A RE-EVALUATION OF THE ROLE OF FATTY ACIDS IN THE PHYSIOLOGICAL REGULATION OF THE PROTON CONDUCTANCE OF BROWN ADIPOSE TISSUE MITOCHONDRIA

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1. Introduction

It is now well established that the enormous thermogenic capacity of brown adipose tissue [1,2] is a consequence of a specific modification to the proton circuit of the mitochondria [3]. An M_r 32 000 protein inserted into the inner mitochondrial membrane [4] functions as a regulatable proton short-circuit allowing respiration to be dissociated from stoicheiometric ATP synthesis [5]. In the absence of endogenous fatty acids the conductance of this protein may be inhibited specifically by addition to the incubation of low concentrations of purine nucleoside di- or triphosphates [5,6], the nucleotides binding non-covalently to the cytosolic face of the protein [7].

The conductance in vivo must be activated synchronously with the induction of thermogenesis and inhibited upon its termination in order to allow efficient energy transduction under non-thermogenic conditions. The mechanism of this acute regulation is unclear. While there is a decrease in the total ADP plus ATP content of brown adipocytes following noradrenaline induction of thermogenesis [8], the extent is slight in relation to the required increase in mitochondrial proton conductance, and it is in any case unclear whether the nucleotide decrease is the cause, or merely a secondary consequence, of the increased C_mH^+.

The fatty acids released by lipolysis, or derivatives such as acyl CoA or acyl carnitine, would provide a simple autoregulation of the proton short circuit if it could be demonstrated that they could reversibly increase C_mH^+ under plausibly physiological conditions. While fatty acids were considered as 'uncoupling' candidates in [9,10], their apparent lack of specificity led to their rejection [3,11], despite the finding that brown adipose tissue mitochondria were some 30-fold more sensitive than liver mitochondria to fatty acid-induced proton conductance increase [12]. More recently, observations that single large additions of palmitoyl CoA reverse the nucleotide-induced inhibition of swelling in KCl plus valinomycin [13] and partially overcome respiratory control [14] have been advanced as evidence for acyl CoA, rather than the free acid, as the physiological modulator of C_mH^+ [11,13,14].

Here, we re-evaluate the ability of fatty acids and their derivatives to reverse the nucleotide-induced inhibition of C_mH^+. By mimicking physiological conditions as closely as possible (i.e., by studying the effects of a controlled transition from a glycolytic substrate (pyruvate) to a controlled infusion of fatty acid) and by monitoring C_mH^+ continuously (by electrode measurements of respiration and $\Delta\Psi$) we conclude that free fatty acids (but not acyl CoA or acyl carnitine) are highly plausible physiological regulators of brown adipose tissue mitochondrial proton conductance.

Abbreviations: palmitoyl CoA, palmitoyl coenzyme A; Na-TES, sodium 2[(2-hydroxy-1,1-bis[hydroxymethyl]-ethyl)amine]-ethane sulphonate; $\Delta\mu_{H^+}$, proton electrochemical potential; $\Delta\Psi$, transmembrane potential; C_mH^+, effective proton conductance of inner mitochondrial membrane; TPP^+, tetraphenylphosphonium cation

2. Methods

Hamster brown adipose tissue mitochondria were prepared from cold-adapted animals as in [5] with a

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final resuspension in 100 mM KCl, 10 mM Na-TES to avoid matrix condensation [15]. A chamber of 1.7 ml capacity was fitted with a Clark-type oxygen electrode, a tetraphenylphosphonium (TPP^+)-selective electrode for membrane potential determination [16], and a port for the infusion of palmitate, palmitoyl CoA or palmitoyl L-carnitine, each as 10 mM ethanolic solutions. Total amounts infused did not exceed 2.5 μl. The incubations each contained mitochondria (0.5 mg protein/ml incubation), 50 mM KCl, 10 mM Na-TES, 1 mM potassium phosphate, 5 mM sodium pyruvate, 2 mM $MgCl_2$, 2 mM D,L-carnitine and 1 μg oligomycin/ml incubation, pH 7.0, 30°C. Under these conditions the predominant end products of β-oxidation are acetyl carnitine and acetate [17].

The approximate effective proton conductance of the membrane, C_mH^+ (in nmol $H^+ . min^{-1} . mg\ protein^{-1} . mV\ \Delta\mu_{H^+}{}^{-1}$ [5]) was calculated assuming a H^+/O stoicheiometry for both pyruvate and palmitoyl carnitine oxidation of 7, and ignoring the contribution of the transmembrane pH gradient to the total $\Delta\mu_{H^+}$.

The TPP^+ electrode was calibrated before each incubation. Binding of TPP^+ to the mitochondria was corrected for in a separate experiment by comparing the accumulation of [^{3}H]TPP^+ and $^{86}Rb^+$ in the presence of valinomycin as in [18].

[^{3}H]TPP^+ was a gift from Dr R. Kaback, Roche Institute (Nutley NJ). All other reagents were of the highest available purity.

3. Results and discussion

In fig.1 the $\Delta\Psi$ maintained by brown adipose tissue mitochondria oxidizing pyruvate is plotted as a function of ATP concentration, ATP being chosen as the predominant cytosolic purine nucleotide [8]. During the initial few minutes of the incubation endogenous fatty acids were oxidized [10], 100 μM ATP being present together with CoA and carnitine. Further ATP was added after this oxidation was complete and the subsequent steady state potential was recorded, with pyruvate as the remaining substrate. It is clear that under these conditions quite high concentrations of ATP are required for development of an optimal $\Delta\Psi$, i.e., for maximal inhibition of C_mH^+.

In fig.2 the effect of a single addition of 23 nmol palmitate/mg protein is shown upon the $\Delta\Psi$ and respiration of mitochondria oxidizing pyruvate in the

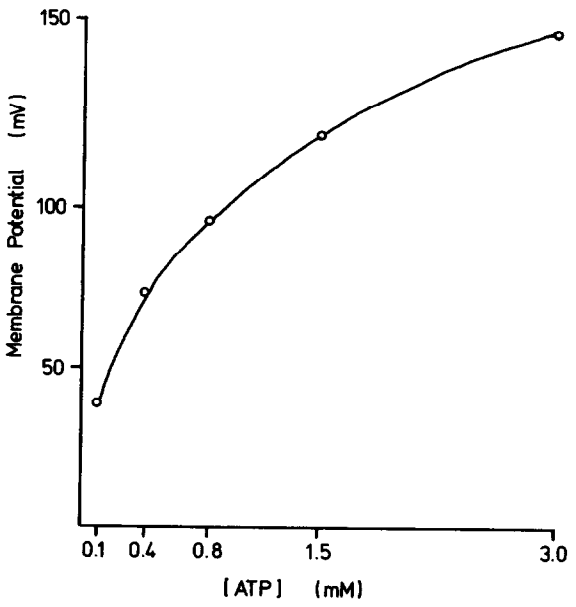

Fig.1. Membrane potential maintained by pyruvate oxidation in the absence of fatty acids. Mitochondria were incubated in the basic medium (see section 2) with the initial addition of 100 μM ATP to allow oxidation of endogenous fatty acids. Further additions of ATP were then made as indicated, and the steady-state $\Delta\Psi$ was determined.

presence of 3 mM ATP. The fatty acid causes a 6-fold increase in respiration and an instantaneous depolarization of the membrane to ~70 mV. If the ΔpH component of $\Delta\mu_{H^+}$ is ignored, this is indicative of an increase in C_mH^+ from 1.6–20 nmol $H^+ . min^{-1} . mg\ protein^{-1} . mV^{-1}$. Clearly therefore a single large addition of palmitate can cause a substantial increase in C_mH^+ even in the presence of 3 mM ATP, an increase which is reversible since a high $\Delta\Psi$ and low respiration are restored when oxidation of the palmitate is complete. This figure also shows that $\Delta\Psi$ rises slowly during the first 3 min when endogenous fatty acids are being oxidized [10].

Although fig.2 shows that palmitate, or one of its metabolites, can effect the proton conductance, the conditions are still unphysiological, since fatty acids would be supplied to the mitochondrion at a steady rate, rather than as a single large aliquot. The experiments shown in fig.3 were therefore designed to study the effect on C_mH^+ of a steady controlled infusion of fatty acid, in order to avoid exposing the mitochondria to unrealistically high free fatty acid concentrations.

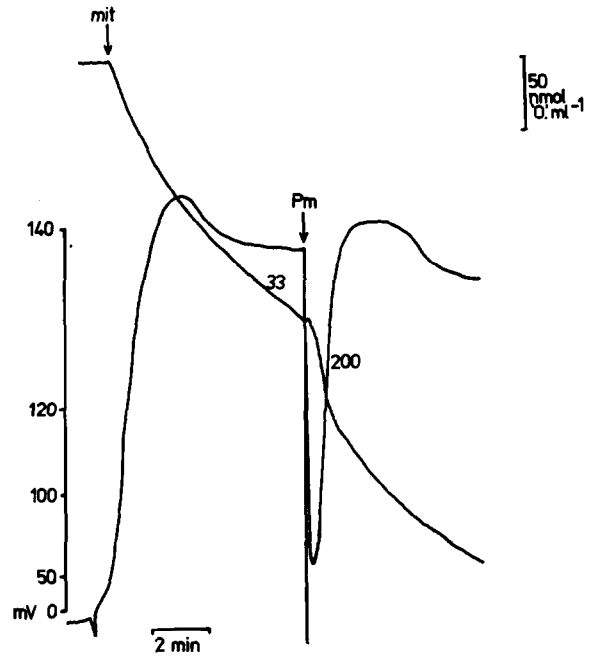

Fig.2. The effect of a single addition of palmitate on $\Delta\Psi$ and respiration. Mitochondria were incubated in the basic medium with addition of 3 mM ATP and allowed to attain a steady-state potential. Palmitate (23 nmol/mg protein) was added where indicated (Pm). Numbers refer to respiration rates (nmol O . min^{-1} . mg $protein^{-1}$).

Palmitate infusion (fig.3a) shows a number of distinctive features:

(i) There is no detectable delay between the initiation of infusion and the start of membrane depolarization. This implies that there is no significant threshold concentration of palmitate (or metabolite) which must be exceeded before an effect on C_mH^+ can be detected;

(ii) There is a perceptable lag before respiration becomes maximal, possibly because of the necessity to accumulate sufficient palmitoyl carnitine for maximal β-oxidation;

(iii) A depolarization of only ~10 mV is sufficient for rapid respiration, in agreement with other studies showing that a modest decrease in $\Delta\mu_{H^+}$ can allow uncontrolled respiration [5,19];

(iv) During the infusion a state is reached at which the respiratory rate and $\Delta\Psi$ become essentially constant, implying a steady state concentration of the 'uncoupling' intermediate responsible for increasing C_mH^+;

(v) As soon as the infusion is stopped, the membrane starts to repolarize, presumably as the concentration of the 'uncoupling' intermediate decreases due to further metabolism;

(vi) Finally respiration returns to pre-infusion rates,

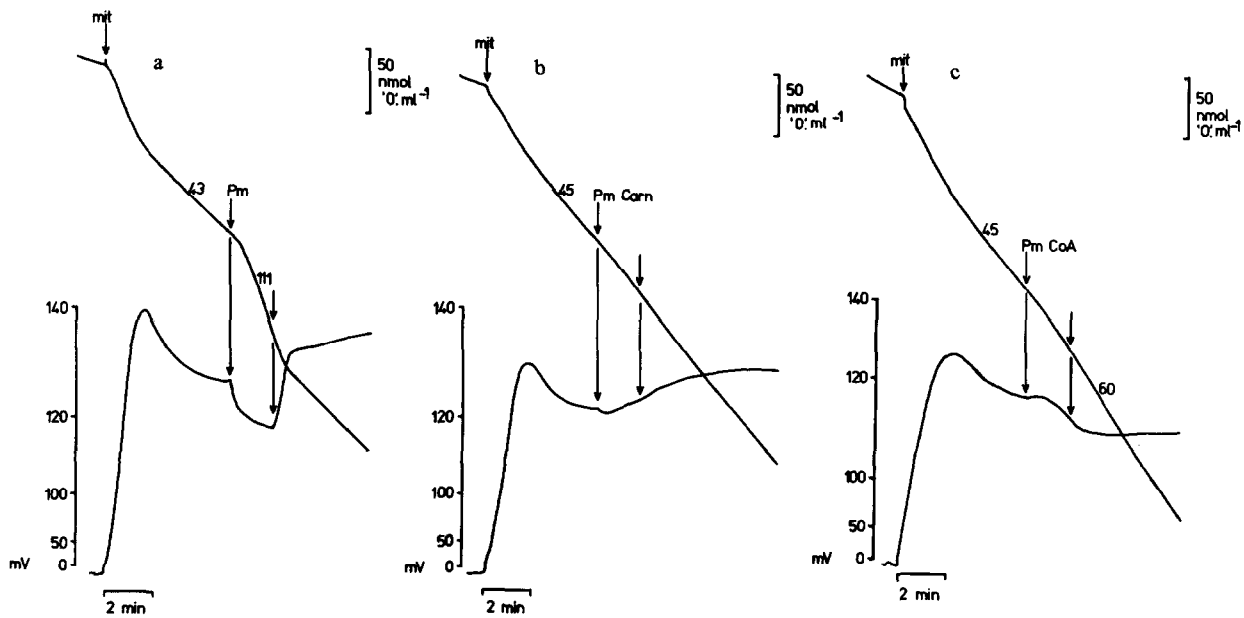

Fig.3. The effect of infusing palmitate, palmitoyl L-carnitine or palmitoyl CoA on $\Delta\Psi$ and respiration. Mitochondria were incubated in the basic medium with addition of 3 mM ATP. Palmitate (Pm), palmitoyl L-carnitine (PmCn) or palmitoyl CoA (PmCoA) were infused at 17 nmol . min^{-1} . mg $protein^{-1}$ for the period between the arrows.

either because the total long chain acyl derivatives are exhausted, or because the 'uncoupling' intermediate has disappeared.

Under these near physiological conditions it therefore appears that fatty acid, or an immediate derivative, is a sufficient messenger to reverse the inhibition of C_mH^+ induced by mM ATP levels. There would therefore appear to be no necessity to search for an independent 'second messenger' to modulate the induction and termination of a high conductance state required for non-shivering thermogenesis.

If the actual 'uncoupling' intermediate were acyl CoA or acyl carnitine, rather than the free fatty acid, it should be possible to obtain an identical conductance increase when these intermediates are infused into the incubation. This is investigated in fig.3a,b. When palmitoyl L-carnitine is infused at the same rate as palmitate only a very slight depolarization is seen, followed by a slow hyperpolarization which is maintained for the duration of the experiment. Most importantly there is little increase in respiration. Palmitoyl L-carnitine appears therefore not to increase the membrane proton conductance. The hyperpolarization, like the overshoot in potential which is seen as the last of the endogenous fatty acids are oxidized (fig.2,3) may suggest that β-oxidation can maintain a slightly higher $\Delta\Psi$ than pyruvate oxidation.

When palmitoyl CoA is infused no immediate depolarization is seen (fig.3c). Instead an initial slight hyperpolarization is followed by a slow depolarization which becomes significant only after 1 min, when 17 nmol acyl CoA . mg protein have been infused. The slight respiratory response implies that palmitoyl CoA is accumulating rather than being oxidized, while the failure of the potential to return upon termination of the infusion is suggestive of detergent-induced membrane damage (contrast the hyperpolarization induced by palmitoyl carnitine under parallel conditions, fig.3b). In any case it is apparent that palmitoyl CoA is much less effective than palmitate in mediating a reversible conductance increase.

The conclusion from these experiments is that free fatty acid is the most plausible 'uncoupling intermediate'. As well as confirming early hypotheses [9,10], this is in agreement with our observation [12] that free fatty acid can increase C_mH^+ in the presence of GDP under conditions where there is no possibility of activation to acyl CoA. It remains to establish whether the fatty acids are interacting directly with the 32 000 M_r protein, and if so by what mechanism.

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