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Carnitine cycle in brown adipose tissue mitochondria as a tool for studying the regulatory role of fatty acids in the uncoupling protein function

Petr Ježek, Inna P. Krasinskaya*, Ira Smirnova* and Zdeněk Drahota

Institute of Physiology, Czechoslovak Academy of Sciences, Vídeňská 1083, 14220 Prague, Czechoslovakia and **Department of Bioenergetics, A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 117234, USSR*

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A concerted function of purine nucleotide (PN) binding and fatty acid (FA) release from the uncoupling protein (UP) resulting in the maximum coupling (potential) of brown adipose tissue (BAT) mitochondria was demonstrated. The uncoupling effect of FA was studied (at 4 mM $MgCl_2$): 17 nmol oleate per mg protein caused a slight uncoupling with 8.9 mM ATP but with ATP below 3.6 mM almost total uncoupling was achieved. This shows that the PN-controlled gate can be stabilized in the closed conformation (with 8.9 mM ATP), also when FA is bound to UP. The sensitivity of the FA effect to ATP proves that oleate directly interacts with UP. The closed conformation of the H^+ channel of UP is then abolished by oleate when a lower free ATP concentration is maintained outside.

Fatty acid uncoupling; Uncoupling protein; Brown adipose tissue mitochondria; H^+ channel

1. INTRODUCTION

Brown adipose tissue mitochondria represent a valuable model in bioenergetics [1]. Apart from other types of mitochondria they possess the uncoupling protein (UP), a unique proton translocator [2,3], converting $\Delta\tilde{\mu}H^+$ into heat. H^+ fluxes through the UP are inhibited by purine nucleotides [4–8] and activated by fatty acids [3,8–10]. Both regulatory mechanisms were also demonstrated in a reconstituted system [3], but the activation by fatty acids was disputed in another laboratory [2]. The direct proof of the existence of the putative FA-binding site on UP is still lacking. FA do not bind at the purine nucleotide-binding site on UP because the effect of their removal on H^+ transport is preserved when the nucleotide binding is prevented by chemical modification [6]. It remains to be determined which of these two regulatory mechanisms acts as the primary initiator of thermogenesis [7,11] and whether they are interrelated. The latter can be deduced from the inability of purine nucleotides to fully inhibit H^+ transport via UP in the presence of FA in respiring BAT mitochondria [1,5,8,12].

The regulatory role of FA, however, is not exclusive to brown adipose tissue. The unique regulation of UP function by FA is only one example of the messenger function of fatty acids. They regulate the degree of coupling in mitochondria in intact liver [13] which indicates the existence of some components which interact directly with FA [14]. Among the possible candidates are the adenine nucleotide translocator [15], proton pumps [14] or bc_1 complex [16]. Moreover, FA may play a role as endogenous natural protonophores in biological membranes [17] which is

Correspondence address: Z. Drahota, Institute of Physiology, Czechoslovak Academy of Sciences, Vídeňská 1083, 14220 Prague, Czechoslovakia

Abbreviations: BAT, brown adipose tissue; DASMPI, 2-(*p*-dimethylaminostyryl)-1-methylpyridinium iodide; FA, fatty acid(s); PN, purine nucleotide

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extremely important in considering dissipative H^+ currents in mitochondria lacking UP. The stronger uncoupling effect of FA in BAT mitochondria clearly reflects activation of H^+ transport through UP and correlates with the exclusive occurrence of UP in BAT [9,10]. In our work we used a controlled removal of endogenous fatty acid from BAT mitochondria by a carnitine cycle [8,9,18–20] in order to estimate the interrelationship between effects of purine nucleotides and fatty acids on UP function.

2. MATERIALS AND METHODS

Most of the chemicals were purchased from Sigma (USA); palmitoyl-L-carnitine was from Otsuka (Japan). 2-(*p*-Dimethylaminostyryl)-1-methylpyridinium iodide was a kind gift from Professor J. Rafael, University of Heidelberg (FRG).

Brown fat mitochondria were isolated from cold-adapted hamsters (at least two weeks at 5°C) according to [20]. The protein content was assayed by Lowry's method with albumin as standard. The membrane potential was monitored by the fluorescent probe DASMPI according to Rafael [21,22] in a medium containing 20 mM KCl, 20 mM Tris-Cl, 4 mM $MgCl_2$, 2 mM KP_i, 7 mM K-malate, pH 6.6 or 7.8. 2 nmol DASMPI per mg mitochondrial protein (1 mg per ml) were added and fluorescence increase resulted from the accumulation of $DASMP^+$ cation in the matrix due to increasing negative charge inside has been taken as a relative measure of membrane potential [21–23]. The carnitine cycle was induced usually with 2.9 mM L-carnitine and 7 μM CoA at various concentrations of ATP. Estimation of mitochondrial respiration rate was done polarographically with a Clark electrode.

3. RESULTS AND DISCUSSION

To establish a state of tight coupling and, consequently, the highest protonmotive force in BAT mitochondria we employed the so-called carnitine cycle [9,18–20,24,25] to oxidize endogenous fatty acids after addition of ATP, CoA and carnitine to mitochondria respiring with malate as the additive substrate. This results in a rapid increase of membrane potential (fig.1). The tight coupling is reversed by uncoupler and negligible uncoupling is observed when palmitoyl-carnitine or palmitoyl-CoA was added (not shown). On decreasing the ATP concentration from 8.9 mM to 3.6 mM at constant 4 mM Mg^{2+} we observed no decrease in the maximum potential reached by the carnitine cycle and when ATP was further diminished to 180 μM the maximum decreased below 100 mV

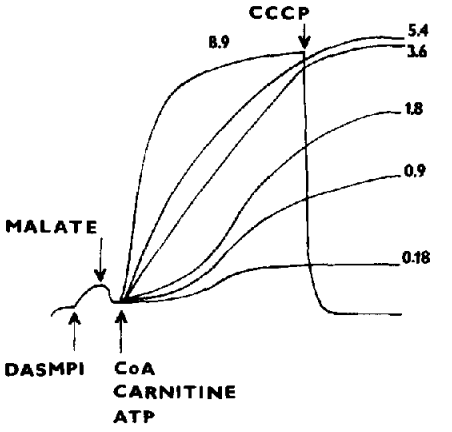

Fig.1. Membrane potential induced by the carnitine cycle in brown adipose tissue mitochondria at various millimolar concentrations of ATP (indicated by numbers) monitored by DASMPI fluorescence. Measured in 20 mM KCl, 20 mM Tris-Cl, 4 mM $MgCl_2$, 2 mM KP_i, 7 mM K-malate, pH 6.6, with 2.9 mM L-carnitine and 7 μM CoA.

(fig.1). The rates of potential elevation were slower with decreasing ATP concentrations (fig.1).

The enzyme removing fatty acids within this cycle, an acyl-CoA synthetase, requires one mol of ATP [24] per mol FA, therefore, one cannot judge whether the potential burst is due to ATP inhibition of the H^+ transport through UP or due to fatty acid removal. However, the following experiments provide evidence that both are required to obtain maximum coupling. When we used 1.8 mM ADP with a comparable inhibitory ability with ATP [1] but not a substrate of acyl-CoA synthetase (1.8 mM AMP was further included to inhibit adenylate kinase) no potential elevation occurred until ATP was added (fig.2a). Similarly, when 2 mM GDP instead of ATP was added a slight fluorescence increase (fig.2b) indicated that the established potential did not exceed 100 mV [21–23], whereas the maximum in tightly coupled BAT mitochondria exceeds 160 mV [5,8,9,21,23]. Subsequent addition of 180 μM ATP, which does not induce the maximum potential (cf. fig.1), resulted in a rapid potential burst to the maximum (fig.2b). Thus neither GDP nor the other purine nucleotides can induce full coupling in BAT mitochondria without removal of FA. In contrast, the simple removal of FA does not result in full coupling as implicated by the in-

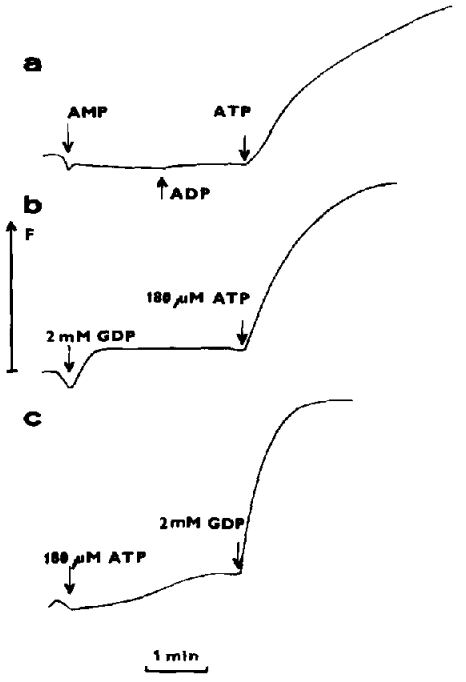

Fig.2. Demonstration of the necessity of both purine nucleotide addition and removal of endogenous fatty acid to obtain a maximum potential in respiring BAT mitochondria. (a) Effect of AMP (1.8 mM) and ADP (1.8 mM), or 2 mM GDP; (b) without removal of endogenous FA; (c) almost exclusive effect of FA removal (at 180 μM ATP). Further additions necessary for attaining a maximum are indicated.

ability of 180 μM ATP to induce the maximum potential. The maximum can be attained only after subsequent addition of 2 mM GDP (fig.2c).

Further, we investigated the uncoupling effect of fatty acids added in this tightly coupled state. Addition of 17 nmol oleate per mg protein resulted in uncoupling of mitochondria [8,9] as indicated by a fluorescence decrease (fig.3a). However, the remaining potential is much higher when a high ATP concentration was used in the cycle, while with lower ATP, uncoupling by oleate suppresses the membrane potential to a low value (fig.3b). Such extensive uncoupling was not reported when a slow infusion of oleate was performed [8]. Recently, an intensive uncoupling of rat liver mitochondria by 200 nmol oleate per mg protein was described; however, it was negligible at 30 nmol oleate per mg protein [14]. Clearly, BAT mitochondria are more sensitive to oleate due to oleate interaction with UP.

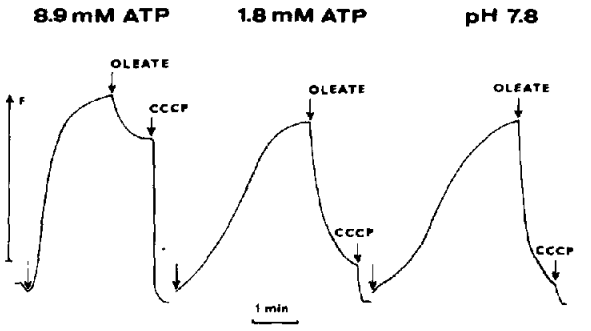

Fig.3. Uncoupling with 17 nmol oleate per mg protein in brown adipose tissue mitochondria coupled by the carnitine cycle with 8.9 mM ATP (a); 1.8 mM ATP, pH 6.6 (b); or with 8.9 mM ATP at pH 7.8 (c). Arrows without specifications indicate addition of carnitine, CoA and ATP; the others indicate addition of 5 μM CCCP.

The existence of such an interaction is also confirmed by disappearance of the intensive oleate uncoupling in the presence of excess of ATP (fig.3a) or GDP. Inhibition of UP H^+ channels by these nucleotides must be retained here in the presence of oleate as they had generated (together with FA removal) the highest potential and as they cannot be metabolized. Hence, the incremental H^+ permeability activated by oleate at lower ATP can be inhibited by higher ATP or GDP which proves a direct interaction of oleate with UP. Thus nucleotides at a high concentration prevent the intensive uncoupling effect of oleate.

To prove that ATP inhibition of UP H^+ channels in the presence of oleate has taken place, disappearance of such inhibition at alkaline pH must be observed because gating by nucleotides is much less intensive with increasing pH [5,7]. Fig.3c shows this to be true. The uncoupling by oleate is very pronounced at pH 7.8 in spite of the presence of 8.9 mM ATP.

We might consider that the small uncoupling effect of oleate at high ATP results predominantly from a protonophoric action of oleate or from other regulatory mechanisms exerted by oleate on other membrane proteins. Such effects are common in all types of mitochondria [14]. The protonophoric action of oleate could be simulated by the addition of 10 nM CCCP.

The remaining higher potential in the presence of oleate and high ATP indicates that the purine-nucleotide-controlled gate of the UP H^+ channel is

stabilized in the closed conformation and that binding of FA to another (putative) binding site on UP does not abolish it. However, when the free ATP concentration is lowered, oleate mediates the opening of a portion of H^+ channels which are probably liganded by nucleotides [12]. Similar binding without inhibition has already been reported [6,26]; this too was reversed to the usual closed channel conformation with bound ligand on increasing the nucleotide concentration. Interaction of oleate with UP would establish a different threshold nucleotide concentration below which the nucleotide is bound but cannot inhibit the H^+ transport through UP.

One can assume the purine-nucleotide-controlled gate and the gate controlled by fatty acid to be placed in series within the structure of the H^+ channel [7]. Their conformations are primarily dependent on the binding (releasing) of the ligands from the corresponding binding sites but can affect each other. Thus a concerted function of two gates of UP (or one gate controlled from two binding sites) leads to the maximum potential established at the membrane and the reversal of such a concerted action is sufficient for total uncoupling.

Our results also suggest that the two regulatory levels are equally important for the regulation of thermogenesis in BAT. Norepinephrine-released FA cannot open the H^+ channels before the ATP concentration decreases to a certain level (given by pH and Mg^{2+}) and a simple ATP increase without metabolism of FA cannot induce the fully coupled (resting) state.

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