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Role of Ca^{2+} ions in the regulation of intramitochondrial metabolism in rat epididymal adipose tissue

Evidence against a role for Ca^{2+} in the activation of pyruvate dehydrogenase by insulin

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1. The sensitivity of rat epididymal-adipose-tissue pyruvate dehydrogenase phosphate phosphatase, NAD^+–isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase to Ca^{2+} ions was studied both in mitochondrial extracts and within intact coupled mitochondria. It is concluded that all three enzymes may be activated by increases in the intramitochondrial concentration of Ca^{2+} and that the distribution of Ca^{2+} across the mitochondrial inner membrane is determined, as in rat heart mitochondria, by the relative activities of a uniporter (which transports Ca^{2+} into mitochondria and is inhibited by Mg^{2+} and Ruthenium Red) and an antiporter (which allows Ca^{2+} to leave mitochondria in exchange for Na^+ and is inhibited by diltiazem). 2. Previous studies with incubated fat-cell mitochondria have indicated that the increases in the amount of active non-phosphorylated pyruvate dehydrogenase in rat epididymal tissue exposed to insulin are the result of activation of pyruvate dehydrogenase phosphate phosphatase. In the present studies, no changes in the activity of the phosphatase were found in extracts of mitochondria, and thus it seemed likely that insulin altered the intramitochondrial concentration of some effector of the phosphatase. 3. Incubation of rat epididymal adipose tissue with medium containing a high concentration of $CaCl_2$ (5mM) was found to increase the active form of pyruvate dehydrogenase to much the same extent as insulin. However, the increases caused by high $[Ca^{2+}]$ in the medium were blocked by Ruthenium Red, whereas those caused by insulin were not. Moreover, whereas the increases resulting from both treatments persisted during the preparation of mitochondria and their subsequent incubation in the absence of Na^+, only the increases caused by treatment of the tissue with insulin persisted when the mitochondria were incubated in the presence of Na^+ under conditions where the mitochondria are largely depleted of Ca^{2+}. 4. It is concluded that insulin does not act by increasing the intramitochondrial concentration of Ca^{2+}. This conclusion was supported by finding no increases in the activities of the other two Ca^{2+}-responsive intramitochondrial enzymes (NAD^+–isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase) in mitochondria prepared from insulin-treated tissue compared with controls.

Studies, mainly with rat heart, have indicated that Ca^{2+} may be an important regulator of intramitochondrial oxidative metabolism in vertebrate tissues (see Denton & McCormack, 1980, 1981). PDHP phosphatase, NAD^+–isocitrate dehydrogenase and the 2-oxoglutarate dehydrogenase complex are all activated by Ca^{2+} with half-maximal effects ($k_{0.5}$ value) at about 1 μM (Denton *et al.*, 1972, 1978*a*; McCormack & Denton, 1979, 1981*b*). Parallel increases in PDH_a and 2-oxoglutarate dehydrogenase activity can be demonstrated within coupled rat heart mitochondria exposed to increases in the extramitochondrial concentration

Abbreviations used: PDH, pyruvate dehydrogenase; PDHP phosphatase, pyruvate dehydrogenase phosphate phosphatase; PDH_a, the active, non-phosphorylated, form of pyruvate dehydrogenase; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $k_{0.5}$ value, the concentration of effector required for a half-maximal response.

of Ca^{2+} (Denton *et al.*, 1980; Hansford, 1981; Hansford & Castro, 1981; McCormack *et al.*, 1982). The addition of either Mg^{2+}, which inhibits the uptake of Ca^{2+} into mitochondria (Crompton *et al.*, 1976), or Na^+, which allows the efflux of Ca^{2+} from heart mitochondria by a Ca/Na translocase (Crompton *et al.*, 1977, 1978), was shown to increase the concentration of extramitochondrial Ca^{2+} required to obtain half-maximal activation of both enzymes. In the presence of both Na^+ and Mg^{2+}, half-maximal activation was observed at about 0.5 μM-Ca^{2+}, and thus it could be argued that changes in cytoplasmic [Ca^{2+}] within the physiological range (see Marban *et al.*, 1980) would be expected to result in changes in intramitochondrial metabolism in the intact heart (Denton *et al.*, 1980). These findings led us to propose that hormones and neurotransmitters which act by increasing the concentration of cytoplasmic Ca^{2+} could also influence intramitochondrial oxidative metabolism through increases in the intramitochondrial concentration of Ca^{2+}, and hence activation of the above enzymes (Denton & McCormack, 1980, 1981). Strong evidence for this view has been obtained in subsequent studies on the effects of β-adrenergic agonists on the rat heart (McCormack & England, 1983; McCormack & Denton, 1984).

The stimulation of fatty acid synthesis caused by the administration of insulin to mammalian tissues is brought about, in part, by an increase in PDH_a (see Wieland, 1983; Denton & Brownsey, 1983). In rat epididymal adipose tissue this increase is 2–3-fold and is complete within 5 min (Jungas, 1970; Denton *et al.*, 1971; Weiss *et al.*, 1971; Denton & Hughes, 1978). The effect persists during the preparation and subsequent incubation of mitochondria under appropriate conditions (Severson *et al.*, 1976; Denton *et al.*, 1984). This persistence greatly simplifies the study of the mechanism whereby insulin might bring about its effect on the PDH system. Previous work has suggested that insulin action involves an increase in PDHP phosphatase activity rather than inhibition of PDH kinase (Hughes & Denton, 1976; Denton *et al.*, 1984). The only known regulators of PDHP phosphatase are Ca^{2+}, Mg^{2+} and possibly changes in the [NAD^+]/[NADH] ratio (Denton *et al.*, 1972; Hucho *et al.*, 1972; Siess & Wieland, 1972; Pettit *et al.*, 1975). Since no changes in either Mg^{2+} or [NAD^+]/[NADH] ratio are found in mitochondria prepared from insulin-treated tissue compared with controls, the possibility that insulin's effects on PDH_a may be brought about by an increase in intramitochondrial Ca^{2+} concentration must be considered (Denton *et al.*, 1978*b*, 1984). Two recent observations have increased the plausibility of this possible mechanism of insulin action on PDH_a. Firstly, it was shown that increasing the concentration of Ca^{2+} in incubation media leads to increases in PDH_a in fat-pads which are comparable with those observed with insulin (results reported in the present paper) and secondly, it became apparent that changes in intramitochondrial [Ca^{2+}] occurring in the intact rat heart owing to exposure to β-adrenergic agonists or high Ca^{2+} concentration in the medium could persist during the preparation and subsequent incubation of mitochondria (McCormack & Denton, 1984). It was realized that if insulin were to increase intramitochondrial Ca^{2+} concentration, then it was unlikely to be secondary to an increase in the cytoplasmic concentration of Ca^{2+}, since insulin does not, in general, result in intracellular effects such as the activation of glycogen breakdown, which would be expected to follow such an increase (see Denton *et al.*, 1981).

Experimental

The details for the incubation of epididymal adipose tissue (from Wistar rats, 170–220 g) are given by Denton *et al.* (1984), except for the instances, indicated in Table legends, where the concentration of $CaCl_2$ in the medium was changed from 1.25 to either 0 or 5 mM. Denton *et al.* (1984) also described the methods used for the preparation of white-adipose-tissue mitochondria and their subsequent incubation in KCl-based medium, followed by their sedimentation for later analysis.

Mitochondria were extracted and assayed for NAD^+–isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase complex activities as described by Denton *et al.* (1978*a*) and McCormack & Denton (1979) respectively. The extraction of mitochondria for the assay of PDHP phosphatase was described by McCormack & Denton (1980); this enzyme was assayed in the incubation media described by McCormack & Denton (1980) (i) by using added pig heart PDH [^{32}P]phosphate (as in McCormack & Denton, 1980) or (ii) by monitoring the increases in PDH_a derived from endogenous PDH phosphate. Mitochondrial samples were extracted and assayed for both the amount of PDH_a and the total amount of PDH present, as described by McCormack & Denton (1980); tissue samples for PDH assays were extracted as described by Stansbie *et al.* (1976) and assayed as described above. A unit of enzyme activity is defined as the amount of enzyme that transforms 1 μmol of substrate/min at 30°C, except for PDHP phosphatase, where units are calculated in terms of μmol of P_i released/min at 30°C.

Estimates of the activities of NAD^+–isocitrate dehydrogenase and the 2-oxoglutarate dehydrogenase complex within intact coupled mito-

chondria were obtained by monitoring oxygen uptake under the incubation conditions given in Figure and Table legends (Denton *et al.*, 1980). Mitochondria were pre-loaded with ^{45}Ca, and the subsequent rates of ^{45}Ca efflux from re-isolated mitochondria were measured by using methods developed by McCormack & Denton (1984) for rat heart mitochondria.

Mitochondrial ATP and protein contents were measured as described by Denton *et al.* (1984).

Ca–EGTA buffers were prepared and used as described by Denton *et al.* (1978*a*). Results are expressed and analysed as described by Denton *et al.* (1984).

Sources of materials are as for Denton *et al.* (1984), except for Ruthenium Red, which was obtained from Sigma, Poole, Dorset, U.K., diltiazem, which was kindly given by Dr. G. Satzinger of Goedecke A.G., 78 Freiburg, Germany, and $^{45}CaCl_2$, which was obtained from Amersham International, Amersham, Bucks., U.K.

Results and discussion

Effects of Ca^{2+} on the activities of PDHP phosphatase, NAD^+-isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase in extracts of mitochondria from rat epididymal adipose tissue

Previous studies have shown that PDHP phosphatase activity in extracts of mitochondria from rat epididymal adipose tissue measured with pig heart PDH phosphate as substrate is activated by Ca^{2+}, with a $k_{0.5}$ value close to 1 μM (Denton *et al.*, 1972; Severson *et al.*, 1974; McCormack & Denton, 1980; Table 1). A similar value was obtained during the course of the present studies, in which PDHP phosphatase activities in extracts of mitochondria from control and insulin-treated tissue were compared (see below).

As expected on the basis of previous studies on other rat tissues (see Denton & McCormack, 1980), the K_m values of NAD^+-isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase for their appropriate substrates were markedly decreased by Ca^{2+}, without any appreciable effects on the relevant $V_{max.}$ values (Table 1). Kinetic constants, measured at 30°C in 50mM-Mops (4-morpholinepropanesulphonic acid) buffer (pH 7.2) containing 1mM-NAD^+, 1mM-ADP, 1mM-dithiothreitol and rotenone (2μg/ml), were very similar to those found previously for the two enzymes in extracts of mitochondria from rat interscapular brown adipose tissue and rat heart, assayed under similar conditions (McCormack & Denton, 1980; Denton *et al.*, 1980; McCormack & Denton, 1981*b*). In particular, the calculated $k_{0.5}$ values for Ca^{2+} activation (measured at 0.1mM-*threo*-D_s-isocitrate and

Table 1. *Summary of $k_{0.5}$ values obtained for the effects of Ca^{2+} on the Ca^{2+}-sensitive intramitochondrial enzymes in (i) mitochondrial extracts and (ii) intact mitochondria incubated under a variety of conditions: comparison of values obtained with mitochondria from rat epididymal adipose tissue with those from rat heart*

Mitochondria from rat epididymal adipose tissue prepared as described in Fig. 1 were then either (i) extracted for the assay of the indicated enzymes as described in the Experimental section or (ii) incubated with the appropriate additions as for Figs. 1(*a*)-1(*c*) for the assay of the same enzymes within intact mitochondria. Where FCCP was present, no 2-oxoglutarate or malate was added, and in the case of the adipose tissue only, 5μM-A23187 and 1μM-valinomycin were also present. Any further additions or changes were as indicated. Where appropriate, results are given as $k_{0.5}$ values, ±S.D. for the numbers of degrees of freedom in parentheses; these were calculated from measurements made over a suitable range of free Ca^{2+} concentrations on samples from at least two different preparations of mitochondria. Other values were taken from: *Denton *et al.* (1980); †McCormack & Denton (1980); ‡Denton *et al.* (1975); §McCormack & Denton (1981*b*). Abbreviation: N.A., not assayed.

Parameter	Additions or changes to incubation media	$k_{0.5}$ values (nM) in samples prepared from: Epididymal adipose tissue	Heart
(*a*) (i) PDHP phosphatase activity	–	1080†	~1000‡
(ii) PDH_a content	None	33±2.4 (42)	39*
	10mM-NaCl	93±13.1 (39)	189*
	1mM-$MgCl_2$	79±6.2 (9)	175*
	10mM-NaCl plus 1mM-$MgCl_2$	419±30.3 (14)	464*
	1μM-FCCP plus 5mM-ATPMg plus 5μM-oligomycin	540†	980*
(*b*) (i) NAD^+-isocitrate dehydrogenase activity	–	1020±130 (15)	1120§
(ii) Oxidation of 50μM-*threo*-D_s-isocitrate	None	36±7.9 (14)	N.A.
	10mM-NaCl	59±7.9 (14)	N.A.
(*c*) (i) 2-Oxoglutarate dehydrogenase complex activity	–	234±64 (10)	160*
(ii) Oxidation of 100μM-2-oxoglutarate	None	13±1.4 (30)	21*
	10mM-NaCl	18±0.8 (30)	82*

Table 2. *PDHP phosphatase activity of mitochondria prepared from control and insulin-treated rat epididymal adipose tissue*
Mitochondria were prepared from paired fat-pads incubated (as in Fig. 1) with or without 50 nM-insulin. Mitochondria were incubated at 30°C for 5 min in KCl-based media containing 5 mM-2-oxoglutarate, 0.2 mM-malate, 2 mM-potassium phosphate and 5 mM-EGTA and then sedimented and rapidly frozen for the subsequent extraction and assay of (*a*) PDH_a (and total PDH activity) and (*b*) PDHP phosphatase activity (with other conditions as indicated) (see the Experimental section for further details). Results in (i) are given as means ± S.E.M. or $k_{0.5}$ values ± S.D. (calculated from observations made over a suitable range of six different Ca^{2+} concentrations) and are derived from either the number of observations made on separate preparations of mitochondria from paired control and insulin-treated tissue (in parentheses), or the number of degrees of freedom [in square brackets]. Results in (ii) are taken from a single experiment; similar results have been obtained on two other pairs of mitochondrial preparations. *$P \leqslant 0.01$ for the effect of insulin pre-treatment compared with the appropriate control value.

		Parameter values derived from mitochondria prepared from:	
Enzyme	Parameter or condition	Control tissue	Insulin-treated tissue
(i)(*a*) PDH_a content (as % of total activity)		19 ± 1.3 (4)	32 ± 2.3 (4)*
(*b*) PDHP phosphatase activity (assayed as release of $[^{32}P]P_i$ from added pig heart PDH [^{32}P]phosphate)	With 5 mM-EGTA (free $[Ca^{2+}] < 1$ nM) as μunits/unit of total PDH activity)	108 ± 21 (4)	117 ± 14 (4)
	Response to Ca^{2+}:		
	$k_{0.5}$ value (μM)	1.2 ± 0.06 [24] (4)	1.04 ± 0.07 [24] (4)
	$V_{max.}$ (μunits/unit of total PDH activity)	287 ± 11.4 [24] (4)	296 ± 16.2 [24] (4)
(ii)(*a*) PDH_a content (as % of total activity)		18	30
(*b*) PDHP phosphatase activity [assayed as increase in endogenous PDH_a (as % of total activity)] in extracts incubated with:	5 mM-EGTA (free $[Ca^{2+}] < 1$ nM) for:		
	2 min	7	5
	5 min	19	26
	5 mM-EGTA plus 5 mM-$CaCl_2$ (free $[Ca^2] \simeq 20$ μM) for:		
	2 min	21	19
	5 min	52	51

0.1 mM-2-oxoglutarate respectively) were 1.02 and 0.23 μM respectively, compared with values of 1.12 and 0.16 μM for the enzymes from rat heart mitochondria (Table 1). Values obtained for the enzymes from rat brown-adipose-tissue mitochondria were 1.6 and 0.2 μM (McCormack & Denton, 1980).

The increases in the amount of PDH_a evident in incubated mitochondria prepared from insulin-treated fat-pads compared with controls appears to be the result of increases in PDHP phosphatase activity within the mitochondria (Hughes & Denton, 1976; Denton *et al.*, 1984). It was therefore important to establish whether or not increases in PDHP phosphatase activity persisted into extracts of mitochondria. Two techniques were used to assay PDHP phosphatase in extracts (Table 2). In the first, activity was followed by monitoring the release of $[^{32}P]P_i$ from fully phosphorylated pig heart PDH [^{32}P]phosphate (Denton *et al.*, 1972; McCormack & Denton, 1980). Although there were clear-cut increases in the amount of endogenous PDH_a in extracts of mitochondria from insulin-treated tissue, no changes in PDHP phosphatase activity were evident by using this technique, either when the enzyme was assayed in the presence of EGTA or when it was assayed at saturating concentrations of Ca^{2+} [Table 2(i)]. Moreover, the sensitivity to Ca^{2+} remained unaltered, at close to 1 μM [Table 2(i)]. The second technique was to follow the increases in PDH_a activity resulting from the dephosphorylation of the endogenous PDH phosphate present in mitochondrial extracts on incubation of the extracts in the presence of $MgCl_2$ with either EGTA (giving < 1 nM free Ca^{2+}) or EGTA plus $CaCl_2$ (giving about 20 μM free Ca^{2+}). Results of a single experiment, which was typical of three separate experiments, are given in Table 2(ii). This technique had the advantage that endogenous fat-pad PDH phosphate was used as substrate, but was less precise than following release of $[^{32}P]P_i$ from added pig heart PDH [^{32}P]phosphate. In particular, the initial concentrations of PDH phosphate

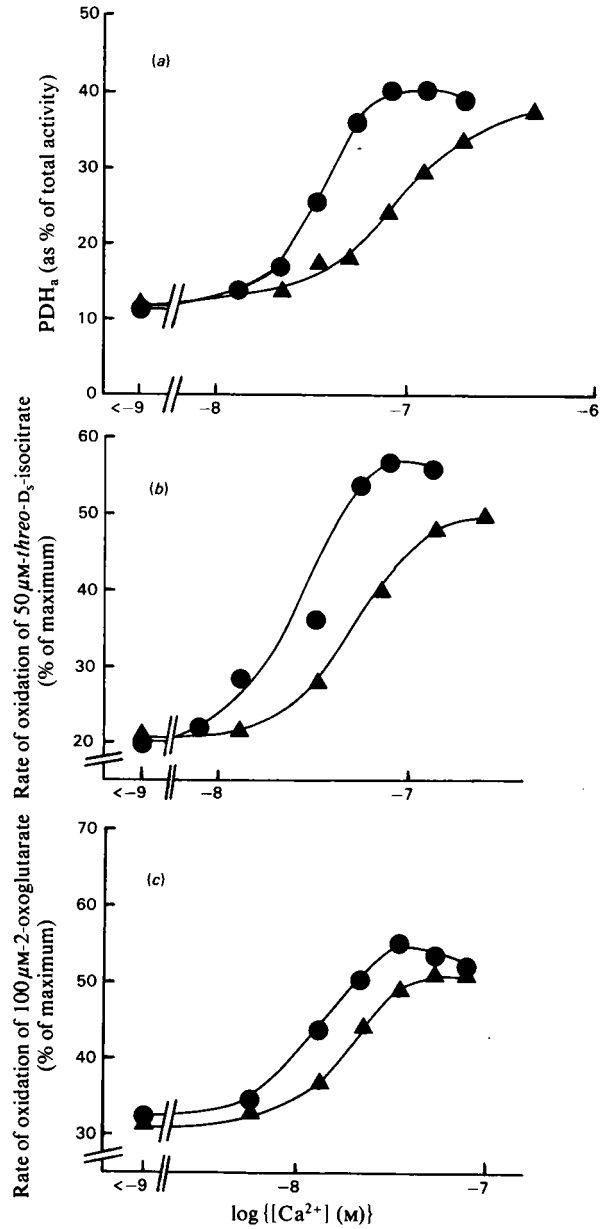

Fig. 1. *Effects of Na^+ on the sensitivity to extramitochondrial Ca^{2+} of (a) the amount of PDH_a in, and the rates of oxidation of non-saturating concentrations of (b) threo-D_s-isocitrate and (c) 2-oxoglutarate by, coupled mitochondria prepared from rat epididymal adipose tissue preincubated under control conditions*

Mitochondria, prepared from fat-pads preincubated at 37°C in gassed (O_2/CO_2, 19:1) bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing 10mM-glucose for 30min, were incubated (at about 0.5mg of protein/ml) at 30°C in KCl-based medium [125mM-KCl/20mM-Tris (pH7.3)/5mM-potassium phosphate] containing 5mM-EGTA, with additions of $CaCl_2$ to give the concentrations of free Ca^{2+} indicated, in either the absence (●) or presence (▲) of 10mM-NaCl. In (*a*), mitochondria were incubated in medium containing the further additions of 5mM-2-oxoglutarate and 0.2mM-malate for 5min before rapid sedimentation and freezing for the subsequent assay of both PDH_a and total PDH activity in extracts of the frozen mitochondria; PDH_a is expressed as a percentage of total PDH activity. In (*b*), mitochondria were incubated in media containing the further additions of 2mM-ADP and 1mM-malate, and then changes in O_2 uptake were monitored by following the additions of 50μM-*threo*-D_s-isocitrate after 2min and 2mM-*threo*-D_s-isocitrate after 4min; increases in O_2 uptake with the sub-saturating concentration (50μM) of *threo*-D_s-isocitrate are expressed as a percentage of those with the saturating concentration (2mM). In (*c*), the procedure was as in (*b*), but the additions of *threo*-D_s-isocitrate were replaced by 100μM- and 5mM-2-oxoglutarate respectively. Values given are means obtained from at least three different preparations of mitochondria in all cases: kinetic constants calculated from (*a*), (*b*) and (*c*) are summarized in Table 1.

were appreciably and unavoidably lower in extracts of mitochondria derived from tissue previously exposed to insulin. Again, no indication of any change in PDHP phosphatase activity was found [Table 2(ii)]. These results suggest that the apparent activation of PDHP phosphatase within intact mitochondria from insulin-treated tissue is probably the result of a change in the concentration of some effector of this enzyme (such as Ca^{2+}) which dissociates during the preparation of mitochondrial extracts.

Effects of Ca^{2+} on the amount of PDH_a in coupled mitochondria prepared from rat epididymal adipose tissue

Mitochondria were prepared from epididymal fat-pads previously incubated in the absence of insulin. As observed in previous studies (Denton *et al.*, 1984), incubation of these mitochondria at 30°C in KCl-based medium containing 2-oxoglutarate and malate as the oxidizable substrates, plus EGTA to lower the extramitochondrial concentrations of Ca^{2+} to below 1nM, resulted in PDH_a values corresponding to about 10% of total activity (Fig. 1*a*). On increasing the extramitochondrial concentration of Ca^{2+} by using EGTA buffers, the amount of PDH_a increased by up to about 4-fold. A comparable increase was observed in the presence of 10mM-NaCl, but higher concentrations of extramitochondrial Ca^{2+} were required (Fig. 1*a*). The calculated $k_{0.5}$ values for the effects of extramitochondrial Ca^{2+} were 33nM in the absence of Na^+, increasing to 93nM in the

Table 3. *Effects of Na^+ and diltiazem on the egress of ^{45}Ca from pre-loaded mitochondria*

Mitochondria were prepared from fat-pads incubated as described in Fig. 1 and were then preincubated (at about 4mg of protein/ml) at 30°C for 2min in KCl-based media (see Fig. 1) containing 5mM-2-oxoglutarate, 0.2mM-malate, approx. 100μM-EGTA and sufficient added $^{45}CaCl_2$ to cause appreciable activation of PDH (at approx. 2μCi/ml and 50μM). After this preincubation, 10vol. of ice-cold sucrose-based isolation medium [250mM-sucrose/20mM-Tris/HCl (pH7.4)/2mM-EGTA] was added and the mitochondria were sedimented and resuspended by the normal preparation method (Denton *et al.*, 1984). The 're-prepared' mitochondria were incubated at 30°C in KCl-based media (see Fig. 1) containing 5mM-2-oxoglutarate, 0.2mM-malate and 5mM-EGTA with further additions as indicated, and then ^{45}Ca content was determined after the times shown. The procedure was based on the method described by McCormack & Denton (1984) for rat heart mitochondria. Results are given as means or means ± S.E.M. for the numbers of observations, each made on different preparations of mitochondria, given in parentheses, and are expressed as the percentage of the ^{45}Ca present in unincubated mitochondria that was lost after incubation. $^*P \leqslant 0.01$ and $^{**}P \leqslant 0.001$ for the effects of Na^+ compared with the appropriate control values.

Additions	Percentage of initial ^{45}Ca content of mitochondria lost after incubation at 30°C for: 1 min	5 min
None (control)	33±6.0 (4)	50±6.0 (4)
10mM-NaCl	82±4.4 (4)**	89±1.3 (4)*
300μM-diltiazem	42 (2)	57 (2)
10mM-NaCl plus 300μM-diltiazem	48 (2)	63 (2)

presence of Na^+ (Table 1). The addition of 1mM-$MgCl_2$ also resulted in an increase in the $k_{0.5}$ value, whereas in the presence of both NaCl and $MgCl_2$ the $k_{0.5}$ value rose to over 400nM (Table 1). Total activity of pyruvate dehydrogenase was unaltered by any of these conditions and was about 80munits/mg of mitochondrial protein, in agreement with Denton *et al.* (1984). The mitochondrial concentration of ATP remained essentially constant (about 40nmol/mg of mitochondrial protein) under all of the conditions studied for Table 1, except when the extramitochondrial concentration of Ca^{2+} was above 400nM in the presence of both NaCl and $MgCl_2$. Under these conditions rather variable ATP concentrations, in the range 20–35nmol/mg of mitochondrial protein, were found.

These observations are very similar to those made previously with coupled rat heart mitochondria (Denton *et al.*, 1980; see Table 1). They are consistent with the distribution of Ca^{2+} ions across the inner membrane of epididymal fat-cell mitochondria being maintained by systems similar to those present in rat heart mitochondria, that is, by a cycle made up of an uptake pathway inhibited by Mg^{2+} and an efflux pathway stimulated by Na^+ (for reviews see Carafoli, 1979; Nicholls & Crompton, 1980).

Effects of Ca^{2+} on the oxidation of sub-saturating concentrations of threo-D_s-isocitrate and 2-oxoglutarate by coupled mitochondria from rat epididymal adipose tissue

The sensitivity of 2-oxoglutarate dehydrogenase within coupled rat heart mitochondria to changes in the extramitochondrial concentration of Ca^{2+} can be conveniently demonstrated by following changes in the rate of oxidation of 2-oxoglutarate added at a sub-saturating concentration (Denton *et al.*, 1980). This approach can be applied to mitochondria from rat epididymal adipose tissue not only for 2-oxoglutarate dehydrogenase but also for NAD^+-isocitrate dehydrogenase (Figs. 1*b* and 1*c*), since, unlike heart mitochondria (Chappell & Robinson, 1968), epididymal fat-cell mitochondria have a very active tricarboxylate carrier in their inner membrane (Martin & Denton, 1970). As shown in Figs. 1(*b*) and 1(*c*), increasing the extramitochondrial concentration of Ca^{2+} resulted in increases in the rate of oxidation of both 50μM-*threo*-D_s-isocitrate and 100μM-2-oxoglutarate of up to 2–3-fold. In both cases, the range of Ca^{2+} concentration required was appreciably greater in the presence of 10mM-NaCl. Calculated $k_{0.5}$ values are given in Table 1. Ca^{2+} had no effect on the rates of O_2 uptake at saturating concentrations of *threo*-D_s-isocitrate or 2-oxoglutarate. It is evident that the oxidation of 2-oxoglutarate is more sensitive to Ca^{2+} than are the oxidation of *threo*-D_s-isocitrate or changes in PDH_a. This may, in part, reflect the lower $k_{0.5}$ value of 2-oxoglutarate dehydrogenase for Ca^{2+} assayed in mitochondrial extracts in the presence of ADP (Table 1).

Effects of Na^+ on ^{45}Ca efflux from pre-loaded mitochondria from rat epididymal adipose tissue

The effects of Na^+ on the sensitivity of PDH_a and the oxidation of sub-saturating concentrations of both *threo*-D_s-isocitrate and 2-oxoglutarate to Ca^{2+} strongly suggest that the efflux of Ca^{2+} from

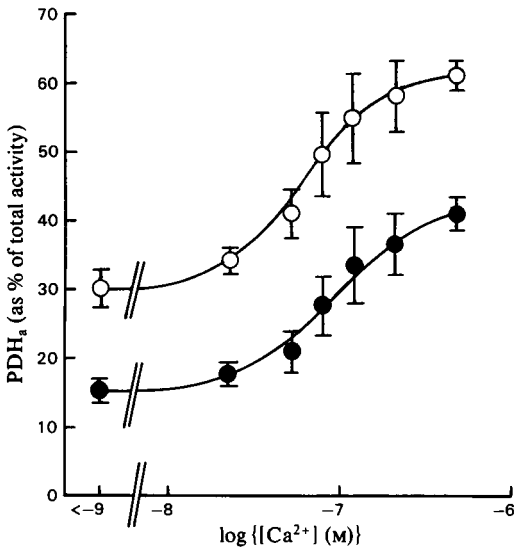

Fig. 2. *Effects of the exposure of rat epididymal fat-pads to insulin on the sensitivity to extramitochondrial Ca^{2+} of the amount of PDH_a in subsequently prepared mitochondria incubated in the presence of Na^+*

Mitochondria were prepared from paired fat-pads in either the absence (●) or presence (○) of 50nM-insulin as described for Table 2. Mitochondria were incubated at 30°C for 5min in KCl-based media containing EGTA, $CaCl_2$, 2-oxoglutarate and malate as described in Fig. 1(*a*), with the additional presence of 10mM-NaCl. Results were obtained, and are expressed, as in Fig. 1(*a*) and are given as means ± S.E.M. (bars) for observations made on five or six different preparations of mitochondria. The values shown for mitochondria prepared from insulin-treated tissue were significantly different ($P \leqslant 0.01$) from controls at each of the concentrations of Ca^{2+} used.

rat epididymal-adipose-tissue mitochondria is stimulated by Na^+. The results given in Table 3 are a more direct demonstration of this. Rat epididymal-adipose-tissue mitochondria were pre-incubated with medium containing ^{45}Ca at a sufficient concentration to cause an appreciable (3-fold), but not maximal, Ca^{2+}-dependent increase in PDH_a and were then sedimented once again before subsequent incubation at 30°C in KCl-based media containing EGTA plus 2-oxoglutarate and malate. In the absence of Na^+, about half of the initial ^{45}Ca content was lost from the mitochondria by 5min, whereas in the presence of 10mM-NaCl nearly 90% was lost; NaCl increased the rate of efflux by nearly 3-fold over the first 1min of incubation (Table 3). As found previously with heart mitochondria from rabbits (Väghy *et al.*, 1982) and rats (McCormack & Denton, 1984), the effect of Na^+ was very largely blocked by the presence of 300μM-diltiazem; diltiazem had little effect on Ca^{2+} egress in the absence of Na^+ (Table 3). It should be noted that essentially similar results to those shown in Table 3 were obtained if the mitochondrial fraction was further purified, after pre-loading with ^{45}Ca, by Percoll-gradient centrifugation as described by Belsham *et al.* (1980). The uptake of ^{45}Ca by the mitochondria was completely inhibited if the Ca-loading part of the procedure was carried out in the presence of Ruthenium Red (5 or 10μg/ml) (results not shown), in agreement with other studies (Severson *et al.*, 1974).

Persistence of differences in PDH_a during the incubation of mitochondria prepared from control and insulin-treated fat tissue in medium containing various concentrations of Ca^{2+} in the presence or absence of added Na^+ and/or Mg^{2+}

If the effect of insulin on PDH_a is due to the activation of PDHP phosphatase by an increase in the intramitochondrial concentration of Ca^{2+}, then the persistent increases in PDH_a observed in mitochondria from insulin-treated tissue compared with controls should be greatly diminished on incubation with medium containing EGTA and 10mM-NaCl, since under these conditions after 5min virtually 90% of the ^{45}Ca in pre-loaded mitochondria is lost from the mitochondria (Table 3). However, incubation of mitochondria under these conditions, in either the presence or the absence of $MgCl_2$, had no discernible effect on the amount of PDH_a in mitochondria prepared from either control or insulin-treated tissue. Results after incubation for 5min are given in Table 4, but similar results (not shown) were obtained after incubation for 10min.

Further experiments were carried out to compare the response of PDH_a within mitochondria to changes in the extramitochondrial concentration of Ca^{2+}. In general, significant increases in PDH_a were evident in the mitochondria prepared from insulin-treated tissue compared with controls at all extramitochondrial concentrations of Ca^{2+} studied. This is illustrated in Fig. 2 for mitochondria incubated with medium containing added NaCl. The fact that significant increases were observed, even at extramitochondrial concentrations that gave a maximal response for Ca^{2+}, again suggests that changes in intramitochondrial Ca^{2+} concentrations are not the basis of the persistent increases in the amount of PDH_a in mitochondria prepared from insulin-treated tissue.

$k_{0.5}$ values for the increases in PDH_a caused by increasing extramitochondrial concentrations of Ca^{2+} were also calculated in these experiments (Table 4). The values obtained for this parameter with mitochondria from insulin-treated tissue were consistently lower than the corresponding control

Table 4. *Effects of the pretreatment of rat epididymal adipose tissue with insulin on the PDH_a content of subsequently isolated mitochondria incubated with EGTA or Ca-EGTA buffers in the absence or presence of Na^+, Mg^{2+} or uncoupler*

Mitochondria were prepared from paired control and insulin-treated fat-pads as described in Table 2. In (*a*) mitochondria were incubated at 30°C for 5min in KCl-based media (see Fig. 1) containing 5mM-2-oxoglutarate, 0.2mM-malate and 5mM-EGTA with further additions of Na^+ and/or Mg^{2+} as indicated, except where FCCP was used; in these latter instances no 2-oxoglutarate or malate was present. In (*b*) mitochondria were incubated as above, but also with the further presence of suitable additions of $CaCl_2$ to cover the range of extramitochondrial Ca^{2+} concentrations to which PDH_a content responded. Results are given as (*a*) means ± S.E.M. and (*b*) $k_{0.5}$ or Max. ΔPDH_a values ± S.D. for the numbers of different paired preparations of mitochondria shown in parentheses (*n*) or the number of degrees of freedom shown in square brackets [d. of f.]. $*P \leqslant 0.01$ for the effects of insulin pretreatment compared with the appropriate control values.

Additions or changes to mitochondrial incubation media	Values derived from mitochondria prepared from paired control or insulin-treated rat epididymal adipose tissue (as indicated): (*a*) PDH_a content (as % of total activity) (at free $[Ca^{2+}] < 1$ nM)		(*b*) response to Ca^{2+}: $k_{0.5}$ (nM)		Max. ΔPDH_a (as % of total activity)	
	Control	Insulin	Control	Insulin	Control	Insulin
None	11.3 ± 1.5	24.7 ± 2.6* (10)	33 ± 2.4	28 ± 2.1	28 ± 1.4	34 ± 1.4 (6) [42]
10mM-NaCl	11.7 ± 1.4	24.8 ± 2.4* (12)	93 ± 13.1	65 ± 3.7	27 ± 2.3	31 ± 1.0 (6) [39]
1mM-$MgCl_2$	14.4 ± 3.2	29.5 ± 2.7* (3)	79 ± 6.2	44 ± 7.8	30 ± 1.6	30 ± 1.1 (1) [9]
10mM-NaCl plus 1mM-$MgCl_2$	14.8 ± 1.8	27.1 ± 2.4* (8)	419 ± 20	229 ± 47	47 ± 1.3	49 ± 4.0 (2) [14]
1 μM-FCCP plus 5 μM-A23187, 1 μM-valinomycin, 5mM-ATPMg and 5 μM-oligomycin	19.1 ± 1.0	21.2 ± 1.5 (5)	940 ± 160	460 ± 110	32 ± 1.9	32 ± 2.4 (5) [38]

values (Table 4). The decrease was quite small in the absence of added NaCl or $MgCl_2$, but was about 30% in the mitochondria incubated with NaCl, and nearly 50% in mitochondria incubated with $MgCl_2$ or $MgCl_2$ plus NaCl. The difference, as a result of insulin treatment, could also be detected in mitochondria incubated with the uncoupler FCCP and the ionophores valinomycin and A23187 plus MgATP and oligomycin (Table 4). If mitochondria are incubated under these uncoupled conditions in the presence of EGTA alone, there is little difference in PDH_a in mitochondria prepared from insulin-treated tissue compared with controls (Denton *et al.*, 1984). However, if the extramitochondrial concentration of Ca^{2+} is increased with Ca–EGTA buffers, then statistically significant differences again become evident at Ca^{2+} concentrations close to the $k_{0.5}$ values. For example, at an extramitochondrial concentration of free Ca^{2+} of about 0·4 μM, PDH_a was 29.6 ± 3.1% of total PDH activity in control mitochondria, compared with 38.4 ± 3.1% in the mitochondria from insulin-treated tissue (means ± S.E.M. for observations on five separate preparations of mitochondria in each case; $P \leqslant 0.01$, by a paired statistical test, for the effect of insulin treatment). The corresponding values at an extramitochondrial Ca^{2+} concentration of about 6.9 μM, which gave a near-maximal response, were 48.1 ± 4.5 and 51.6 ± 4.0% respectively. Since under these conditions of incubation it is unlikely that there is a concentration gradient of Ca^{2+} across the inner membranes of the mitochondria, these results suggest that there may be a decrease in the $k_{0.5}$ value of PDHP phosphatase for Ca^{2+} within intact mitochondria as a result of insulin treatment of the tissue. It should be noted that no corresponding change in $k_{0.5}$ values for Ca^{2+} was observed when the enzyme was assayed in extracts of mitochondria with pig heart PDH phosphate as a substrate (Table 2).

Oxidation of non-saturating concentrations of threo-D_s-isocitrate and 2-oxoglutarate by mitochondria prepared from control and insulin-treated fat tissue

Rates of oxidation of 50 μM-*threo*-D_s-isocitrate by mitochondria incubated in the presence of EGTA (free $[Ca^{2+}] < 1$ nM) were not significantly different if the mitochondria were prepared from control or insulin-treated tissue (Table 5). Furthermore, both sets of mitochondria responded similarly to the presence of 23nM-Ca^{2+} in the extramitochondrial medium. There was also no evidence of

Table 5. *Oxidation of non-saturating concentrations of threo-D_s-isocitrate and 2-oxoglutarate in the presence of EGTA or 23nM-free Ca^{2+} by mitochondria prepared from control and insulin-treated epididymal adipose tissue*

Mitochondria were prepared from paired control and insulin-treated fat-pads as described for Table 2. Mitochondria were incubated at 30°C in KCl-based media containing 2mM-ADP and either 5mM-EGTA alone (free $[Ca^{2+}] < 1$nM) or together with 1.5mM-$CaCl_2$ (to give free $[Ca^{2+}] \simeq 23$nM; see Fig. 1 and Table 1) as indicated with further additions, and by following the procedure, as given in Figs. 1(*b*) and 1(*c*). Results are expressed as in Figs. 1(*b*) and 1(*c*) and are given as means ± S.E.M. for observations made on the number of different paired preparations of mitochondria given in parentheses. The rates of oxidation of saturating concentrations of either *threo*-D_s-isocitrate or 2-oxoglutarate (see Fig. 1) were unaltered by insulin pretreatment. The PDH_a content of fresh mitochondria from the same preparations used in this Table was increased by 106 ± 20% (mean ± S.E.M. for nine observations) as a result of insulin treatment.

Substrate	Free $[Ca^{2+}]$ (nM)	Rate of oxidation at non-saturating substrate concentrations (as % of rate at saturating substrate concentrations) in mitochondria prepared from: Control tissue	Insulin-treated tissue
threo-D_s-Isocitrate (50μM)	<1	33 ± 5.5	28 ± 4.7 (5)
	≃23	51 ± 5.5	59 ± 6.3 (5)
2-Oxoglutarate (100μM)	<1	32 ± 1.0	29 ± 1.5 (4)
	≃23	51 ± 2.8	50 ± 2.5 (4)

any changes in the rates of oxidation of 100μM-2-oxoglutarate as a result of insulin treatment (Table 5). This is further evidence that insulin does not result in an increase in the intramitochondrial concentration of Ca^{2+}. It should be noted that no Na^+ was present in the incubation medium for the experiments of Table 5, and that there was a statistically significant ($P \leqslant 0.01$) increase in the amount of PDH_a in the mitochondria prepared from insulin-treated tissue compared with controls; this increase was measured immediately after preparation. The oxidation of *threo*-D_s-isocitrate and 2-oxoglutarate was followed in medium containing 2mM-ADP; under these conditions PDH_a approaches 100% of total PDH activity in mitochondria prepared from both control and insulin-treated tissue (Denton *et al.*, 1984).

Comparison of the effects on PDH_a resulting from the incubation of rat epididymal adipose tissue in medium containing a high concentration of $CaCl_2$ with those obtained from incubation in medium containing insulin

Perfusion of rat hearts with medium containing a raised concentration of $CaCl_2$ (5–6mM) has been found not only to increase contraction and phosphorylase *a* content, but also to increase the amount of PDH_a in the tissue (McCormack & Denton, 1981*a*; McCormack & England, 1983). There is strong evidence that this increase in PDH_a is due to an increase in the intramitochondrial concentration of Ca^{2+} occurring as a result of an increase in the cytoplasmic concentration. Firstly, the effect on PDH_a is no longer apparent if Ruthenium Red, an inhibitor of Ca^{2+} transport into isolated mitochondria, is present in the perfusion medium, whereas the effects on contraction and phosphorylase *a* content are largely unaffected (McCormack & England, 1983). Secondly, the increases in PDH_a persist during the preparation of mitochondria and their subsequent incubation in KCl-based medium containing respiratory substrates and EGTA alone, but are lost if the incubation medium also contains Na^+ to cause egress of the maintained intramitochondrial Ca^{2+} (McCormack & Denton, 1984).

Very similar effects as a result of incubation in medium containing 5mM-$CaCl_2$ can be observed on PDH_a in adipose tissue (Tables 6 and 7). As found in previous studies (Severson *et al.*, 1976), removal of added $CaCl_2$ from the bicarbonate-based medium had little or no effect on tissue PDH_a values. However, incubation for 30 or 60min in medium containing 5mM-$CaCl_2$ caused increases of about 2-fold in PDH_a compared with control values, which were comparable with or perhaps slightly less than the increases caused by insulin in the same experiments (Table 6). However, the effects of Ruthenium Red on the increases caused by the two agents were markedly different. Whereas the increases in PDH_a in pads caused by incubation in medium containing 5mM-$CaCl_2$ were very greatly diminished by the presence of Ruthenium Red, there was no diminu-

Table 6. *Effects of Ruthenium Red on the increases in PDH_a caused by the incubation of rat epididymal adipose tissue in medium containing insulin or a raised concentration of Ca^{2+}*

In Expt. 1, paired fat-pads were incubated for 30min in standard bicarbonate-buffered medium (Krebs & Henseleit, 1932) with or without insulin as in Table 2, or in modified Krebs medium in which the phosphate concentration was decreased to 0.2mM and there was either no added $CaCl_2$ or 5mM-$CaCl_2$, in the presence or absence of Ruthenium Red (10μg/ml). In Expt. 2 there was a 20min preincubation in the absence or presence of Ruthenium Red alone in standard Krebs medium, followed by a 60min incubation under the conditions shown. The fat-pads were then blotted on filter paper and quickly frozen in liquid N_2 for the subsequent extraction and assay of frozen tissue for both PDH_a and total PDH content as described by Stansbie *et al.* (1976); total PDH activity was essentially unaltered by any of the treatments used and ranged from 0.15 to 0.3unit/g wet wt. of tissue. Results are given as means $\pm$ S.E.M. for six different fat-pads in each case. $*P \leqslant 0.01$ for the effects of insulin or a raised concentration of medium Ca^{2+} compared with the appropriate controls (i.e. down columns); $\dagger P \leqslant 0.01$ for the effect of Ruthenium Red compared with the appropriate value in its absence (i.e. across rows). The differences in the PDH_a values under control conditions in the two experiments below are likely to be due to differences in the season or to slight alterations in nutritional status of the animals rather than to the differences in incubation times. The two experiments were carried out in late summer and mid-winter respectively.

	Additions or changes to incubation media	PDH_a (as % of total activity) in epididymal adipose tissue preincubated (Expt. 2) and incubated (Expts. 1 and 2) in: Absence of Ruthenium Red	Presence of Ruthenium Red
Expt. 1	None	45±4.1	35±5.3
	50nM-insulin	88±5.6*	98±2.4*
	0.2mM-phosphate; no added Ca^{2+}	40±5.2	34±4.4
	0.2mM-phosphate; 5mM-$CaCl_2$	76±8.5*	35±5.1†
Expt. 2	None	22±3.9	24±3.9
	50nM-insulin	58±3.6*	62±5.1*
	0.2mM-phosphate; no added Ca^{2+}	19±2.1	23±6.4
	0.2mM-phosphate; 5mM-$CaCl_2$	39±4.4*	29±5.2

Table 7. *Effects of Na^+ on the persistent increases in PDH_a in incubated mitochondria from rat epididymal adipose tissue resulting from the pre-treatment of tissue with media containing a raised Ca^{2+} concentration*

Mitochondria were prepared from paired fat-pads incubated in the absence or in the presence of a raised concentration of Ca^{2+} in the medium as described in Table 6. In (*a*) freshly prepared mitochondria were sedimented in cold isolation media. In (*b*) mitochondria were incubated at 30°C for 5min in KCl-based media (see Fig. 1) containing 5mM-2-oxoglutarate, 0.2mM-malate and 5mM-EGTA, with further additions as indicated. Results are given as means $\pm$ S.E.M. for observations made on eight different paired preparations of mitochondria in each case. $*P \leqslant 0.05$ by a paired statistical test, and $**P \leqslant 0.01$ by an unpaired statistical test, for the effects of pre-treatment of tissue with a raised concentration of Ca^{2+} in the medium compared with untreated values.

	Additions to incubation media	PDH_a content (as % of total activity) of mitochondria prepared from tissue pre-treated with: No additions	High Ca^{2+} in medium
(*a*) Fresh mitochondria	None	40±5.8	62±7.8**
(*b*) Incubated mitochondria	None	23±2.9	31±4.5*
	10mM-NaCl	22±3.3	23±2.8
	0.2mM-potassium pyruvate	33±3.3	51±4.1**
	0.2mM-potassium pyruvate plus 10mM-NaCl	33±4.0	39±3.9

tion in the increases in PDH_a caused by incubation of pads with insulin. In an earlier study, we had found evidence for some decrease in the effect of insulin in PDH_a in pads incubated with Ruthenium Red, but the decrease was modest and inconsistent (Severson *et al.*, 1974). We have no satisfactory explanation for these previous observations, except that perhaps some of the prepara-

tions of Ruthenium Red used in the earlier studies may have contained an impurity which inhibited insulin action.

Increases in PDH_a as a result of incubating tissue in a medium containing 5mM-$CaCl_2$ persisted during the preparation of mitochondria and their subsequent incubation in media containing EGTA but no added Na^+. The difference from control values after 5min incubation was smaller than in the original tissue; this probably reflects the partial loss of Ca^{2+} that occurs under these conditions (see Table 3). If NaCl was added to the incubation media to stimulate the efflux of Ca^{2+} from the mitochondria, then the effects of the pretreatment of fat-pads with medium containing high $CaCl_2$ on PDH_a disappeared. These results are, in general, similar to those found with mitochondria prepared from rat hearts perfused with medium containing a high $CaCl_2$ concentration (McCormack & Denton, 1984). On the other hand, they are in marked contrast with results obtained with mitochondria from fat-pads exposed to insulin. The increases in PDH_a in these latter mitochondria are fully preserved during their incubation under comparable conditions, in both the presence and the absence of added NaCl (Table 4).

General conclusions

The transport systems determining the distribution of Ca^{2+} across the inner membrane of fat-cell mitochondria appear to be very similar to those found in the mitochondria of rat heart. In both cases, therefore, the influx of Ca^{2+} occurs on a uniporter inhibited by Mg^{2+} and Ruthenium Red, whereas the efflux of Ca^{2+} appears to occur largely via a Na/Ca antiporter plus a second uncharacterized process which, although Na-independent, must also be charge-compensated (for reviews, see Carafoli, 1979; Nicholls & Crompton, 1980). Also in each case, the Na/Ca antiporter is inhibited by diltiazem (see McCormack & Denton, 1984). Our findings with isolated fat-cell mitochondria also suggest that increases in the extramitochondrial concentration of Ca^{2+} within the expected physiological range can cause parallel activation of PDHP phosphatase (and thus an increase in PDH_a), NAD^+–isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase. This view is given some support by the Ruthenium Red-sensitive increases in PDH_a observed in rat epididymal fat-pads incubated with medium containing a high concentration of $CaCl_2$. It follows that any hormone that increases the cytoplasmic concentration of Ca^{2+} in fat-cells would probably also increase the intramitochondrial concentration of Ca^{2+} and thus increase PDH_a as well as the activities of NAD^+–isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase at sub-saturating concentrations of their respective substrates.

The main objective of the present studies was to establish whether or not the increase in PDH_a caused by the exposure of rat epididymal adipose tissue to insulin was the result of an increase in the intramitochondrial concentration of Ca^{2+}, perhaps as the result of insulin causing changes in the activity of one or more of the systems involved in the transfer of Ca^{2+} across the inner membrane of the fat-cell mitochondria. This objective has been realized, and we can now conclude with confidence that this is not the basis of this action of insulin. Not only do the increases in PDH_a as a result of insulin action persist through to isolated mitochondria incubated with medium containing EGTA and Na^+, but they also persist when the mitochondria are incubated in media containing sufficient Ca^{2+} to cause a maximal increase in PDH_a. In addition, we could find no evidence for any increases in the activity of NAD^+–isocitrate dehydrogenase or 2-oxoglutarate dehydrogenase within mitochondria from insulin-treated tissue compared with controls; these enzymes are at least as sensitive to increases in the intramitochondrial concentration of Ca^{2+} as is PDHP phosphatase. Finally, incubation of intact fat-pads with Ruthenium Red did not cause any decrease in the effects of insulin on PDH_a, whereas in a parallel set of experiments it blocked the effects of incubation with medium containing 5mM-$CaCl_2$.

Observations made in the course of the present studies suggest that insulin treatment of intact tissue may result in quite complex changes in the activity of PDHP phosphatase within the fat-cell mitochondria. Activity is apparently increased in Ca-depleted mitochondria, as discussed above, but in addition it appears that the sensitivity of PDHP phosphatase to Ca^{2+} within intact mitochondria prepared from insulin-treated tissue is also enhanced. However, we could find no evidence for the persistence of such changes in Ca^{2+}-sensitivity into extracts of mitochondria. These observations are consistent with the alterations in PDHP phosphatase activity being brought about by a change in the intramitochondrial concentration of an effector of the enzyme (other than Ca^{2+}). One possibility is the low-molecular-weight mediator proposed by several workers (see, e.g., Jarett & Seals, 1979; Seals & Czech, 1980; Kiechle *et al.*, 1981) to be released from the plasma membrane of fat-cells by insulin, but their observations have proved difficult to reproduce (Wieland, 1983; Denton *et al.*, 1984), and a number of major criticisms of the experimental conditions and assays used in their studies can be made (Wieland, 1983; Denton *et al.*, 1984).

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