mitochondria were recorded by the polarographic technique as described by Martin & Denton (1970). ATP content was measured as given by Martin *et al.* (1972).

^{45}Ca incorporation into isolated fat-cell mitochondria. Isolated mitochondria were suspended in 0.25M-sucrose–20mM-Tris–HCl–2% (w/v) defatted albumin–10mM-GSH, pH7.4 (treated with Chelex-100 to remove contaminating Ca^{2+} as described by Randle *et al.*, 1974). The mitochondrial protein concentration was approx. 1mg/ml. Mitochondria (50μl of the above suspension) were incubated at 37°C in 450μl of 125mM-KCl–20mM-Tris–HCl, pH7.4, containing [^{45}Ca]$CaCl_2$ (0.1mM, approx. 2μCi/μmol) and other additions given in the text or Tables. Incorporation of ^{45}Ca into mitochondria was terminated by filtration through Millipore filters (pore diameter 0.45μm); the filters were immediately washed with 5ml of 0.25M-sucrose–20mM-Tris–HCl, pH7.5, dried, and placed in 5ml of scintillation fluid [4g of 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole in 1 litre of toluene]. Radioactivity was determined in a Nuclear-Chicago mark I liquid-scintillation spectrometer.

Total calcium and magnesium content of fat-cell mitochondria. Calcium and magnesium concentrations in fat-cell mitochondria were determined with an EEL 240 atomic-absorption spectrophotometer. Mitochondrial pellets were dissolved in conc. HNO_3 and diluted into 2.5mM-disodium EDTA to prevent interference by phosphate. Measurements with appropriate reagent blanks were performed for each metal. Protein was determined by the method of Lowry *et al.* (1951) with bovine albumin as a standard.

Calculations

Ca^{2+} and Mg^{2+} concentrations. Concentrations of Ca^{2+} and Mg^{2+} in the presence of EGTA, phosphate and sulphate were calculated by using a computer program (Feldman *et al.*, 1972). The following dissociation constants (at pH7.0) were used: CaEGTA 2.07×10^{-7}; calcium phosphate 6.13×10^{-3}; $CaSO_4$ 5.25×10^{-3}; MgEGTA 2.45×10^{-2}; magnesium phosphate 3.3×10^{-3}; $MgSO_4$ 5.37×10^{-3}. In earlier papers the binding of these metals by phosphate (Denton *et al.*, 1972) or sulphate (Randle *et al.*, 1974) was not included in calculations of free Ca^{2+} and Mg^{2+}. It should be noted that, in this paper, Ca^{2+} and Mg^{2+} refer to the free ionized forms of the respective metal ions. When referring to the sum of free and complexed species, the terms total calcium and total magnesium have been used.

Enzyme activities. These are given as units of enzyme activity (1μmol of substrate converted/min = 1 unit) measured at 30°C. One unit of phosphatase activity hydrolyses 1μmol of protein-bound phosphate/min.

Since glutamate dehydrogenase is a purely mitochondrial enzyme (Martin & Denton, 1970), the activity of this enzyme has been used as a recovery index for activities of pyruvate dehydrogenase or pyruvate dehydrogenase phosphate phosphatase in fat-cells. Adipose tissue contains approx. 1 unit of glutamate dehydrogenase/g wet wt. of tissue.

Results and Discussion

Properties of adipose-tissue pyruvate dehydrogenase phosphate phosphatase

Previous experiments on extracts of fat-cell mitochondria broken by freezing and thawing showed that pyruvate dehydrogenase phosphate phosphatase requires Mg^{2+} for activity (Denton *et al.*, 1972). In the absence of Mg^{2+}, Ca^{2+} did not activate the enzyme, but at saturating concentrations of $MgCl_2$ (16mM), activity was inhibited by 10mM-EGTA, and increased by CaEGTA buffer giving a computed Ca^{2+} concentration of 10μM (10mM-EGTA+9.75mM-$CaCl_2$). By using a partially purified preparation of phosphatase free of endogenous pyruvate dehydrogenase, these findings have been confirmed and quantified.

In the presence of Ca^{2+}, the K_m of the phosphatase for Mg^{2+} was 0.52±0.09mM, measured at eight Mg^{2+} concentrations between 0 and 5mM in 20mM-potassium phosphate buffer, pH7.0, containing 5mM-mercaptoethanol, 0.2mM-EGTA and 100–200μM-Ca^{2+}. In this experiment spectrographically characterized $CaCl_2$ and $MgSO_4$ were used.

As shown in Fig. 1, at 32mM-$MgCl_2$ increasing the [Ca^{2+}] from 0 (EGTA alone) to 0.1mM led to a threefold activation of the phosphatase; the K_m for Ca^{2+} was approx. 1μM. The K_m of the adipose-tissue phosphatase for pig heart pyruvate dehydrogenase phosphate was approx. 3μM-protein-bound phosphate at saturating concentrations of Mg^{2+} and Ca^{2+}.

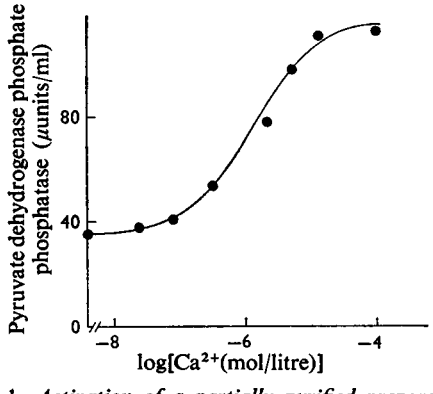

Fig. 1. *Activation of a partially purified preparation of adipose-tissue pyruvate dehydrogenase phosphate phosphatase by Ca^{2+}*

Activities were assayed in the presence of 20mM-potassium phosphate buffer, pH7.0, containing EGTA (10mM), mercaptoethanol (5mM), $MgCl_2$ (32mM) and various concentrations of $CaCl_2$ (0, 0.75, 1.95, 4.88, 8.13, 9.10, 9.75, 9.85mM). The concentration of pig heart pyruvate dehydrogenase [^{32}P]phosphate was 2.6μM. Ca^{2+} concentrations were calculated from the binding constants of Ca^{2+} and Mg^{2+} for phosphate and EGTA given in the Experimental section.

Table 1 compares the effects of Ca^{2+}, Sr^{2+} and Ni^{2+} on the activity of adipose-tissue phosphatase at saturating concentrations of Mg^{2+}. Twofold activation in the presence of 0.2mM-EGTA was produced by 0.2mM-$CaCl_2$ (Ca^{2+} = 6μM) or by 0.5mM-$SrCl_2$. Increasing $CaCl_2$ to 0.4mM (Ca^{2+} = 27μM) or adding 0.5mM-$SrCl_2$ to 0.2mM-$CaCl_2$ did not increase the activity further. $NiCl_2$ inhibited the activation by $CaCl_2$ but had no inhibiting effect in the absence of $CaCl_2$.

In summary, the properties of the phosphatase from adipose tissue appear to be very similar to those found in this laboratory for the pig heart and rat heart enzymes (Denton *et al.*, 1972; Randle *et al.*, 1974; H. T. Pask, P. J. Randle & R. M. Denton, unpublished work).

Ca^{2+} uptake by isolated fat-cell mitochondria

The effects of Ca^{2+} on pyruvate dehydrogenase phosphate phosphatase described above suggest the possibility that changes in mitochondrial Ca^{2+} content might alter the activity of this phosphatase in fat-cell mitochondria, thereby changing the ratio of phosphorylated (inactive) to non-phosphorylated (active) pyruvate dehydrogenase. Therefore it seemed important to compare the processes for Ca^{2+} uptake into fat-cell mitochondria with those already described for mitochondria from other mammalian tissues (Lehninger *et al.*, 1967; Lehninger, 1970). Uptake was studied by monitoring both O_2 consumption (Fig. 2) and the incorporation of ^{45}Ca into isolated fat-cell mitochondria (Tables 2 and 3).

Fat-cell mitochondria prepared by the method of Martin & Denton (1970) showed a P/O ratio of nearly 3 and a respiratory-control ratio (Chappell & Hansford, 1969), of about 5 with pyruvate and malate as substrates (Fig. 2, trace *a*). The addition of $CaCl_2$ to

Table 1. *Effect of $NiCl_2$ and $SrCl_2$ on activity of partially purified preparation of adipose-tissue pyruvate dehydrogenase phosphate phosphatase*

Assays were performed in 20mM-potassium phosphate buffer, pH7.0, containing mercaptoethanol (5mM), EGTA (0.2mM) and $MgCl_2$ (32mM) and other additions as indicated. The concentration of pyruvate dehydrogenase [^{32}P]phosphate (pig heart) was 2.7μM. Results are the mean of triplicate determinations with a single preparation of phosphatase.

$CaCl_2$ added (μM)	Calculated [Ca^{2+}] (μM)	Phosphatase activity (μunits/ml) in the presence of: No addition	$SrCl_2$ (0.5mM)	$NiCl_2$ (0.5mM)
None	—	66	121	54
200	6.4	116	122	59
400	27	119	—	46

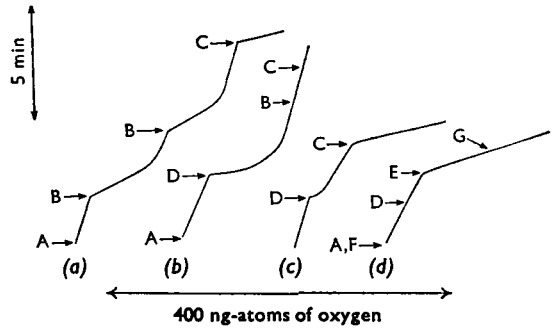

Fig. 2. *Effects of $CaCl_2$ on O_2 uptake by isolated fat-cell mitochondria*

Mitochondria (approx. 0.5mg of protein) were incubated at 30°C in KCl (125mM)–Tris–HCl (20mM) medium, pH7.4, containing pyruvate (2mM) and L-malate (0.5mM) in a total volume of 1ml. Additions were as follows: A, potassium phosphate (2μmol); B, ADP (0.2μmol); C, carbonyl cyanide *m*-chlorophenylhydrazone (2μg); D, $CaCl_2$ (0.2μmol); E, ADP (1μmol); F, Ruthenium Red (0.25μg); G, Ruthenium Red (25μg). The traces shown are typical of those found with three separate preparations of mitochondria.

Table 2. *Incorporation of Ca^{2+} into fat-cell mitochondria*

Incorporation of ^{45}Ca into fat-cell mitochondria (approx. 50μg of protein) was determined after 2min incubation at 37°C in KCl–Tris–HCl medium containing 0.1mM-$^{45}CaCl_2$ and other additions as indicated. Further details are given in the Experimental section. Results are the mean of duplicate determinations with a single preparation of mitochondria. Qualitatively similar results have been obtained with two additional preparations.

Additions to mitochondrial incubation medium	^{45}Ca incorporated (ng-atoms)
None	0.21
Pyruvate (2mM), malate (0.5mM)	2.64
Pyruvate (2mM), malate (0.5mM), phosphate (10mM)	0.04
Pyruvate (2mM), malate (0.5mM), oxalate (10mM)	14.3
Pyruvate (2mM), malate (0.5mM), ADP (4mM)	10.5
Pyruvate (2mM), malate (0.5mM), ADP (4mM), phosphate (10mM)	8.2
ADP (4mM)	1.05
ATP (4mM)	11.0
ATP (4mM), phosphate (10mM)	10.1
ATP (4mM), oxalate (10mM)	11.4

fat-cell mitochondria incubated in the presence of the permeant anion, phosphate, produced a marked transient increase in rate of respiration. It also resulted in subsequent inactivation of respiration; thus subsequent additions of ADP and uncoupler did not induce the usual increase in the rate of O_2 uptake (Fig. 2, trace *b*). The addition of a similar amount of $CaCl_2$ to mitochondria incubated in the absence of phosphate produced a much smaller transient increase in O_2 uptake; in this case the mitochondria appeared to be still functional, as subsequent addition of uncoupler increased the rate of respiration (Fig. 2, trace *c*). Ruthenium Red has been shown to be a potent inhibitor of mitochondrial Ca^{2+} uptake (Vasington *et al.*, 1972). Ruthenium Red (0.25μg/ml) completely prevented stimulation of O_2 uptake of fat-cell mitochondria by $CaCl_2$ in the presence of phosphate without appreciably affecting ADP-stimulated respiration, even when the Ruthenium Red concentration was increased 100-fold (Fig. 2, trace *d*).

Table 3. *Effects of uncouplers, oligomycin and Ruthenium Red on respiration-dependent and ATP-dependent incorporation of Ca^{2+} into fat-cell mitochondria*

Details are as given in Table 2. Respiration-dependent incorporation was measured in the presence of pyruvate (2mM) and malate (0.5mM) and ATP-dependent incorporation in the presence of ATP (4mM) and $MgCl_2$ (4mM). Results are the mean of duplicate determinations with a single preparation of mitochondria.

Additions to mitochondrial incubation medium	^{45}Ca incorporated (ng-atoms)	
	Respiration-dependent	ATP-dependent
None	1.75	5.17
Dinitrophenol (10μM)	0.94	3.87
Dinitrophenol (100μM)	0.08	2.20
m-Chlorocarbonyl cyanide phenylhydrazone (1μM)	0.35	3.57
m-Chlorocarbonyl cyanide phenylhydrazone (10μM)	0.09	0.25
Oligomycin (2μg/ml)	1.50	0.78
Ruthenium Red (0.1μg/ml)	0.16	0.26

Addition of pyruvate and L-malate increased the incorporation of ^{45}Ca into isolated mitochondria more than tenfold (Table 2). The further addition of permeant anions, phosphate and oxalate, produced opposite effects: phosphate decreased incorporation, whereas oxalate increased incorporation. The diminution of ^{45}Ca incorporation in the presence of phosphate was largely overcome by the presence of ADP (Table 2). Incorporation was also stimulated by the addition of ATP but not by ADP in the absence of respiratory substrates and phosphate; the further addition of phosphate or oxalate under these conditions had little or no effect (Table 2).

The uptake of Ca^{2+} by fat-cell mitochondria can thus be induced either by respiratory substrates or by ATP. Further, as with liver mitochondria (Lehninger *et al.*, 1967), the respiration-dependent uptake was inhibited to a greater extent by uncouplers than was

the ATP-dependent uptake, whereas only the ATP-dependent uptake of Ca^{2+} was sensitive to inhibition by oligomycin (Table 3). In agreement with results obtained by using liver mitochondria (Vasington *et al.*, 1972), Ruthenium Red eliminated the incorporation of ^{45}Ca both in the presence of respiratory substrates and in the presence of ATP (Table 3).

The presence of phosphate greatly enhanced the transient increase in O_2 consumption on addition of $CaCl_2$ (Fig. 2), whereas it greatly diminished the incorporation of ^{45}Ca under similar conditions (Table 2). The probable explanation of these apparently conflicting results is that massive uptake of both Ca^{2+} and phosphate under these conditions so damages the mitochondria that Ca^{2+} is lost and respiration abolished. Rossi & Lehninger (1964) have observed that the addition of phosphate to rat liver mitochondria resulted in loss of Ca^{2+}-stimulated respiratory control and that this effect was reversed by the presence of ADP.

Effects of the ionophore A23187 on the total calcium and magnesium content and pyruvate dehydrogenase phosphate phosphatase activity of isolated fat-cell mitochondria

Activation of pyruvate dehydrogenase phosphate phosphatase from pig kidney and pig heart by Ca^{2+} appears to be associated with a substantial decrease in the K_m of the phosphatase for its substrate pyruvate dehydrogenase phosphate. Thus Ca^{2+} lowers the K_m of the pig kidney enzyme from about 58μM- to 2.9μM-protein-bound phosphate (Pettit *et al.*, 1972) and that of the pig heart enzyme from about 30 to 1.6μM (Randle *et al.*, 1974). Since the fat-cell enzyme appears to resemble closely these enzymes in many aspects, including its K_m for pyruvate dehydrogenase phosphate in the presence of Ca^{2+}, it seems reasonable to suppose that as the concentration of pyruvate dehydrogenase phosphate increases, the degree of Ca^{2+} activation would diminish. Estimates of the concentration of pyruvate dehydrogenase phosphate suggest that its concentration could exceed 20μM in fat-cell mitochondria and thus it is possible that the enzyme *in situ* would not be sensitive to Ca^{2+}. To study the sensitivity of the phosphatase to Mg^{2+} and Ca^{2+} in intact mitochondria, it was first necessary to deplete the mitochondria of these bivalent ions, and this was accomplished by using the ionophore A23187.

A23187 is a carboxylic antibiotic which acts as a bivalent-cation ionophore; its effects on liver mitochondria have been described in detail by Reed & Lardy (1972) and Wong *et al.* (1973). The effects of A23187 on the total calcium and magnesium contents of fat-cell mitochondria are shown in Table 4. Exposure of the mitochondria to the ionophore for 10min at 30°C in the presence of EGTA resulted in a loss of more than 90% of mitochondrial total calcium and more than 70% of total magnesium. Incubation of the mitochondria in the absence of A23187 but in the presence of EGTA resulted in the loss of some 60% of the mitochondrial calcium but no loss of magnesium.

Table 4. *Effect of A23187 on the total calcium and magnesium content of fat-cell mitochondria*

Mitochondria were suspended in 125mM-KCl–20mM-Tris–HCl, pH7.4, medium containing potassium phosphate (2mM) and EGTA (5mM) to give a final concentration of approx. 4.5 mg of protein/ml. Samples (1.0ml) were centrifuged immediately to obtain mitochondrial pellets for the determination of initial contents of calcium and magnesium. Other samples were incubated at 30°C in the absence and in the presence of A23187 (5μM) for 10min before centrifugation. All pellets were analysed for calcium and magnesium as described in the Experimental section. Results are expressed as the mean±S.E.M. of three determinations for calcium and the mean of two determinations for magnesium.

	Mitochondrial content (ng-atoms/mg of protein)	
	Calcium	Magnesium
Initial	80.2±7.4	63.9
Incubated	23.5±2.3	70.5
Incubated+A23187	4.9±2.0	16.0

The effect of A23187 on ^{45}Ca incorporation into fat-cell mitochondria is shown in Fig. 3. The presence of the ionophore at 5μM completely inhibited the incorporation of ^{45}Ca that was dependent on ATP or pyruvate and L-malate. Addition of A23187 to the mitochondrial incubation medium after incorporation had proceeded for 2min led to complete loss of the incorporated ^{45}Ca within 3min.

The activity of pyruvate dehydrogenase in fat-cell mitochondria, prepared by the normal procedure, can be decreased by incubation with oxidizable substrates such as 2-oxoglutarate and malate, presumably because ATP synthesis leads to phosphorylation of pyruvate dehydrogenase (Martin *et al.*, 1972). The subsequent addition of pyruvate increases the activity of pyruvate dehydrogenase, presumably through inhibition of the kinase, thus indicating that the phosphatase is active in these mitochondria (Martin *et al.*, 1972). These experimental conditions have been used to examine the effects of $MgCl_2$ and $CaCl_2$ on the activity of the phosphatase in mitochondria.

In Fig. 4(*a*), fat-cell mitochondria were incubated with 2-oxoglutarate and L-malate leading to the expected decrease in the activity of pyruvate dehydrogenase. After 5min, additions of $MgCl_2$ (2mM), A23187 (5μM), or A23187 (5μM) plus $MgCl_2$ (2mM) to samples of these mitochondria had no significant effect on pyruvate dehydrogenase activity after a

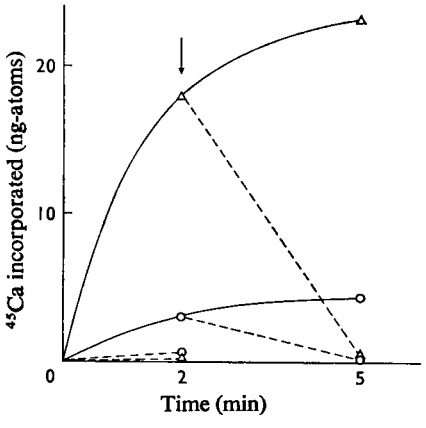

Fig. 3. *Effect of A23187 on the time-course of ^{45}Ca incorporation by fat-cell mitochondria*

Details are as given in legend to Table 4 except that EGTA and phosphate were omitted and that calcium binding was measured after 2 and 5min of incubation with pyruvate (2.5mM) and L-malate (0.5mM) (○) or ATP (4mM) (△). A23187 (5mM) was added to the incubation medium at zero time or at 2min (as indicated by the arrow); the presence of the ionophore is indicated by the broken lines.

further 10min of incubation. At the end of the 15-min period, pyruvate was added to all samples of mitochondria. In samples to which no addition had been made, or $MgCl_2$ alone had been added after 5min, the activity of pyruvate dehydrogenase showed the expected increase. However, in samples to which A23187 had been added after 5min no increase in pyruvate dehydrogenase activity was observed. Moreover, in those mitochondria exposed to A23187 plus $MgCl_2$ after 5min the rate of increase in pyruvate dehydrogenase activity was much less than in the controls. Unfortunately, because of the presence of phosphate and EGTA in the medium, it was not possible to test the effects of adding $CaCl_2$ under these conditions without causing damage and loss of enzymes from the mitochondria. The experimental design was modified to allow the examination of the effects of both added $CaCl_2$ and $MgCl_2$. Mitochondria were preincubated with A23187 in the presence of 2-oxoglutarate and L-malate, centrifuged and resuspended in KCl medium containing pyruvate, L-malate, A23187 and a small amount of EGTA (Fig. 4*b*). On incubation of the mitochondria in this medium no increase in pyruvate dehydrogenase activity was apparent, i.e. the phosphatase was apparently inactive. If $MgCl_2$ (2mM) was present

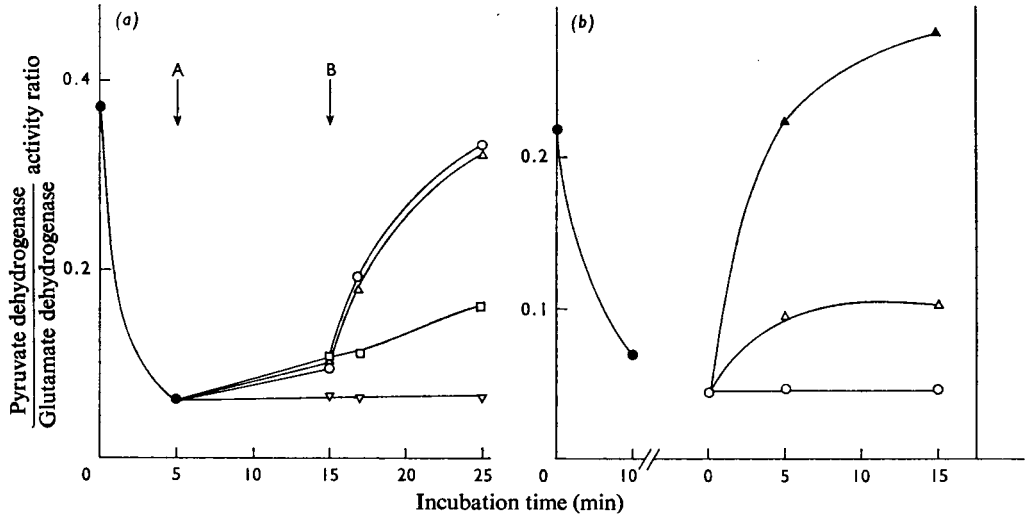

Fig. 4. *Effects of treatment with A23187 on pyruvate dehydrogenase activity in fat-cell mitochondria*

(*a*) Mitochondria (approx. 1mg of protein) were incubated in medium [KCl (125mM), Tris–HCl (20mM), potassium phosphate (2mM), EGTA (5mM), pH7.4] containing 2-oxoglutarate (5mM) and L-malate (0.5mM) at 30°C (●). After 5min, at point A the following additions were made to 1ml samples of mitochondria: ▽, A23187 (5μM); □, A23187 (5μM) plus $MgCl_2$ (2mM); △, $MgCl_2$ (2mM); ○, none. After 10min, at point B pyruvate (1mM) was added to all samples. (*b*) Mitochondria were preincubated (●) in the KCl medium described above, also containing 2-oxoglutarate (5mM), L-malate (0.5mM) and A23187 (5μM), for 10min at 30°C. After centrifugation the mitochondria were then resuspended in KCl (125mM)–Tris–HCl (20mM), pH7.4, containing pyruvate (2mM), L-malate (0.5mM), EGTA (40μM), together with the following: ○, none; △, $MgCl_2$ (2mM); ▲, $MgCl_2$ (2mM) plus $CaCl_2$ (0.1mM).

Table 5. *Effects of $CaCl_2$ and $MgCl_2$ on the activity of pyruvate dehydrogenase in A23187-treated fat-cell mitochondria under various conditions*

Mitochondria were preincubated with A23187 as described in the legend to Fig. 4(*b*), centrifuged and resuspended in KCl (125mM), Tris–HCl (20mM), pH7.4, containing EGTA (40μM), A23187 (5μM) and other additions as indicated and incubated for 0, 4 or 10min at 30°C. Results presented in each experiment were obtained with a different preparation of mitochondria.

Expt. no.	Additions to mitochondrial incubation medium	Period of incubation (min)	Mitochondrial pyruvate dehydrogenase/glutamate dehydrogenase activity ratio after incubation in the presence of: 0mM-$CaCl_2$	0.1mM-$CaCl_2$	1.0mM-$CaCl_2$
1	None	0	0.025		
	Pyruvate, malate	10	0.016	0.019	0.020
	Pyruvate, malate, $MgCl_2$ (1mM)	10	0.135	0.246	0.230
2	None	0	0.054		
	None	4	0.064		
	$MgCl_2$ (1mM)	4	0.183	0.380	
	Pyruvate, malate	4	0.047		
	Pyruvate, malate, $MgCl_2$ (1mM)	4	0.115	0.242	
3	None	0	0.039		
	Pyruvate, malate	4	0.016		
	Pyruvate, malate, CCCP* (10μM)	4	0.025		
	Pyruvate, malate, $MgCl_2$ (1mM)	4	0.067	0.230	
	Pyruvate, malate, CCCP* (10μM), $MgCl_2$ (1mM)	4	0.110	0.263	
4	None	0	0.051		
	None	4	0.044	0.044	
	$MgCl_2$ (0.5mM)	4	0.093	0.263	
	$MgCl_2$ (1mM)	4	0.188	0.231	
	$MgCl_2$ (2mM)	4	0.176	0.257	
	$MgCl_2$ (5mM)	4	0.173	0.293	
	$MgCl_2$ (10mM)	4	0.210	0.306	

* Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

some increase in pyruvate dehydrogenase activity was seen. However, the full rate of increase in pyruvate dehydrogenase activity was only observed if both $MgCl_2$ (2mM) and $CaCl_2$ (0.1mM) were added. Thus the phosphatase within the mitochondrion does appear to be sensitive to activation by $CaCl_2$. This activation by $CaCl_2$ is not apparent in the absence of $MgCl_2$, is perhaps a little less marked at high $MgCl_2$ (10mM), and is maximal when 100μM-$CaCl_2$ is added ($CaCl_2$ concentration in the medium would be about 60μM) (Table 5, Expts. 1 and 4). Pyruvate and L-malate were included in the incubation medium in initial experiments to provide an energy source for mitochondrial Ca^{2+} uptake in addition to inhibiting the pyruvate dehydrogenase kinase. However, it became clear that Ca^{2+} was not taken up by an energy-dependent process in the A23187-treated mitochondria, as activation was observed in the presence of uncoupler and was apparently accelerated in the absence of substrate (Table 5, Expts. 2 and 3). Indeed, the A23187-treated mitochondria incubated in the absence of phosphate contained little ATP (even in the presence of pyruvate and malate the concentration of ATP was less than 25% of that of the untreated mitochondria incubated with 2-oxoglutarate and L-malate or pyruvate and L-malate in the presence of phosphate). Further, preliminary experiments indicated that Ruthenium Red did not inhibit the uptake of Ca^{2+} by A23187-treated mitochondria incubated without phosphate. Thus it is to be expected that the intramitochondrial concentrations of Ca^{2+} and Mg^{2+} in experiments with A23187 will be no greater than their concentrations in the incubation medium.

In some experiments (Table 5, Expts. 2, 3 and 4) samples of the extracts of mitochondria were incubated with pig heart pyruvate dehydrogenase phosphate phosphatase (1.5 units/ml) in the presence of $MgCl_2$ (25mM) and $CaCl_2$ (1mM) for 10min to convert all the pyruvate dehydrogenase phosphate in the extract into the active form. No differences in total activity were found before and after treatment with A23187 or before and after incubation under the various conditions used in these experiments (total activity as ratio of pyruvate dehydrogenase/glutamate dehydrogenase was 0.55 ± 0.02; mean$\pm$S.E.M. for 21 observations).

Table 6. *Effects of $NiCl_2$ and $SrCl_2$ on the activation by $CaCl_2$ and $MgCl_2$ of pyruvate dehydrogenase activity in A23187-treated mitochondria*

Mitochondria were preincubated with A23187 as described in the legend to Fig. 4(*b*), centrifuged and resuspended in KCl (125mM), Tris–HCl (20mM), pH7.4, containing EGTA (40μM), A23187 (5μM) and other additions as indicated. Enzyme activities were measured after incubation at 30°C for 4min. Results were obtained with a single preparation of mitochondria.

Other additions to incubation medium	Pyruvate dehydrogenase/glutamate dehydrogenase activity ratio in the presence of: No addition	$SrCl_2$ (1mM)	$NiCl_2$ (1mM)
None	0.026	0.032	0.026
$MgCl_2$ (1mM)	0.102	0.222	0.026
$MgCl_2$ (1mM) plus $CaCl_2$ (0.1mM)	0.193	0.210	0.026

The effects of $NiCl_2$ and $SrCl_2$ on the activity of pyruvate dehydrogenase in the A23187-treated mitochondria are shown in Table 6. Neither could replace $MgCl_2$, but $SrCl_2$ mimicked the effect of $CaCl_2$ and $NiCl_2$ antagonized the action of both $MgCl_2$ alone and of $CaCl_2$ plus $MgCl_2$.

In summary, the metal-ion requirement of the phosphatase within mitochondria appears to be very similar to that of the isolated enzyme in spite of the much higher concentration of pyruvate dehydrogenase phosphate in mitochondria. The phosphatase in mitochondria requires Mg^{2+} for activity and is further activated by Ca^{2+}; Sr^{2+} will replace Ca^{2+} and Ni^{2+} is a potent inhibitor.

The depletion of total calcium which occurs during incubation of mitochondria with EGTA is not sufficient to inhibit the phosphatase (Fig. 3*a*), despite the fact that over 60% of the total calcium content is lost (Table 4). On the other hand, the residual total calcium and magnesium concentrations of A23187-treated mitochondria are approx. 5 and 16mM respectively (assuming that the metal ions are freely distributed in an intramitochondrial space of approx. 1μl/mg of mitochondrial protein). Apparently Mg^{2+} and Ca^{2+} are not available to pyruvate dehydrogenase phosphate phosphatase at these concentrations, since the phosphatase is inactive after A23187 treatment, and can be reactivated by addition of only 100μM-$CaCl_2$ and 1mM-$MgCl_2$ to the extramitochondrial incubation medium.

Effects of insulin on total pyruvate dehydrogenase activity and pyruvate dehydrogenase phosphate phosphatase activity in epididymal fat-pads

Previous studies have indicated that the increase in pyruvate dehydrogenase activity observed after brief exposure of adipose tissue to insulin is solely the result of an increase in the proportion of pyruvate dehydrogenase in the active non-phosphorylated form and does not involve any change in the total amount of the complex (Weiss *et al.*, 1971; Coore *et al.*, 1971). Further evidence in support of this is given in Table 7. In these experiments the total activity of pyruvate dehydrogenase in extracts was measured after incubation of the extracts with pig heart pyruvate dehydrogenase phosphatase in the presence of Mg^{2+} (25mM-$MgCl_2$) and Ca^{2+} (1mM-$CaCl_2$) for 15min at 30°C. Sufficient phosphatase activity was added so that complete activation was obtained well within this time (5min being sufficient). No change in the total activity of the enzyme as a result of insulin action was found in extracts of tissues incubated for 5 or 20min in the presence and absence of the hormone. In tissues incubated for 20min, insulin more than doubled the concentration of the active form of pyruvate dehydrogenase; after 5min exposure to insulin the increase was less than 30%.

We have used two approaches to measure pyruvate dehydrogenase phosphate phosphatase in fat-pads (Table 7). In the first approach, extracts were prepared from tissues incubated for 5min in the presence or absence of insulin. The increase in pyruvate dehydrogenase activity in these extracts during a subsequent 5min incubation at 30°C with addition of $MgCl_2$ and $CaCl_2$ was taken as a measure of the activity of the phosphatase in the extract acting on endogenous adipose-tissue pyruvate dehydrogenase phosphate in the extract. The extracts were prepared from tissue exposed to insulin for only 5min, so that differences in the concentration of the substrate (pyruvate dehydrogenase phosphate) were minimized. No effect of insulin on the activity of the phosphatase was found by this technique. In the second approach, phosphatase activity was measured by using exogenous substrate, namely pig heart pyruvate dehydrogenase [^{32}P]phosphate. Activity was measured with 25mM-$MgCl_2$ and with either EGTA (8mM) or CaEGTA (8mM-EGTA–7.8mM-$CaCl_2$) in extracts of pads incubated with or without insulin for 5 or 20min. Again no effects of insulin were found. There was no evidence of any marked change in the sensitivity of the phosphatase to Ca^{2+} after insulin treatment.

Our findings given in Table 7 appear to conflict directly with those reported by Sica & Cuatrecasas (1973). These authors presented evidence that exposure of fat-pads to insulin increased the total activity of pyruvate dehydrogenase and also increased the activity of pyruvate dehydrogenase phosphate phosphatase. No such changes have been detected in our experiments. It should be pointed out that Sica & Cuatrecasas (1973) did not add exogenous phosphatase in the measurement of total pyruvate dehydrogenase activity, and hence conversion into the active

Table 7. *Effect of insulin on activity of pyruvate dehydrogenase and pyruvate dehydrogenase phosphate phosphatase in rat epididymal fat-pads*

Fat-pads (in paired groups of three) were preincubated for 30min at 37°C in bicarbonate-buffered medium containing fructose (2mg/ml) and then incubated in fresh medium containing fructose (2mg/ml) with or without insulin (5munits/ml) for 5 or 20min. Pads were frozen and extracted as described in the Experimental section. Pyruvate dehydrogenase activity was measured in the initial extract and also after incubation of the extract at 30°C with pig heart pyruvate dehydrogenase phosphate phosphatase (1 unit/ml), $MgCl_2$ (25mM) and $CaCl_2$ (1mM) for 15min to give total activity. (Conversion into the active form was complete within 5min; no change in activity was observed if the extracts were incubated without addition for 15min.) Pyruvate dehydrogenase phosphate phosphatase activity was assayed by using exogenous pig heart pyruvate dehydrogenase [^{32}P]phosphate in the absence of added Ca^{2+} or in the presence of 7μM-Ca^{2+} as described in the Experimental section. Phosphatase activity was also assayed as the increase in pyruvate dehydrogenase activity derived from endogenous pyruvate dehydrogenase phosphate caused by incubation of tissue extracts with $MgCl_2$ (25mM) and $CaCl_2$ (1mM) for 5min at 30°C. All results are given as mean±S.E.M. of four observations.

	Expt. 1		Expt. 2			
Additions to incubation medium ...	None	Insulin	None	Insulin	None	Insulin
Time of incubation ...	20min	20min	5min	5min	20min	20min
Pyruvate dehydrogenase activity (munits/g wet wt. of tissue)						
On extraction	78 ± 6.8	203 ±12.4*	89 ± 5.4	117 ± 9.1	78 ±10.3	162 ± 4.5*
Total activity (after treatment of extract with phosphatase+ Mg^{2+}+Ca^{2+})	330 ±55	353 ±51	429 ±33	441 ±15	451 ±11	446 ±15
Pyruvate dehydrogenase phosphate phosphatase activity						
Assayed with exogenous pyruvate dehydrogenase phosphate (μunits/g wet wt. of tissue)						
In the absence of Ca^{2+}	32.3± 1.6	31.3± 2.0	38.9± 2.3	39.8± 2.2	40.0± 2.4	36.5± 1.0
In the presence of Ca^{2+}	69.3± 2.0	62.0± 9.1	65.3± 3.3	67.7± 4.0	72.3± 5.1	64.8± 1.8
Assayed with endogenous pyruvate dehydrogenase phosphate [as the increase in pyruvate dehydrogenase activity (munits/g wet wt. of tissue) on incubation of tissue extract for 5min with $MgCl_2$ +$CaCl_2$]	—	—	26.3± 3.2	28.3± 5.4	—	—

* $P<0.01$ versus appropriate control value.

form may not have been complete in their experiments. In the measurement of phosphatase activity by Sica & Cuatrecasas (1973), a small amount of exogenous rat adipose-tissue pyruvate dehydrogenase phosphate (less than 0.01μM) was added to extracts prepared from tissue exposed to insulin for 60min. Phosphatase activity was taken as the increase in pyruvate dehydrogenase activity derived from the added pyruvate dehydrogenase phosphate during 20min incubation in the presence of about 8mM-$MgCl_2$ and unknown Ca^{2+} (neither EGTA nor $CaCl_2$ was added); allowance was made for the increase in the activity seen in the absence of added pyruvate dehydrogenase phosphate. The concentration of pyruvate dehydrogenase phosphate in the extracts appears to have been comparable with that added, and of course the concentrations would have been less in those extracts prepared from tissue exposed to insulin. This difference, or the low concentrations of Mg^{2+} and Ca^{2+} or the lengthy assay time, may all have contributed to the finding of an apparent increase in phosphatase activity with insulin.

These experiments (Table 7) show no evidence for an action of insulin in adipose tissue leading to the conversion of the phosphatase into a form which retains increased activity after extraction under the conditions used. Moreover, there was no evidence for any gross change in Ca^{2+} requirement as a result of insulin action. These findings provide no conclusive evidence for or against the possibility that insulin may activate the phosphatase through changes in the concentrations of an effector, such as Ca^{2+}, though they are compatible with such a mechanism.

Table 8. *Effect of Ruthenium Red on pyruvate dehydrogenase activity and glycerol output of rat epididymal fat-pads incubated with and without fructose or insulin*

Fat-pads (in paired groups of three) were preincubated for 30min in bicarbonate-buffered medium with or without Ruthenium Red (5μg/ml); in Expt. 1 fructose (2mg/ml) was also present. Pads were transferred to fresh medium containing, where indicated, fructose (2mg/ml), insulin (10munits/ml), Ruthenium Red (5μg/ml) and incubated for 30min. Results are given as the mean±S.E.M. for the number of observations given in parentheses.

	Pyruvate dehydrogenase activity (munits/g wet wt. of tissue)	Pyruvate dehydrogenase/glutamate dehydrogenase activity ratio	Glycerol output (μmol/h per g)
Expt. 1			
Fructose (control)	83± 9 (8)	0.112±0.019 (8)	0.95±0.09 (8)
Fructose plus insulin	158±12* (8)	0.193±0.024* (8)	0.51±0.03* (8)
Fructose plus Ruthenium Red	94± 8 (8)	0.127±0.012 (8)	0.76±0.04 (8)
Fructose plus Ruthenium Red plus insulin	102± 7 (8)	0.140±0.020 (8)	0.52±0.03* (8)
Expt. 2			
None (control)	16± 1 (4)	0.024±0.003 (4)	—
Fructose	58± 5* (4)	0.085±0.007* (4)	—
Ruthenium Red	18± 1 (4)	0.029±0.002 (4)	—
Fructose plus Ruthenium Red	61± 8* (4)	0.085±0.006* (4)	—

* $P<0.01$ versus appropriate control.

Table 9. *Effects of Ruthenium Red, $NiCl_2$, $MnCl_2$ and A23187 on the activation of rat epididymal fat-pad pyruvate dehydrogenase by insulin*

Fat-pads (in paired groups of three) were preincubated for 30min in bicarbonate-buffered medium containing fructose (2mg/ml) and then incubated in fresh medium containing fructose (2mg/ml) and other additions as indicated at the following concentrations: insulin (5–10munits/ml), Ruthenium Red (5–10μg/ml), $NiCl_2$ (1mM), $MnCl_2$ (0.6mM), A23187 (5μg/ml). Results are given as mean±S.E.M. for the number of observations given in parentheses.

Expt. no.	Additions to incubation medium		Pyruvate dehydrogenase activity (munits/g wet wt. of tissue)	Increase with insulin (%)
1	None	(28)	83± 5	69±15
	Insulin	(28)	137± 6*	
	Ruthenium Red	(28)	87± 5	16±12
	Ruthenium Red plus insulin	(28)	103± 5	
2	None	(8)	126±11	55±10
	Insulin	(8)	191±13*	
	$NiCl_2$	(8)	141±16	7± 5
	$NiCl_2$ plus insulin	(8)	150±10	
3	None	(3)	101±15	140±36
	Insulin	(3)	233± 7*	
	$MnCl_2$	(3)	133±10	29±21
	$MnCl_2$ plus insulin	(3)	168±13	
	A23187	(3)	88±11	168±14
	A23187 plus insulin	(3)	233±32*	

* $P<0.01$ versus appropriate control incubated without insulin.

Effects of Ruthenium Red, $NiCl_2$, $MnCl_2$ and A23187 on the activation of pyruvate dehydrogenase in fat-pads by insulin

Ruthenium Red inhibits the uptake of Ca^{2+} by mitochondria (see above) and may inhibit the transport of Ca^{2+} across the plasma membrane (Cittadini *et al.*, 1973). $NiCl_2$ and $MnCl_2$ may inhibit the uptake of Ca^{2+} into cells across the plasma membrane (Kohlhardt *et al.*, 1973). $NiCl_2$ is also an inhibitor of pyruvate dehydrogenase phosphate phosphatase in isolated mitochondria (see above), whereas $MnCl_2$ can substitute for $MgCl_2$ as an activator of this enzyme (Hucho *et al.*, 1972; Randle *et al.*, 1974). Because of the possibility that insulin may activate pyruvate dehydrogenase phosphate phosphatase by increasing mitochondrial Ca^{2+} it was decided to investigate effects of Ruthenium Red, $NiCl_2$ and

$MnCl_2$ on the activation of pyruvate dehydrogenase by insulin in fat-pads. Since A23187 may be expected to facilitate entry of Ca^{2+} into fat-cells, it was decided to investigate effects of the ionophore on pyruvate dehydrogenase activity in the presence and absence of insulin. These results are shown in Tables 8 and 9.

As shown in Table 8, Ruthenium Red can selectively block the activation by insulin of pyruvate dehydrogenase in fat-pads incubated in the presence of fructose. The antilipolytic effect of insulin, as measured by glycerol output, was not affected by Ruthenium Red, and nor was the activation of pyruvate dehydrogenase in fat-pads induced by fructose. This effect of Ruthenium Red on activation of pyruvate dehydrogenase by insulin has not been entirely consistent. Ruthenium Red inhibited the effect of insulin in only five out of nine experiments. When the results of all nine experiments were combined (in all there were 28 observations in each group) significant inhibition of the insulin effect by Ruthenium Red was evident (Table 9). The effect of insulin on pyruvate dehydrogenase in fat-pads was also inhibited by $NiCl_2$ and by $MnCl_2$ (Table 9). However, these compounds also increased pyruvate dehydrogenase in the absence of insulin in addition to decreasing activity in the presence of the hormone. The effect of $NiCl_2$ on the conversions of [U-^{14}C]glucose into fatty acids shows much the same pattern (K. de Rivaz, unpublished work in this laboratory). The ionophore A23187 had no effect on the activity of pyruvate dehydrogenase in fat-pads in the presence of insulin (Table 9).

Conclusions

The studies with phosphatase extracted from adipose tissue have shown that the enzyme required Mg^{2+} and that in the presence of Mg^{2+} the enzyme is activated by Ca^{2+} or by Sr^{2+}. With both Mg^{2+} and Ca^{2+} the enzyme is inhibited by Ni^{2+}. The K_m values were 0.5mM (for Mg^{2+}), 1μM (for Ca^{2+}) and 3μM (for protein-bound phosphate in pyruvate dehydrogenase phosphate at saturating [Mg^{2+}] and [Ca^{2+}]). The properties of the adipose-tissue phosphatase are thus qualitatively and quantitatively similar to those of the pig heart enzyme (Randle *et al.*, 1974).

The metal-ion requirements of the phosphatase in intact fat-cell mitochondria have been studied by using the bivalent-metal ionophore A23187 to deplete mitochondria of total magnesium and calcium. These studies have shown that the phosphatase in mitochondria acting on its physiological substrate is sensitive to both Mg^{2+} and Ca^{2+}, that Sr^{2+} can substitute for Ca^{2+} and that Ni^{2+} is inhibitory. These properties of the intramitochondrial phosphatase are qualitatively similar to those of the extracted enzyme. Also the results appear to show that it is intramitochondrial Mg^{2+} and Ca^{2+} to which the enzyme is sensitive.

Detailed studies with pig heart pyruvate dehydrogenase phosphatase have shown that activation by Ca^{2+} is complex. It is dependent on Mg^{2+} and results in a lowering of the K_m for pyruvate dehydrogenase phosphate (Randle *et al.*, 1974) and of the K_m for Mg^{2+} (Denton *et al.*, 1972; H. T. Pask, P. J. Randle & R. M. Denton, unpublished work). Studies with fat-cell mitochondria (Table 5) have now shown that the effects of Ca^{2+} on intramitochondrial phosphatase require Mg^{2+} and have also suggested that Ca^{2+} may lower the K_m of the phosphatase for Mg^{2+}. Thus higher extramitochondrial Mg^{2+} was required for intramitochondrial phosphatase activity in the absence of Ca^{2+} (1mM-$MgCl_2$) than in the presence of 0.1mM-$CaCl_2$ (0.5mM-$MgCl_2$) (Table 5, Expt. 4). These studies with mitochondria depleted of total magnesium or calcium do not indicate whether Ca^{2+} affects the K_m of the phosphatase for its substrate in the mitochondria. They do, however, show that the mitochondrial concentration of pyruvate dehydrogenase phosphate is not so high that the phosphatase is insensitive to Ca^{2+}.

The present studies have also confirmed that fat-cell mitochondria, as isolated, contain sufficient Mg^{2+} and Ca^{2+} to support phosphatase activity (Martin *et al.*, 1972). This is still the case if fat-cells are broken in the presence of EGTA or EGTA and Ruthenium Red (D. L. Severson, R. M. Denton & P. J. Randle, unpublished work) to minimize uptake of Ca^{2+} by mitochondria during their isolation. Analyses of mitochondrial total calcium in the present study have shown that removal of 60% of mitochondrial calcium by incubation *in vitro* with EGTA does not materially influence the activity of the phosphatase. The mitochondrial phosphatase does, however, become totally dependent on extramitochondrial $CaCl_2$ when 94% of mitochondrial total calcium is removed by incubation with A23187. These experiments with isolated mitochondria may therefore show that Ca^{2+} is an essential cofactor for the phosphatase, but they have not provided evidence that Ca^{2+} is a regulator of phosphatase activity. Such negative findings do not exclude the possibility that Ca^{2+} can regulate in the intact cell, for it is possible that the very active uptake system in fat-cell mitochondria may lead to the incorporation of substantial amounts of Ca^{2+} during their isolation in spite of the precautions that have been taken.

Our interest in the properties of pyruvate dehydrogenase phosphate phosphatase is particularly concerned with the possibility that regulation of this enzyme may contribute to activation of pyruvate dehydrogenase by the action of insulin in adipose tissue. We have found no evidence with insulin for the conversion of the phosphatase into a form that is more active in extracts of the tissue. Indirect evidence that insulin may activate the phosphatase by increasing intramitochondrial [Ca^{2+}] is afforded by the

inhibitory effects of Ruthenium Red, $NiCl_2$ and $MnCl_2$. These agents diminished the action of insulin on adipose-tissue pyruvate dehydrogenase. The question as to whether these agents act by diminishing incorporation of Ca^{2+} into mitochondria in fat-cells can only be resolved by direct measurements.

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