Mitochondrial membrane potential monitored in situ within isolated guinea pig brown adipocytes by a styryl pyridinium fluorescent indicator

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2-(Dimethylaminostyryl)-1-methyl pyridinium, a fluorescent indicator of mitochondrial membrane potential, is accumulated up to 400-fold by intact, isolated brown adipocytes from cold-adapted guinea pigs. The indicator can be optically localized within the in situ mitochondria. Ionophores which would be predicted to lower or increase the in situ mitochondrial membrane potential lead to decreased and increased uptake, respectively, of the radio-labelled indicator. Noradrenaline induces a 15 mV decrease in the sum of mitochondrial and plasma membrane potentials. The results indicate that the mitochondria within isolated brown adipocytes maintain a substantial membrane potential in the resting state, rather than being artifactually depolarized.

Brown adipocyte   *Mitochondrial membrane potential*   *Fluorescent indicator*   *Styryl pyridinium dye*
            Non-shivering thermogenesis   *Mitochondria*

1. INTRODUCTION

The currently accepted mechanism (review [1]) for the thermogenic ability of brown adipose tissue (BAT) was first proposed in 1969 [2] and involves a reversible uncoupling of mitochondria by a pathway sensitive in vitro to purine nucleotides and fatty acids. A protein [3] in the inner membrane of BAT mitochondria provides the proton conductance pathway required by the chemiosmotic modification [4] of the original hypothesis, and correlates in amount with the thermogenic capacity of the tissue [5].

While isolated mitochondria maintain a high $\Delta\psi$ as monitored with the tetraphenyl phosphonium (TPP) electrode [4,6] our experience has been that the electrode fails to register a significant potential with intact brown adipocytes (unpublished). This is in contrast to the extensive accumulation into

Abbreviations: DSMP, 2-(dimethylaminostyryl)-1-methyl pyridinium; $\Delta\psi_p$, plasma membrane potential; $\Delta\psi_m$, mitochondrial membrane potential; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone

hepatocytes [7], synaptosomes [8] or white adipocytes [9], and indicates either that the mitochondria within the isolated BAT cells are permanently (and presumably artifactually) depolarized, in which case they cannot be employed to study the regulation of the uncoupling protein in situ, or that the phosphonium ions fail to permeate the plasma membrane and equilibrate with the mitochondrial $\Delta\psi$.

We have investigated the uptake by brown adipocytes of an alternative indicator of membrane potential DSMP. Styryl pyridinium dyes have been used as indicators of the action potential of the giant axon [10] and can permeate isolated tadpole heart cells and become accumulated to a high concentration within individual mitochondria [11]. The dye has been introduced as a quantitative indicator of $\Delta\psi$ of isolated mitochondria [11–13].

Here we show that the cation is accumulated by the mitochondria within brown adipocytes from cold-adapted guinea pigs, and that a substantial mitochondrial membrane potential exists responsive to ionophores and inhibitors and possibly noradrenaline.

2. MATERIALS AND METHODS

Weaned Duncan-Hartley strain guinea pigs (3–4 weeks old) were transferred to a 4–7°C environment and sacrificed after 18–24 days of adaptation.

Adipocytes were prepared from the interscapular and perirenal brown fat as in [11]. Cell suspensions were counted in a Burker chamber, and incubations were standardized by cell number.

DSMP uptake by adipocytes was quantitated by incubating cells (2×10^5/ml incubation) in a shaking water bath at 37°C and pH 7.4 in a medium containing 110 mM NaCl, 20 mM Na_2HPO_4, 5.5 mM KCl, 1.5 mM KH_2PO_4, 1.4 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM $NaHCO_3$, 10 mM D-glucose, 10 mM fructose and 0.64 mM albumin. After varying incubation times, 200-μl aliquots were centrifuged at $10 \times g$ for 3 min in Eppendorf centrifuge tubes. The samples were immediately frozen in methanol–dry ice and the centrifuge tubes were cut underneath the cell layer. The radioactivity in the infranatant was determined.

To determine the total intra-cellular aqueous space, cells were suspended in incubation medium in the presence of 10 μCi ^3H_2O/ml and 2.5 μCi [^{14}C]sucrose/ml. Aliquots (250 μl) were taken, 500 μl of a dinonyl phthalate–Dow Corning silicone oil 550 mixture (60:40, v/v) was layered on top, and the cells were spun up through the oil by centrifugation at $10 \times g$ for 3 min. The cell layer was removed, extracted with 12% (v/v) perchloric acid, neutralized and counted. A mean space of 4.0 μl/10^6 cells was found. The mitochondrial protein per 10^6 cells was estimated by comparison of the specific activity of NAD-linked isocitric dehydrogenase activity of cells and isolated mitochondria. A value of 1.3 mg mitochondrial protein/10^6 cells was found.

Cell fluorescence was monitored in a Leitz Orthoplan with Ploem fluorescence attachment, with a <490 nm primary filter and a >510 nm secondary. Cells were incubated with 1.5 μM DSMP for 15 min prior to examination.

[^{3}H]DSMP was synthesized as in [13,15]. FCCP, oligomycin, rotenone and valinomycin were obtained from Sigma (Poole, Dorset). Nigericin was a gift from Dr P. Heytler, Eli Lilly Corp. All other reagents were of the highest purity commercially available.

3. RESULTS AND DISCUSSION

Fig.1 plots the uptake of [^{3}H]DSMP into brown adipocytes from cold-adapted guinea pigs. A steady state accumulation is attained after 20 min, the unstimulated adipocytes concentrating DSMP about 400-fold.

Fig.2 shows phase-contrast and fluorescent micrographs of typical multi-locular and unilocular adipocytes isolated from the brown fat of a cold-adapted guinea pig and incubated for 15 min with DSMP. The dye is accumulated by both types of cell, and in unilocular cells it is possible to distinguish brightly fluorescing individual mitochondria in the narrow gap between the
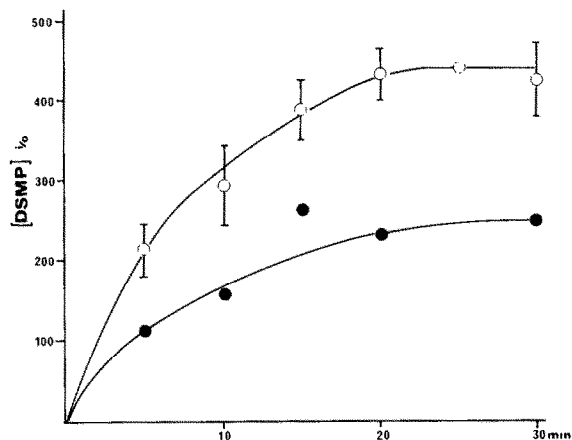

Fig.1. The time-course of uptake of [^{3}H]DSMP by brown adipocytes − the effect of noradrenaline. Cells from cold-adapted guinea pigs were incubated as described in section 2 in the presence of 0.3 μM [^{3}H]DSMP, and in the absence (○) or presence (●) of 2 μM noradrenaline. Results are the means of 5 and 2 experiments, respectively.

Fig.2. Phase-contrast (A,C) and fluorescent (B,D,E) micrographs of brown adipocytes from cold-adapted guinea pigs incubated for 15 min in the presence of 1 μM DSMP. A and B show the same multilocular cell under, respectively, phase contrast and fluorescent illumination. C and D are of the same unilocular cell. E shows a higher magnification of an individual unilocular cell with groups of highly fluorescent mitochondria.
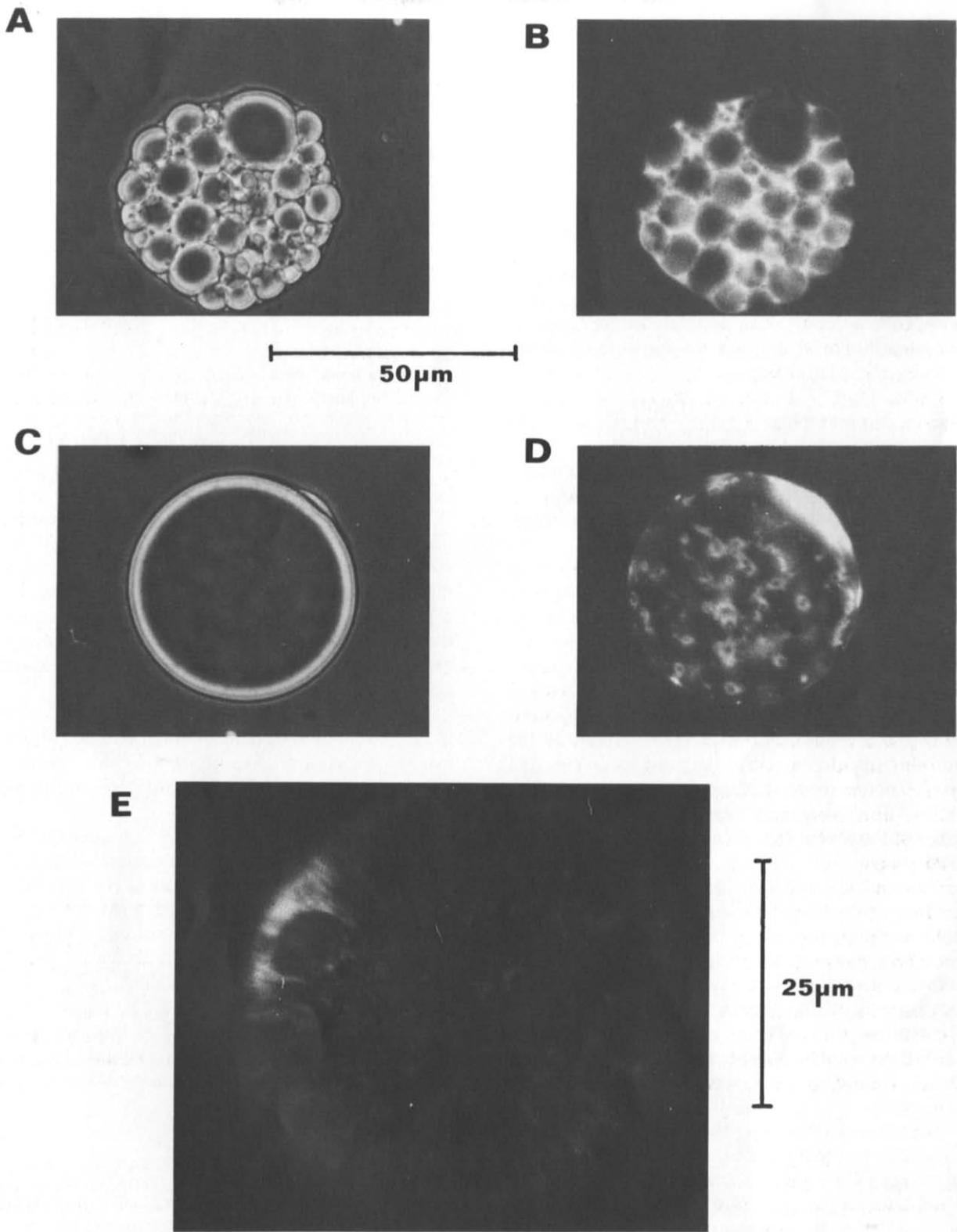
plasma membrane and the fat droplet (fig.2E). Individual mitochondria cannot be distinguished in the multilocular cells, due presumably to their greater proliferation.

As with other cells, the overall accumulation of the lipophilic cation would be predicted to be a function of both the plasma and mitochondrial membrane potentials. The fluorescence microscopy suggests that the concentration of DSMP within the mitochondria is much greater than that free in the cytosol. If cytosolic DSMP is neglected, the overall accumulation ratio of the indicator within the adipocyte can be related to the sum of the mitochondrial and plasma membrane potentials by the relationship:

$$\Delta\psi_m + \Delta\psi_p = 60 \log \left\{ \frac{A \cdot V_c \, [\text{DSMP}]_{in}}{V_m \, [\text{DSMP}]_{out}} \right\} \qquad (1)$$

where A is a factor to account for the differences in the activity coefficients of the indicators in the different compartments, V_m is the mitochondrial volume, V_c is the volume of the cytosol.

Based on the mitochondrial content for these cells of 1.3 mg protein/10^6 cells and a matrix volume of the isolated mitochondria of 0.85 μl/mg protein [4], a 400-fold accumulation implies that the sum of $\Delta\psi_m$ and $\Delta\psi_p$ is 190 mV in the control adipocytes (before correction for activity factors).

The membrane potentials across both mitochondrial and plasma membranes are collapsed by the addition of valinomycin in the presence of elevated [K$^+$]. Table 1 shows that this decreases the association of the dye to less than 10%, indicating that potential-independent binding is of minor importance.

Valinomycin added to cells in the presence of normal extra-cellular [K$^+$] depolarizes the in situ mitochondria, due to the high cytosolic [K$^+$], but would be expected to increase the plasma membrane potential to the value of the K-diffusion potential [8,16]. As seen in table 1, valinomycin in the presence of 7 mM K$^+$ causes a 60% decrease in DSMP accumulation, suggesting that the abolition of a mitochondrial membrane potential is dominant.

Nigericin diminishes the pH gradient and enhances the membrane potential of mitochondria in a high [K$^+$] milieu. Table 1 shows that 0.5 μM ionophore causes a 20% enhancement in DSMP accumulation, consistent with an enhanced

Table 1

The accumulation ratios of [^{3}H]DSMP within brown adipocytes from cold-adapted guinea pigs: the effects of modifying agents

Addition	[DSMP]$_i$/[DSMP]$_o$	% Control
Control	408	
5 μM valinomycin + 70 mM KCl	40	9.8
5 μM valinomycin + 7 mM KCl	145	35.5
0.5 μM nigericin	502	123.0
10 μM rotenone	244	59.8
2 μM noradrenaline	232	56.9
2 μM noradrenaline + 10 μM oligomycin	194	47.5

Brown adipocytes (200 000 cells/ml incubation) were incubated with 0.57 μM [^{3}H]DSMP for 20 min in the presence of the additions detailed above. Each result is the mean of 3 experiments

mitochondrial membrane potential in situ. Since nigericin's ability to exchange both Na$^+$ and K$^+$ would tend to depolarize the plasma membrane, the overall increase in cellular DSMP uptake suggests that it is responsible for the majority of the accumulation.

More direct evidence for a mitochondrial membrane potential is obtained with rotenone. Inhibition of mitochondrial respiration by this inhibitor reduces the accumulation of DSMP by 40% (table 1).

A drop in $\Delta\psi_m$ of only 20 mV is needed for the transition from controlled to uncontrolled respiration by isolated BAT mitochondria [14,17]. Fig.1 shows that noradrenaline, which provides the β-adrenergic stimulus for lipolysis and enhanced respiration (review [1]) leads to a decreased steady-state uptake of DSMP, equivalent to a 15 mV decrease in the sum of $\Delta\psi_m$ and $\Delta\psi_p$ (eq.1). If all the decrease were concentrated at the mitochondrial membrane, this could account for the stimulation of respiration which is observed with noradrenaline.

4. CONCLUSIONS

The 400-fold accumulation of the styryl pyridinium dye, the ability to locate the indicator

within the mitochondria under the fluorescent microscope, and the responsiveness to ionophores all indicate that the mitochondria within BAT cells maintain a substantial membrane potential in unstimulated brown adipocytes. Thus the isolated brown adipocyte preparation is not artifactually de-energized (as suggested by the failure to accumulate phosphonium cations) but is a viable preparation for bioenergetic studies of the action of the 32 kDa uncoupling protein in situ.

Respiration, and hence the mitochondrial proton current, increases several fold on stimulation of the cold-adapted guinea pig brown adipocyte with noradrenaline [14]. Although the present results do not establish the site of the 15 mV depolarization seen with the hormone, it is unlikely to increase (and may decrease). This implies that the effective proton conductance of the inner mitochondrial membrane must rise. As oligomycin potentiates the depolarization (table 1), the increased conductance does not involve the ATP synthetase and is consistent with the activation of the 32 kDa uncoupling protein. This work thus provides evidence for the regulated operation of the uncoupling protein within the intact brown adipocyte.

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