Mitochondrial Ca^{2+} transport in lean and genetically obese (*ob/ob*) mice

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Isolated mitochondria from liver or brown adipose tissue of obese *ob/ob* mice demonstrated increased rates of Ca^{2+} uptake and release compared with those of lean mice. This enhanced transport activity was not found in mitochondria from kidney or skeletal muscle. Respiration-induced membrane potential was the same in mitochondria from lean and *ob/ob* mice. It is therefore concluded that the increased Ca^{2+} uptake rates reflect an activation of the Ca^{2+} uniporter rather than a change in the electrophoretic driving force. As mitochondria from pre-obese *ob/ob* mice did not show elevated rates of Ca^{2+} transport, the activated transport in the obese animals was thus a consequence of the state of obesity rather than being a direct effect of the *ob/ob* genotype. It is suggested that the enhanced activity of the Ca^{2+} -transport pathways in liver and brown adipose tissue may alter metabolic functions in these tissues by modifying cytoplasmic or intramitochondrial Ca^{2+} concentrations.

The energy-dependent uptake of Ca^{2+} by mitochondria and its subsequent release provides a mechanism for Ca^{2+} recycling across the mitochondrial inner membrane. Because Ca^{2+} uptake takes precedence over ADP phosphorylation in competition for the energy of respiration (Rossi & Lehninger, 1964), the cyclic transport of Ca^{2+} by mitochondria dissipates energy (Stucki & Ineichen, 1974) and is therefore potentially a thermogenic process of physiological significance. Increased rates of Ca^{2+} influx and efflux have been reported in skeletal-muscle mitochondria of cold-acclimated rats (Greenway & Himms-Hagen, 1978), which would be compatible with Ca^{2+} cycling having some role in non-shivering thermogenesis. Similarly, the stimulation of mitochondrial Ca^{2+} cycling by halothane *in vitro* (Grist & Baum, 1975) and the enhanced Ca^{2+} -efflux capacity in anaerobic muscle mitochondria from halothane-sensitive pigs (Cheah & Cheah, 1981) implicate mitochondrial Ca^{2+} cycling in the uncontrolled heat production of halothane-induced malignant hyperthermia.

Genetically obese (*ob/ob*) mice have an impaired capacity for non-shivering thermogenesis which is related to their ability to accumulate excess energy as triacylglycerol in white adipose tissue (Trayhurn & James, 1978). This thermogenic defect in obese mice is demonstrated in brown adipose tissue

(Himms-Hagen & Desautels, 1978; Thurlby & Trayhurn, 1980), where mitochondria are less able than in lean mice to generate heat by controlled dissipation of the proton electrochemical gradient (Nicholls, 1979). To determine whether any thermogenic cycling of Ca^{2+} might also be defective in the obese animals, the rates of uptake and release of Ca^{2+} have now been measured in isolated mitochondria from various tissues of lean and *ob/ob* mice. The results indicate that, far from being impaired, the Ca^{2+} -transport capacity is greatly enhanced in mitochondria of liver and brown adipose tissue in the obese state.

Experimental

Animals

Female 'Aston' *ob/ob* and lean mice were reared at 21°C on a standard rodent diet (Spillers–Spratts Rodent Breeding Diet 1; Spratts Patent Ltd., Barking, Essex, U.K.) with drinking water available *ad libitum*. For most studies mature mice aged 2–3 months were used. Pre-obese *ob/ob* mice at 13 days after birth were identified by their fall in body temperature when placed individually at 21°C for short periods (Trayhurn *et al.*, 1977). Such pre-weanling animals were then used at 14 days of age.

For some experiments, diabetic-obese (*db/db*) mice with the *db* gene on the C57BL/ks background (Goodbody & Trayhurn, 1981) were compared with their lean littermates. The effect of

Abbreviation used: TPMP⁺, methyltriphenylphosphonium.

cold-acclimation on mitochondrial calcium transport was also examined in lean mice and female golden hamsters (*Mesocricetus auratus*, outbred MB strain, obtained from Intersimian Ltd., Abingdon, Oxon., U.K.). Both mice and hamsters were cold-acclimated at 5°C, or warm-acclimated at either 30°C (hamsters) or 33°C (mice) for 3–4 weeks as described by Trayhurn (1980).

Mitochondrial preparation

Mitochondria were released from liver or kidney by homogenization at 0°C in 250 mM-sucrose, buffered to pH 7.4 with 2 mM-Hepes [4-(2-hydroxy-ethyl)-1-piperazine-ethanesulphonic acid]/Tris and 0.5 mM-EGTA/Tris. After conventional differential centrifugation, the mitochondrial preparation was washed twice in 250 mM-sucrose/2 mM-Hepes/Tris, pH 7.4, and resuspended in this buffered medium to a concentration of 3–6 mg of mitochondrial protein/ml. Brown adipose tissue, pooled from the interscapular, subscapular, cervical and axillary regions of three to six mice, was homogenized, and mitochondria were prepared as described by Cannon & Lindberg (1979). Hindleg skeletal muscle from groups of three mice was treated with bacterial protease (Type VII, Nagarse; Sigma Chemical Co., Poole, Dorset, U.K.), and mitochondria were isolated by the procedure of Makinen & Lee (1968). All mitochondrial preparations were kept at 0°C for a maximum of 3 h before measurement of Ca^{2+} transport. Protein concentration was determined by a biuret method (Gornall *et al.*, 1949) against a standard of bovine serum albumin (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.).

Measurement of Ca^{2+} -transport rates

The initial rate of Ca^{2+} uptake by respiring mitochondria was measured with $^{45}\text{Ca}^{2+}$ (200 nmol/ml) by the Ruthenium-Red/EGTA quench-sampling technique and rapid centrifugation (Reed & Bygrave, 1975). For liver and kidney mitochondria, Ca^{2+} uptake was determined at 10°C in a simple medium of 250 mM-sucrose/2 mM-Hepes/Tris, 1 mM- KH_2PO_4 and 2 mM-succinate/Tris, pH 7.4. The effect of calcium concentration on initial uptake rate was examined with liver mitochondria in nitrilotriacetate/calcium buffers (Reed & Bygrave, 1975). Ca^{2+} uptake by brown-adipose-tissue mitochondria was measured at 20°C in a Na^+ -free medium at pH 7.2 containing 100 mM-sucrose/2 mM-Hepes/Tris, 5 mM-L- α -glycerophosphate [di(monocyclohexylamine) salt], 0.2% (w/v) bovine serum albumin (essentially fatty acid-free), 1 mM-GDP (Tris salt), 1 mM- KH_2PO_4 and 2.4 μM -rotenone. Skeletal-muscle mitochondria were incubated in a Na^+ -free medium at pH 7.2 containing 250 mM-sucrose/2 mM-Hepes/

Tris, 2 mM-succinate/Tris, 0.1% bovine serum albumin (essentially fatty acid-free), 1 mM- MgCl_2 , 1 mM- KH_2PO_4 , 15 mM-KCl, 0.2 mM-ATP (potassium salt), 2.4 μM -rotenone and 2 μg of oligomycin/ml.

The initial Ca^{2+} -influx rate during the first 30 s was calculated for mitochondria from all tissues by linear-regression analysis (Dorman *et al.*, 1975) from 50 μl samples taken at 10 s intervals. The rate of release of Ca^{2+} from mitochondria was estimated over 6–8 min at 20°C after preloading with 80–90 nmol of $^{45}\text{Ca}^{2+}$ /mg of protein by incubation with 100 nmol of $^{45}\text{Ca}^{2+}$ /ml added 5 min before release was measured. Samples (100 μl) of incubation mixture were filtered under vacuum through 0.45 μm -pore-size Millipore filters, and the concentration of released $^{45}\text{Ca}^{2+}$ was measured in the mitochondria-free filtrate.

For liver mitochondria the Ca^{2+} -release rate was originally measured after adding 2.5 mM-EGTA/Tris and 2 mM-acetoacetate (potassium salt) to bring the mitochondrial nicotinamide nucleotides into the oxidized state (Fiskum & Lehninger, 1979). During the course of the present study it became known that this procedure leads to collapse of the mitochondrial membrane potential (Nicholls & Brand, 1980), with a consequent loss of Ca^{2+} by reversal of the uptake uniporter (Puskin *et al.*, 1976). Hence the conditions for measuring Ca^{2+} efflux were subsequently modified. Liver and kidney mitochondria were incubated in a medium of 100 mM-KCl/25 mM-Tes (2-[[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]-amino]ethanesulphonic acid) (sodium salt), pH 7.0, at 20°C containing 0.1% bovine serum albumin (fatty acid-free), 2 mM-succinate/Tris, 0.2 mM-ATP, 1 mM- KH_2PO_4 , 1 mM- MgCl_2 , 2.4 μM -rotenone and 2 μg of oligomycin/ml. $^{45}\text{Ca}^{2+}$ was preloaded as before and its release was monitored after adding 2.5 mM-EGTA/Tris and 0.27 μM -Ruthenium Red, with or without 2 mM-acetoacetate (lithium salt).

Mitochondria from brown adipose tissue and from muscle were preloaded with $^{45}\text{Ca}^{2+}$ in the respective media used for Ca^{2+} -uptake studies. As Ca^{2+} release is stimulated by Na^+ in mitochondria from brown adipose tissue (Al-Shaikhly *et al.*, 1979) and from skeletal muscle (Crompton *et al.*, 1978), the efflux rate in these tissues was determined after adding 20 mM- Na^+ together with EGTA and Ruthenium Red, as above.

For studies on both uptake and release of Ca^{2+} , mitochondria were incubated at a concentration of 1–1.5 mg of protein/ml in a final volume of 1 ml. Rapid mixing and efficient aeration was obtained by agitation with a magnetic stirrer. Radioactivity was measured in a Triton X-100/toluene scintillator solution in a Packard model 3375 liquid-scintillation spectrometer calibrated with a series of quenched ^{45}Ca standards.

Measurement of mitochondrial membrane potential

The membrane potential ($\Delta\psi$) was determined from the distribution of a lipophilic cation, [^3H]-TPMP $^+$ (New England Nuclear Chemicals G.m.b.H., Dreieich, Germany), across the mitochondrial inner membrane in the presence of tetraphenylboron (Scott & Nicholls, 1980; Nicholls & Brand, 1980). After a 3 min incubation period, 200 μl samples were filtered under vacuum through 0.65 μm -pore-size cellulose acetate filters (Sartorius G.m.b.H., D-3400 Göttingen, W. Germany). The addition of [$\text{U-}^{14}\text{C}$]sucrose at 0.4 $\mu\text{Ci/ml}$, 30 s before filtration (Crompton & Heid, 1978), enabled calculation of extramitochondrial [^3H]-TPMP $^+$ retained on the filter. Radioactivity on the filter and in samples of the filtrate was measured with Triton X-100/toluene scintillant in two channels of a Packard model 2650 liquid-scintillation spectrometer. Corrections were made for non-specific binding of TPMP $^+$ to the filter. Matrix volumes for liver and brown-adipose-tissue mitochondria from lean and *ob/ob* mice were obtained from the $^3\text{H}_2\text{O}$ -permeable [$\text{U-}^{14}\text{C}$]sucrose-impermeable space, and $\Delta\psi$ was derived from the ratio of TPMP $^+$ concentrations (Nicholls, 1974).

Materials

Except where otherwise indicated, organic reagents were purchased from Sigma.

Results *Ca^{2+} -transport rates*

It was repeatedly observed that there was a faster rate of Ca^{2+} uptake by mitochondria from liver and brown adipose tissue of *ob/ob* animals than with mitochondria from the same tissues of their lean littermates (Fig. 1). The mean initial uptake rates were 115% and 63% higher, respectively, in liver and brown-adipose-tissue mitochondria of the *ob/ob* mice, and the increased rate with liver mitochondria was found even in the absence of added phosphate. In contrast, there was no apparent difference in these uptake rates in mitochondria from kidney and skeletal muscle (Table 1).

When Ca^{2+} release from mitochondria was examined the same pattern was observed, with liver and brown adipose tissue of *ob/ob* mice having faster release rates than in lean mice (Fig. 2), whereas there was no clear difference in mito-

Fig. 1. Uptake of Ca^{2+} by isolated mitochondria from liver and brown adipose tissue of lean and *ob/ob* mice. The curves are plotted from mean values $\pm$ S.E.M. (bars) for six preparations of liver mitochondria (a) and nine preparations of brown-adipose-tissue mitochondria (b). Incubation conditions are described in the Experimental section. The curves indicate Ca^{2+} uptake by mitochondria from lean mice (O) and from *ob/ob* mice (●).

chondria from kidney and skeletal muscle (Table 2). With acetoacetate treatment, there was a marked increase in the rate of Ca^{2+} efflux from liver mitochondria of *ob/ob* mice. However, this rate of efflux was greatly decreased when the collapse of $\Delta\psi$ was prevented by including ATP and Mg^{2+} in the incubation medium (Figs. 2a and 2b). Nevertheless, even under these conditions, and with EGTA and Ruthenium Red inhibiting re-uptake of Ca^{2+} , the spontaneous efflux rate was still twice as fast in liver mitochondria of *ob/ob* than of lean mice. In brown-adipose-tissue mitochondria, with adequate provision for maintaining $\Delta\psi$, the mean rate of Na^+ -stimulated Ca^{2+} release was 50% higher with *ob/ob* mice (Fig. 2c). Hence it was concluded that in

mitochondria from these two tissues the obese mutants had a genuinely increased transport capacity for both uptake and release of Ca^{2+} .

The question then arises as to whether the altered mitochondrial Ca^{2+} -transport rates are secondary to the obese state or whether this phenomenon is more directly related to the primary gene defect in the mutant mouse. As diabetic-obese (*db/db*) mice also showed enhanced Ca^{2+} transport in brown-adipose-tissue mitochondria (Table 3), at least two independent mutations can cause this change in mitochondrial function. On the other hand, in

Table 1. Initial Ca^{2+} -uptake rates by isolated mitochondria from lean and *ob/ob* mice

The Ca^{2+} -uptake rates for each type of mitochondria were measured as described in the Experimental section. Initial extramitochondrial Ca^{2+} concentration was $200\text{ }\mu\text{M}$. Mean values $\pm$ S.E.M. are given, with the numbers of experiments indicated in parentheses. The statistical significance of differences between values for lean and *ob/ob* mice ($*P < 0.01$) was estimated by Student's *t* test for paired data.

Mitochondria	Mice	Ca^{2+} -uptake rate (nmol/min per mg of mitochondrial protein)	
		Lean	<i>ob/ob</i>
Liver			
+ 1 mM-phosphate		75.2 ± 8.2 (6)	161.5 ± 23.8 (6)*
No added phosphate		35.1 ± 1.4 (7)	61.2 ± 4.0 (6)*
Brown adipose tissue		53.7 ± 7.6 (9)	87.4 ± 9.8 (9)*
Kidney		138.8 ± 48.3 (3)	106.3 ± 31.6 (3)
Skeletal muscle		120.2 (2)	102.3 (2)

Table 2. Rate of Ca^{2+} release from Ca^{2+} -preloaded mitochondria from lean and *ob/ob* mice

$^{45}\text{Ca}^{2+}$ was preloaded to a value of 80–90 nmol/mg of mitochondrial protein and the rate of its release was measured over 8–12 min as described in the Experimental section. Mean values $\pm$ S.E.M. are given, with the numbers of experiments indicated in parentheses. Values significantly different from those for lean mice are indicated by * ($P < 0.01$) or ** ($P < 0.001$).

Mitochondria	Mice	Rate of Ca^{2+} release (nmol/min per mg of mitochondrial protein)	
		Lean	<i>ob/ob</i>
Liver			
+ acetoacetate		1.32 ± 0.15 (5)	4.06 ± 0.46 (5)**
EGTA/Ruthenium Red only		0.54 ± 0.14 (4)	1.19 ± 0.45 (4)
Brown adipose tissue		2.57 ± 0.14 (6)	3.84 ± 0.38 (6)*
Kidney			
+ acetoacetate		1.60 ± 0.21 (3)	1.89 ± 0.18 (3)
EGTA/Ruthenium Red only		1.24 ± 0.03 (3)	1.44 ± 0.14 (3)
Skeletal muscle		3.83 (2)	4.04 (2)

Fig. 2. Release of Ca^{2+} by isolated mitochondria from liver and brown adipose tissue of lean and *ob/ob* mice. Ca^{2+} release was measured from mitochondria pre-loaded with 80–90 nmol of $^{45}\text{Ca}^{2+}$ /mg of protein under conditions described in the Experimental section. The curves are plotted from mean values $\pm$ S.E.M. (bars) for: (a) five preparations of liver mitochondria with acetoacetate-induced release; (b) four preparations of liver mitochondria under conditions maintaining high $\Delta\psi$; (c) six preparations of brown-adipose-tissue mitochondria with Na^+ -induced release. The curves indicate Ca^{2+} release by mitochondria from lean mice (O) and from *ob/ob* mice (●).

pre-obese *ob/ob* mice there was no increase in the rate of Ca²⁺ uptake in either liver or brown-adipose-tissue mitochondria, although with the latter mitochondria a small but statistically significant increase was found in the rate of Ca²⁺ release (Table 3). Therefore, taken together, these results suggest that mitochondrial Ca²⁺-transport rates are modified after the development of obesity and are unlikely to be a primary metabolic abnormality of the obese genotype.

Effect of cold-acclimation

As some mitochondria from thermogenically defective *ob/ob* or *db/db* mice have an increased capacity for Ca²⁺ uptake and release, these pro-

cesses were also measured in lean animals when thermogenesis had been stimulated by low environmental temperature. For liver mitochondria, exposure of mice to cold for either long or short periods had little effect on the Ca²⁺-uptake rate, although both treatments noticeably depressed the rate of Ca²⁺ release (Table 4). In contrast, in brown-adipose-tissue mitochondria both long-term and short-term cold exposure caused a noticeable stimulation of the Ca²⁺-uptake rate, with no effect on the Ca²⁺-release rate. Similarly, an enhanced Ca²⁺-uptake rate was found, with no change in Ca²⁺ release, in brown-adipose-tissue mitochondria from cold-acclimated hamsters. Hence the stimulation of thermogenesis is associated in brown-adipose-tissue mitochondria with a faster rate of Ca²⁺ influx. If

Table 3. Ca²⁺-transport rates in mitochondria from lean and diabetic-obese (*db/db*) mice and from 14-day-old lean and pre-obese *ob/ob* mice

Mitochondrial Ca²⁺-uptake and -release rates were measured as described in the Experimental section. Mitochondria from pooled brown adipose tissue of six lean and six diabetic-obese mice were used. For the 14-day-old mice, mean values $\pm$ s.e.m. from four separate experiments are given for brown-adipose-tissue mitochondria. Values significantly different from those for lean mice are indicated by * ($P < 0.05$). Values for liver are for one experiment only.

Ca ²⁺ -transport rates (nmol/min per mg of mitochondrial protein)				
Ca ²⁺ uptake		Ca ²⁺ release		
		Lean	<i>db/db</i>	
Lean and <i>db/db</i> mice				
Brown adipose tissue		107.9	174.7	
		Lean	<i>ob/ob</i>	
14-day-old mice				
Brown adipose tissue		93.4 $\pm$ 27.3	87.0 $\pm$ 7.3	
Liver		103.5	101.4	
		Lean	<i>ob/ob</i>	
		2.5 $\pm$ 0.4	3.1 $\pm$ 0.3*	
		0.82	0.70	

Table 4. Effect of cold-exposure of mice or hamsters on mitochondrial Ca²⁺-transport rates

Mitochondrial Ca²⁺-uptake and -release rates were measured as described in the Experimental section. Lean mice were acclimated either at 33°C or at 5°C for 2–4 weeks (long-term exposure) or lean mice acclimated at 33°C were placed at 5°C for 1 h (short-term exposure). Hamsters were acclimated either at 30°C or at 5°C for 3 weeks. Lean and *ob/ob* mice were acclimated at 12°C for 3–4 weeks. Values are for single experiments or are means of up to four separate experiments, indicated in parentheses.

Ca ²⁺ -transport rates (nmol/min per mg of mitochondrial protein)					
		Ca ²⁺ uptake		Ca ²⁺ release	
Environmental conditions		Warm control	Cold	Warm control	Cold
Lean mice: liver mitochondria	Long-term cold	76.9 (2)	86.4 (2)	3.2 (1)	0.4 (1)
	Short-term cold	106.8 (1)	128.2 (1)	4.1 (1)	2.1 (1)
Lean mice: brown-adipose-tissue mitochondria	Long-term cold	69.8 (2)	215.4 (3)	3.5 (2)	3.1 (4)
	Short-term cold	79.4 (1)	115.7 (1)	4.8 (1)	4.4 (1)
Hamster: brown-adipose-tissue mitochondria	Long-term cold	447.9 (2)	981.9 (2)	3.6 (2)	3.3 (2)
		Ca ²⁺ uptake		Ca ²⁺ release	
		Lean	<i>ob/ob</i>	Lean	<i>ob/ob</i>
Lean and <i>ob/ob</i> mice at 12°C					
Brown-adipose-tissue mitochondria (3)		96.1 $\pm$ 14.3	269.8 $\pm$ 64.5	2.5 $\pm$ 0.2	3.1 $\pm$ 0.3

ob/ob mice were acclimated at 12°C (the minimum temperature to which they can adapt), the high rates of Ca²⁺ uptake and release by brown-adipose-tissue mitochondria which were previously found in this genotype were still observed compared with 12°C-acclimated lean mice. It is therefore apparent that, in the *ob/ob* mouse, altered mitochondrial Ca²⁺ transport is distinct from that accompanying increased thermogenesis.

Mitochondrial membrane potential

Two separate mechanisms could account for elevated Ca²⁺-uptake rates by mitochondria. Either a Ca²⁺ carrier (or channel) through the inner membrane is induced or activated, or alternatively there may be an increase in the electrophoretic driving force (membrane potential) for the inward flow of Ca²⁺. Kinetic analysis of Ca²⁺ uptake by liver mitochondria indicated that the K_m value in *ob/ob* mice was significantly higher than that in lean mice (Table 5). Nevertheless, the difference is not great, and the higher V_{max} of *ob/ob*-mouse liver mitochondria is, from these values, unlikely to be due simply to a change in affinity of a Ca²⁺-carrier system.

Mitochondrial membrane potential was measured in the two types of mitochondria with altered Ca²⁺ transport rates. There was no significant difference between lean and *ob/ob* mice in the volume of the matrix space in liver mitochondria (1.24 ± 0.10 and $1.45 \pm 0.10 \mu\text{l}/\text{mg}$ of protein for lean and obese, respectively; mean values $\pm$ S.E.M. for eight determinations). However, mitochondria from brown adipose tissue of obese *ob/ob* mice had a significantly larger matrix volume than those of lean mice (0.84 ± 0.05 and $1.09 \pm 0.01 \mu\text{l}/\text{mg}$ of protein for lean and obese, respectively; mean values $\pm$ S.E.M. for eight determinations, $P < 0.01$), in

agreement with their changed morphology (Hogan & Himms-Hagen, 1980). At 14 days after birth, before obesity has developed, brown-adipose-tissue mitochondria from both *ob/ob* and lean mice have the same matrix volume of $0.74 \mu\text{l}/\text{mg}$ of protein (Goodbody, 1983). In all instances, mitochondria from mature and pre-weanling mice, under the conditions used for measuring Ca²⁺ transport, gave very similar values of $\Delta\psi$ when comparing lean and *ob/ob* genotypes (Table 6). Therefore the increased Ca²⁺-uptake rate found in mitochondria of *ob/ob* mice is unlikely to be a result of elevated $\Delta\psi$. Furthermore, as $\Delta\psi$ had only fallen by 13–17 mV at 30 s after the addition of Ca²⁺ to brown-adipose-tissue mitochondria from both lean and *ob/ob* mice (results not shown), a precipitous fall in $\Delta\psi$ could not explain the difference in Ca²⁺-uptake rates. The most probable explanation for the increased rate is that a true activation of the membrane Ca²⁺-uptake system occurs in liver and brown-adipose-tissue mitochondria of obese *ob/ob* mice.

On the other hand, short-term cooling of lean mice at 5°C before they were killed did produce an increase in $\Delta\psi$ of mitochondria from brown adipose tissue (142.1 ± 2.8 versus 155.4 ± 9.6 mV for warm- and cold-exposed mice, respectively; mean values $\pm$ S.E.M. for three determinations). As this treatment also stimulated the initial rate of Ca²⁺ uptake by these mitochondria (Table 4), it is likely that it is the greater electrophoretic driving force rather than an activation of the Ca²⁺ uniporter which accounts for the enhanced rate of uptake. This reasoning is supported by the negative observation that, in liver mitochondria, environmental cooling neither stimulated Ca²⁺ influx (Table 4) nor led to a raised $\Delta\psi$ (150.2 ± 2.3 versus 148.0 ± 2.0 mV for warm- and cold-exposed mice, respectively; mean values $\pm$ S.E.M. for three determinations).

Table 5. Kinetic analysis of Ca²⁺ uptake by liver mitochondria from lean and *ob/ob* mice

The initial Ca²⁺-uptake rate was obtained with extra-mitochondrial Ca²⁺ concentrations varying over the range 0.46–23.0 μM . The data from four experiments were represented in Eadie–Hofstee plots (Lehninger, 1975) to derive the kinetic parameters of Ca²⁺ uptake. Results are given as mean values $\pm$ S.E.M. Values significantly different from those for lean mice are indicated by * ($P < 0.02$) or ** ($P < 0.01$).

	K_m (μM)	V_{max} (nmol/10 s per mg of protein)
Lean	3.4 ± 0.4	2.0 ± 0.6
<i>ob/ob</i>	$5.1 \pm 0.3^*$	$3.5 \pm 0.7^{**}$

Table 6. Membrane potential ($\Delta\psi$) of respiring mitochondria from liver and brown adipose tissue of mature and 14-day-old lean and *ob/ob* mice

$\Delta\psi$ was determined as described in the Experimental section. Mean values are given $\pm$ S.E.M. where the number of separate experiments (indicated in parentheses) was three or more.

Mitochondria	Mitochondrial $\Delta\psi$ (mV)	
	Lean	<i>ob/ob</i>
Liver		
Mature mice	144.3 ± 1.7 (3)	146.0 ± 3.0 (3)
14-day-old mice	146.5 (1)	140.5 (1)
Brown adipose tissue		
Mature mice	134.1 ± 4.4 (4)	135.6 ± 4.3 (4)
14-day-old mice	147.0 (2)	144.9 (2)

Discussion

In order to conclude that mitochondrial Ca^{2+} -transport capacity has changed in response to a particular stimulus, the experimental design for determining the transport rates must be critically assessed. For every set of measurements in the present study, mitochondria were prepared from both control and experimental mice, and Ca^{2+} -transport rates for both were compared. Although there was variation between experiments, within each individual experiment liver and brown-adipose-tissue mitochondria from the obese mice had consistently higher transport rates compared with those from lean mice. Moreover, as the specific activities of cytochrome oxidase in mitochondria from brown adipose tissue are the same in lean and *ob/ob* mice (A. E. Goodbody & P. Trayhurn, unpublished work), enhanced Ca^{2+} -transport rates are found in these mitochondria whether expressed per mg of protein or per unit of enzyme activity.

Initial-rate measurements to assess the mitochondrial Ca^{2+} -uptake pathway have been criticised for not distinguishing whether the rate-limiting factor is the activity of the Ca^{2+} carrier or whether it is the activity of the H^{+} -translocating system required to maintain electroneutrality (Heaton & Nicholls, 1976). In the present study, the initial extramitochondrial Ca^{2+} concentration ($200\mu\text{M}$) was greater than that usually assumed for the cytoplasm of the intact cell ($0.1\text{--}10\mu\text{M}$) (Bygrave, 1978). Hence the supply of Ca^{2+} to the uptake pathway would have ensured full expression of the transport activity. Although H^{+} translocation was not measured, respiration-dependent $\Delta\psi$ was the same in mitochondria from lean and *ob/ob* tissues. Thus differences in the initial rates of Ca^{2+} uptake were unlikely to be caused by variation in the initial electrophoretic driving force. Similarly, because the respiratory rates of brown-adipose-tissue mitochondria from lean and *ob/ob* mice are the same in the presence of GDP (Goodbody, 1983), the higher Ca^{2+} -uptake rate found with the mitochondria from the obese mice cannot be a result of differences in the activity of the respiratory chain.

The relatively high concentration of Ca^{2+} ($80\text{--}90\text{nmol/mg}$ of protein) loaded into mitochondria before measurement of the efflux rate might also be considered to have an unphysiological influence on the release pathway. However, because $\Delta\psi$ was maintained during Ca^{2+} release and because the rate of release was not precipitous and remained steady over the 6–8 min assay period, it is concluded that the efflux rates were an effective measure of the capacity of the Ca^{2+} -release pathway. Furthermore, with phosphate-containing mitochondria the Ca^{2+} -efflux rate has been shown to be independent of matrix Ca^{2+} content over the range used here (Zoccarato & Nicholls, 1982).

Because elevated mitochondrial Ca^{2+} -transport rates were found in obese *ob/ob* and *db/db* mice, but not in pre-obese *ob/ob* mice, this change in function appears to be a consequence of the obese state rather than of the obese genotype. As the plasma of genetically obese mice has a higher concentration (compared with lean mice) of a number of hormones, notably insulin and corticosterone (Bray & York, 1979), it is possible that the modified Ca^{2+} -transport rates are related to the altered endocrine status. Insulin is reported to stimulate the rate of Ca^{2+} influx (Dorman *et al.*, 1975), whereas the corticosteroid dexamethasone enhances the release of Ca^{2+} from liver mitochondria (Kimura & Rasmussen, 1977). Similarly, α -adrenergic agonists can stimulate Ca^{2+} uptake by liver mitochondria (Taylor *et al.*, 1980) and cardiac mitochondria (Kessar & Crompton, 1981) as well as stimulating release of Ca^{2+} from mitochondria in liver (Murphy *et al.*, 1980) and brown adipose tissue (Nedergaard, 1981).

In most of these previous Ca^{2+} -transport experiments $\Delta\psi$ was not measured, so the mechanism by which the rate of Ca^{2+} influx was altered is uncertain. However, in cardiac mitochondria α -adrenergic stimulation of Ca^{2+} uptake occurs without an increase in $\Delta\psi$ (Kessar & Crompton, 1981), thus indicating an activation of the Ca^{2+} uniporter. Because in the present study the enhanced rates of Ca^{2+} influx and efflux in mitochondria from liver and brown adipose tissue of *ob/ob* mice occurred with no change in $\Delta\psi$, it is likely that here too there is an increase in the capacity of the Ca^{2+} -transport pathways.

This contrasts with the stimulation of the Ca^{2+} -influx rate into brown-adipose-tissue mitochondria after acute exposure of mice to cold. In this situation the observed increase in $\Delta\psi$ suggests that the stimulated uptake of Ca^{2+} results from a greater protonmotive force rather than from a change in the capacity of the uniporter. As acute cold-exposure causes glucagon secretion (Seitz *et al.*, 1981), and as this hormone stimulates the mitochondrial respiratory chain in liver, with a consequent elevation of $\Delta\psi$ (Halestrap, 1978), the increased Ca^{2+} -uptake rate found in the cold-exposed animals could have been a response to glucagon.

From present data it is not possible to identify definitely the cause of the increased transport capacity for Ca^{2+} by liver and brown-adipose-tissue mitochondria of *ob/ob* mice. Nevertheless, it is likely that this altered activity will itself influence other aspects of cell metabolism. Control of the transport of Ca^{2+} into and out of mitochondria has been postulated to be a mechanism for regulating extra-mitochondrial (Nicholls, 1978) and intramitochondrial (Denton & McCormack, 1980) Ca^{2+} concentrations. With a change in the activity of the

transport machinery, the concentrations of Ca^{2+} in intracellular compartments would be modified, thus affecting the activity of Ca^{2+} -sensitive reactions. An example of this may be the impaired response to vasopressin whereby the Ca^{2+} -dependent stimulation of fatty acid oxidation appears to be absent from liver cells of *ob/ob* mice and may reflect an abnormality in mobilizing intracellular Ca^{2+} (Edwards *et al.*, 1981). Abnormalities have also been found in the calcium-dependent secretion of insulin by pancreatic β -cells of *db/db* mice (Siegel *et al.*, 1980) and in whole-body calcium metabolism in *ob/ob* mice (Kawashima & Castro, 1981), both of which could indicate modified calcium homeostasis in cells other than those from liver and brown adipose tissue.

As well as influencing cell calcium concentrations, it is still possible that an enhanced capacity for transporting Ca^{2+} into and out of mitochondria might also be a minor compensatory mechanism for generating heat through increased cycling of mitochondrial Ca^{2+} in the thermogenically defective *ob/ob* mice. With their increased capacity for Ca^{2+} uptake, it can be predicted that liver and brown-adipose-tissue mitochondria from *ob/ob* mice would tend to accumulate Ca^{2+} . Measurements of $\Delta\psi$ made during Ca^{2+} uptake (Lötscher *et al.*, 1980) do indeed indicate that Ca^{2+} cycling increases as the mitochondrial Ca^{2+} load rises.

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