Characterisation of Ca^{2+} transport activity by white adipose tissue mitochondria

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Ca^{2+} transport in mitochondria isolated from rat white adipocytes has been examined and many of the properties found to be similar to those reported for mitochondria isolated from rat liver. Ca^{2+} transport is ruthenium red-sensitive (K_i ~5 pmol.mg protein^{-1}), the affinity for free Ca^{2+} is high (K_m ~3.3 μM) and the V_{max} is 135 nmol Ca^{2+}.min^{-1}.mg protein^{-1} at 4°C with 0.2 mM P_i present. Ca^{2+} transport is stimulated by increasing the medium [P_i], and is inhibited when ATP or Mg^{2+} is added to the incubation system and in contrast to brown adipocyte mitochondria, Ca^{2+} efflux is not promoted by Na^+. White adipocyte mitochondria may play a rôle in the regulation of total cell calcium in this tissue.

Calcium-transport *Mitochondria* *Adipocyte, white* *Ruthenium red sensitivity* *(Rat liver)*

1. INTRODUCTION

A redistribution of fat cell Ca^{2+} has been reported following incubation of adipocytes with insulin [1–4] and the ion has been proposed as a possible second messenger for insulin action in adipose tissue [5,6].

Several studies have been carried out to determine which subcellular fractions might be important in regulating the redistribution of intracellular Ca^{2+} in adipose tissue. The experiments in [7] suggested that the ability of mitochondria isolated from adipocytes to transport Ca^{2+} was limited; maximal rates of Ca^{2+} transport achieved were only a few nmol.min^{-1}.mg mitochondrial protein^{-1}. In [3,4,8], the potential role of the endoplasmic reticulum and plasma membrane in insulin-induced Ca^{2+} fluxes has been emphasised in fat cells.

Because of the importance ascribed to mitochondria in the regulation of intracellular Ca^{2+} in many tissues (see [9–13]) we considered it worthwhile to re-examine the ability of adipocyte mitochondria to transport Ca^{2+}. Our results show that when due regard is given to the conditions of measurement of Ca^{2+} transport in vitro, the general characteristics of such transport are similar to those observed with a mitochondrial preparation from a well-studied tissue like rat liver. As a consequence we suggest the role of mitochondria in Ca^{2+} homeostasis in white adipose tissue may require reconsideration.

2. EXPERIMENTAL

2.1. *Preparation of fat cells and cell fractionation*

Epididymal fat pads were obtained from male Wistar rats (180–240 g body wt) fed ad libitum. The pads were placed in bicarbonate-buffered saline [14] containing 1.28 mM $CaCl_2$ at 20°C and adipocytes were isolated essentially as in [15]. Prior to homogenisation, adipocytes were washed and suspended in calcium-free bicarbonate-buffered saline containing 1% (w/v) defatted bovine serum albumin [16] previously dialyzed against Ca^{2+}-free bicarbonate buffer.

Cells were homogenised at 20°C essentially as in [17]. The homogenisation medium contained

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0.5 M sucrose, 20 mM Tris–HCl (pH 7.4), 0.5 mM EGTA, 10 mM glutathione and 2% (w/v) defatted bovine serum albumin. Cells were mixed with ~0.5 vol. homogenisation medium and disrupted by vigorous agitation on a vortex mixer for 90 s.

The disrupted cells were maintained at 4°C for 5 min, centrifuged at 3000 × *g* for 30 s and after a further 3 min at 4°C, the infranatant 'homogenate fraction' was transferred to a Corex centrifuge tube prior to differential centrifugation at 4°C in a Sorvall RC 2-B centrifuge fitted with an SS 34 angle rotor.

The fat-cell homogenate was centrifuged at 3000 × *g* for 1 min to yield a 'low speed pellet'. The supernatant (minus lipid) from this step was centrifuged at 12100 × *g* for 10 min and the resultant 'mitochondrial pellet' was resuspended to 10–15 mg/ml in 0.25 M sucrose, 20 mM Tris–HCl (pH 7.4) and 0.5 mM dithiothreitol, with the aid of a 0.1 ml glass tissue homogeniser (Vitro, USA).

2.2. *Protein estimation*

Protein was determined using a modification [18] of the Lowry method [19] using crystalline bovine serum albumin as standard.

2.3. *Assay for $^{45}Ca^{2+}$ transport*

Mitochondrial Ca^{2+} uptake was measured using the membrane filtration technique as in [20]. Assays were performed at 4°C in 0.3 ml final vol. containing 230 mM sucrose, 5 mM Hepes (pH 7.4), 5 mM KCl, 1 mM succinate and unless otherwise specified, 0.2 mM P_i. After a temperature equilibration period of 1 min, 0.3 mg mitochondrial protein and 1.0 μM rotenone were added. A further 150 s later the reaction was initiated by the addition of 50 μM total $CaCl_2$ containing 0.3 μCi $^{45}CaCl_2$. At the times specified, 50 μl samples of the reaction medium were removed and filtered under vacuum through Millipore membrane filters (HAWP, 0.45 μm porosity) which were prewashed in 0.25 M sucrose, 10 mM Hepes (pH 7.4). Filters were rinsed with 2 ml of the sucrose/Hepes wash medium, dried and dissolved in toluene:2-methoxyethanol (3:2, v/v) containing 6 g/l butyl PBD and 2% Triton X-100. $^{45}Ca^{2+}$ radioactivity was determined by liquid scintillation spectrometry using a Beckmann LS-330.

2.4. *Calcium electrode studies*

Ca^{2+}-Transport was monitored using a Radiometer F2112 calcium-sensitive electrode coupled to an Orion model 901 microprocessor ion-analyzer and a Spectra-Physics SP4100 computing integrator [21]. Assays were performed at 25°C in a 3 ml water-jacketed vessel with continuous stirring.

Mitochondria (2 mg/ml) were incubated for 3 min in 450 μl containing 230 mM sucrose, 5 mM Hepes/KOH buffer (pH 7.4), 5 mM KCl, 0.2 mM P_i, 1 μM rotenone and 5–50 μM free Ca^{2+}. The reaction was initiated after 3 min by the addition of 1 mM succinate.

The electrode was calibrated as in [22] and the calcium content of buffers was determined by atomic absorption spectroscopy [21]. Nitrilotriacetic acid buffers were used to generate accurate concentrations of free Ca^{2+} for kinetic studies [23].

2.5. *Enzyme assays*

Cytochrome *c* oxidase activity was determined by a modification [24] of the method in [25] and NADPH-cytochrome *c* reductase activity was determined as in [26].

2.6. *Materials*

$^{45}CaCl_2$ was purchased from the Radiochemical Centre (Amersham, Bucks). Dithiothreitol (Cleland's Reagent) was from Calbiochem (USA) and collagenase (crude bacterial preparation) was obtained from Worthington Biochem. (Freehold NJ). Enzymes, bovine serum albumin (fraction V) and other chemicals were from Sigma Chemical Co. (St Louis MO) or were of similar analytical-reagent grade. Corex tubes were obtained from Du Pont Instr. (Newtown CN). Ruthenium red (Calbiochem) was recrystallized prior to use [27].

3. RESULTS AND DISCUSSION

3.1. *Initial rate of $^{45}Ca^{2+}$ transport*

The experimental conditions employed in this study differed in several aspects from those employed in [7]. Here, the isolated mitochondria were resuspended in a medium of 0.25 M sucrose, 20 mM Tris–HCl (pH 7.4) and 0.5 mM dithiothreitol at 10–15 mg protein/ml (cf. 0.5 M sucrose, 20 mM Tris–HCl (pH 7.4), 2.0 mM

EGTA, 10 mM glutathione and 2% defatted bovine serum albumin and mitochondria resuspended at ~1 mg protein/ml in [7].

Another major difference was in the conditions for measuring Ca^{2+} transport in vitro. Here, a medium containing 230 mM sucrose, 5 mM KCl, 5 mM Hepes (pH 7.4), 1 mM succinate, 0.2 mM P_i, 50 μM added Ca^{2+} and 1 mg mitochondrial protein/ml was used. Incubation was at 4°C. The medium in [7] contained 125 mM KCl, 20 mM Tris–HCl (pH 7.4), 10 mM oxalate, 2 mM pyruvate, 0.5 mM malate, 100 μM Ca^{2+} and 0.11 mg mitochondrial protein/ml; incubation temperature was 37°C in [7]. Thus the Ca^{2+}:protein ratio was 910, a value which especially in the presence of a high anion concentration and temperature could well lead to early uncoupling of their mitochondria [28]. Moreover, in initial experiments we observed low rates of Ca^{2+} transport when the medium contained high [KCl] (not shown).

Fig.1a clearly indicates that mitochondria isolated from rat adipose tissue transport Ca^{2+} at high rates when isolated and assayed under the conditions described here. Initial rates of Ca^{2+} transport, calculated on the basis of an extrapolation of the amount of Ca^{2+} transported during the first 15 s of incubation, were 70.3 ± 2.4 nmol Ca^{2+} transported.min^{-1}.mg $protein^{-1}$ (n = 19). This compares with a rate of 3.6 nmol.min^{-1}.mg $protein^{-1}$ that can be calculated from the data in [7].

Fig.1a shows also that the majority of the Ca^{2+} transported was prevented when 1 nmol ruthenium red/mg protein was present in the medium. In the experiments where $^{45}Ca^{2+}$ was used (below) the ruthenium red-insensitive component of Ca^{2+} transport was subtracted from the total Ca^{2+} transport to give the value for ruthenium red-sensitive Ca^{2+} transport.

In the presence of oxidizable substrate, white adipocyte mitochondria rapidly buffer extra-

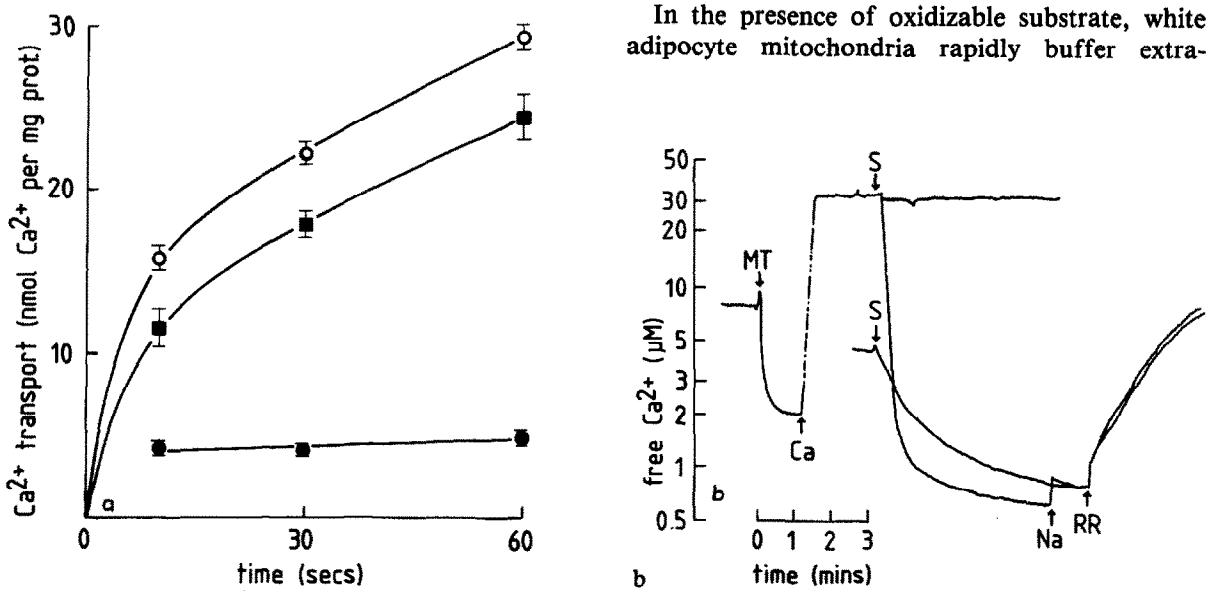

Fig.1. Calcium transport in a mitochondria-rich fraction from white adipocytes. In (a) fat cell mitochondria (1 mg/ml) were incubated in a sucrose medium at 4°C. 50 μM $^{45}Ca^{2+}$ was added and aliquots of the reaction mixture taken at the times indicated. Mitochondria were separated from the incubation medium by filtration and washed free of non-sequestered Ca^{2+} prior to assay for $^{45}Ca^{2+}$ radioactivity. Ca^{2+} transport assays were performed in the absence (○) (n = 19) or presence (●) (n = 9) of 1 nmol ruthenium red/mg protein. Results were obtained using 15 different mitochondrial preparations. Ruthenium red-sensitive Ca^{2+} transport (■) was defined as the difference between total (ruthenium red absent) and ruthenium red-insensitive (1 μg ruthenium red present) Ca^{2+} transport. In (b), fat cell mitochondria (2 mg/ml) were preincubated for 3 min at 25°C with ~5 or 35 μM free Ca^{2+}. Succinate-induced Ca^{2+} uptake was monitored using a Ca^{2+}-sensitive electrode. Additions: MT, mitochondria; S, 1 mM succinate; Na, 5 mM NaCl; RR, 3 μM ruthenium red. For further details see section 2.

mitochondrial Ca^{2+} to sub-μM levels (fig.1b). Ca^{2+} uptake is abolished by 3 μM ruthenium red which, within seconds, induces a release of sequestered Ca^{2+} indicative of a high rate of Ca^{2+}-cycling across the inner membrane of these mitochondria.

The lack of a discernible effect of Na^+ upon the rate of Ca^{2+} efflux suggests that unlike brown adipose tissue mitochondria, a Na^+-induced Ca^{2+}-efflux mechanism may not operate in mitochondria from white adipocytes (review [29]).

3.2. *Affinity for Ca^{2+}, sensitivity to ruthenium red and effect of P_i*

The affinity of the adipocyte mitochondrial Ca^{2+} transport system for Ca^{2+} can be assessed from fig.2a. In the absence of added Mg^{2+}, a hyperbolic plot (initial rate *vs* $[Ca^{2+}]$) was observed when the initial rate of $^{45}Ca^{2+}$ transport was measured as a function of the free $[Ca^{2+}]$. Half-maximal rates of Ca^{2+} transport occurred at a medium free $[Ca^{2+}]$ of 3.2–3.4 μM. Near-maximal rates (135 nmol $Ca^{2+}.min^{-1}.mg\ protein^{-1}$), were observed at 15–20 μM free Ca^{2+}. A similar high affinity for Ca^{2+} has been observed in rat liver mitochondria in the absence of added Mg^{2+} [30] and in mitochondria isolated from a variety of other tissues and species [9]. The presence of 2 mM Mg^{2+} or of 1 mM ATP reduced the initial rate of Ca^{2+} transport by ~50% (not shown). The affinity for Ca^{2+} was not determined under these circumstances.

Ca^{2+} transport activity in the adipocyte subcellular fraction studied is sensitive to very low concentrations of ruthenium red (K_i ~5 pmol/mg protein). Maximal inhibition (75–80%) of the initial rate of $^{45}Ca^{2+}$ transport was observed using >20 pmol ruthenium red/mg protein (fig.2b) suggesting that, similar to mitochondria isolated from other tissues, Ca^{2+} transport by adipocyte mitochondria proceeds largely by a ruthenium red-sensitive carrier. The data also indicate that the bulk of the Ca^{2+} transported in the subcellular fraction studied is attributable to mitochondria and not to other organelles (see below).

Ruthenium red-sensitive Ca^{2+} transport was

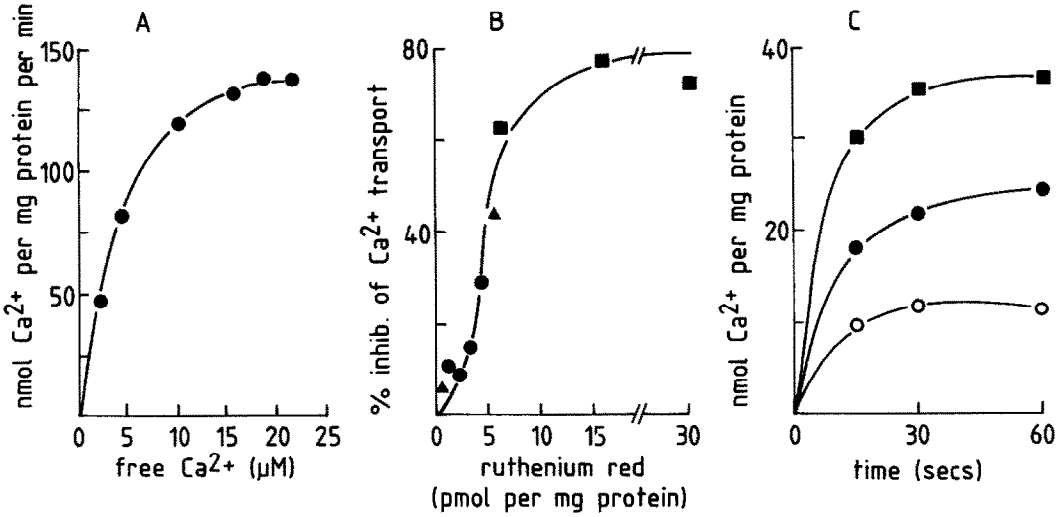

Fig.2. Characterisation of Ca^{2+} transport by white adipocyte mitochondria. (a) Ca^{2+} transport assays were performed (using a single preparation) at 28°C in the presence of 10 mM nitrilotriacetic acid. Following preincubation of mitochondria, the reaction was initiated by the addition of $^{45}Ca^{2+}$ at the concentrations indicated and the initial rate of Ca^{2+} transport determined. (b) Ca^{2+} transport assays were performed in the presence of different concentrations of ruthenium red as indicated. The initial rates of Ca^{2+} transport were determined and expressed as the percentage inhibition of control rates (i.e., with ruthenium red absent). Data were obtained from experiments performed with 3 (●, ■, ▲) different preparations of mitochondria. (c) Ruthenium red-sensitive Ca^{2+} transport by adipocytes was determined as described in the text. Incubations were performed in the absence (○), or in the presence of 0.2 mM (●) and 1.0 mM (■) phosphate.

stimulated by increasing the medium [P_i] (fig.2c). Similar findings have been reported for mitochondria from all vertebrate species studied (see reviews mentioned in section 1) although effects of phosphate on succinate oxidation cannot be excluded [31].

3.3. *Subcellular distribution of Ca^{2+} transport activity in adipocytes*

The initial rate of $^{45}Ca^{2+}$ transport was determined in 3 subcellular fractions prepared from adipocytes, the low speed pellet, the mitochondrial pellet and the post-mitochondrial supernatant (see section 2). A correlation was made between the distribution of ruthenium red-sensitive and -insensitive Ca^{2+} transport activity and enzyme markers for mitochondria (cytochrome *c* oxidase) and endoplasmic reticulum (NADPH-cytochrome *c* reductase) (see [32]).

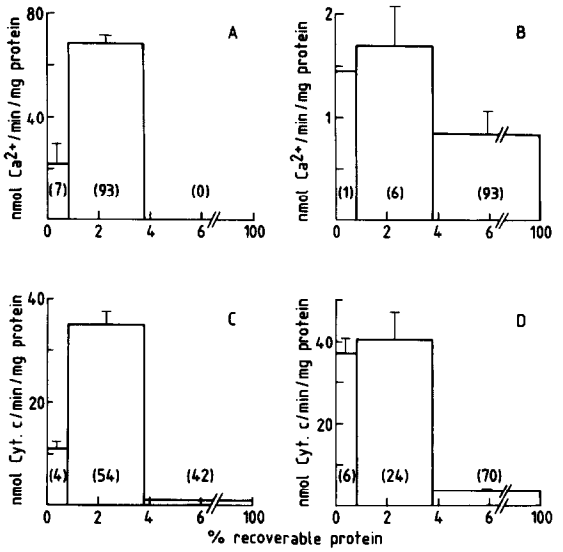

Fig.3. Subcellular distribution of ruthenium red-insensitive and -sensitive Ca^{2+} transport, cytochrome *c* oxidase and NADPH-cytochrome *c* reductase activities in various fractions from adipocytes. Subcellular fractions were isolated and Ca^{2+} transport and enzyme assays carried out as in section 2. The fractions studied were (left to right) low speed pellet, mitochondrial fraction, post mitochondrial supernatant. The activities presented are: (a) ruthenium red-sensitive Ca^{2+} transport; (b) ruthenium red-insensitive Ca^{2+} transport; (c) cytochrome *c* oxidase; (d) NADPH-cytochrome *c* reductase. Homogenate values in fig.3(a–d) are 1.2 ± 0.4, 1.0 ± 0.1, 33 ± 6 and 5.3 ± 0.9 units, respectively. Figures in brackets represent percentage of total recoverable activity present in each fraction. The data represent the mean ± SEM of up to 19 individual determinations.

In initial experiments it was found that Ca^{2+} transport by adipocyte homogenates was inhibited ~55% by ruthenium red indicating that, similar to rat liver homogenates [33], mitochondria may comprise the greatest source of in vitro Ca^{2+} accumulation in fat cell homogenates.

The fraction designated as 'mitochondrial' was found to be highly enriched in mitochondria using cytochrome oxidase activity as an index of mitochondrial recovery (fig.3c) and 93% of recoverable ruthenium red-sensitive Ca^{2+} transport activity was also located in the mitochondrial fraction (fig.3a).

Most of the recoverable NADPH-cytochrome *c* reductase activity and ruthenium red-insensitive Ca^{2+} transport activity was localised within the post-mitochondrial supernatant (fig.3b,d).

4. CONCLUDING COMMENTS

This study has shown that mitochondria isolated from rat white adipose tissue are capable of transporting Ca^{2+} at higher rates than previously realised. In most respects the general properties follow those of perhaps the most well characterised species, rat liver mitochondria. These circumstances provide a valid reason to reassess the role of mitochondrial Ca^{2+} transport in the regulation of intracellular Ca^{2+} in this tissue.

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