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Alkylsulfonates activate the uncoupling protein UCP1: implications for the transport mechanism

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Abstract

Fatty acids activate the uncoupling protein UCP1 by a still controversial mechanism. Two models have been put forward where the fatty acid operates as either substrate (‘‘fatty acid cycling hypothesis’’) or prosthetic group (‘‘proton buffering model’’). Two sets of experiments that should help to discriminate between the two hypothetical mechanisms are presented. We show that undecanosulfonate activates UCP1 in respiring mitochondria under conditions identical to those required for the activation by fatty acids. Since alkylsulfonates cannot cross the lipid bilayer, these experiments rule out the fatty acid cycling hypothesis as the mechanism of uncoupling. We also demonstrate that without added nucleotides and upon careful removal of endogenous fatty acids, brown adipose tissue (BAT) mitochondria from cold-adapted hamsters respire at the full uncoupled rate. Addition of nucleotides lower the respiratory rate tenfold. The high activity observed in the absence of the two regulatory ligands is an indication that UCP1 displays an intrinsic proton conductance that is fatty acid-independent. We propose that the fatty acid uncoupling mediated by other members of the mitochondrial transporter family probably involves a carrier to pore transition and therefore has little in common with the activation of UCP1.

Keywords: UCP1; Uncoupling protein; Mitochondria; Fatty acid; Brown adipose tissue; Permeability transition

1. Introduction

The uncoupling proteins (UCPs) are a family of mitochondrial transporters that mediate a regulated discharge of the proton gradient generated by the respiratory chain (reviewed in Refs. [1,2]). Mitochondrial uncoupling can serve functions like thermogenesis, maintenance of the redox balance or diminishing the production of reactive oxygen species (ROS). Genes coding for UCPs are widely distributed both in animals and plants (reviewed in Ref. [1]). The UCP from brown adipose tissue (BAT) UCP1 has a fundamental role in adaptative thermogenesis. Discovered 25 years ago, it is the best characterized member of the family. The physiological regulation of the protein is well established (reviewed in Ref. [3]). Under non-thermogenic conditions, purine nucleotides maintain UCP1 inhibited. Noradrenaline stimulation of the brown adipocyte initiates a lipolytic cascade and the released fatty acids serve two functions: they are substrates for respiration and activators of UCP1. Fatty acids override the nucleotide inhibition of UCP1 to activate proton transport and thus initiate thermogenesis.

The molecular mechanism of transport is still a matter of debate. The earliest experiments on the bioenergetic properties of BAT mitochondria revealed an unusual permeability to halide anions that could be inhibited with purine nucleotides [4]. This anion permeability was soon related to the thermogenic pathway and it was rationalized by proposing that UCP1 was a hydroxyl ion transporter rather than a proton carrier. When the transport properties were reinvestigated years later, it was shown that UCP1 could catalyze the translocation of a wide variety of anions [5] and led to the proposal that UCP1 could also transport the fatty acid anion. A mechanism was then proposed (‘‘the fatty acid cycling hypothesis’’) to explain the dissipation of the proton gradient: the protonated fatty acid would flip-flop

Abbreviations: AAC, ADP/ATP carrier; AGC, aspartate/glutamate carrier; BAT, brown adipose tissue; BSA, bovine serum albumin; C5S, pentanesulfonate; C11S, undecanosulfonate; DiC, dicarboxylate carrier; PiC, phosphate carrier; UCP, uncoupling protein

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in the membrane to deliver a proton to the matrix side and UCP1 would catalyze the return of the fatty acid anion to the cytosolic side of the membrane [6,7]. However, there is no consensus on the physiological relevance of the anion transport activity. Other groups have proposed that UCP1 is a proton carrier and that fatty acids increase its proton conductance [8,9]. According to this alternative mechanism (''proton buffering model'') fatty acids would act as a prosthetic group in UCP1: the carboxylate would bind protons and deliver them to a site from which they are translocated to the other side of the membrane. In this model, the anion permeability would have no physiological relevance.

In the present paper, we re-examine two issues that we consider critical to discriminate between the two models for the regulation of UCP1. The first one addresses the transport properties of UCP1 without fatty acids and nucleotides. We demonstrate that in the absence of nucleotides and despite thorough albumin treatments to remove traces of endogenous fatty acids, the uncoupling activity of UCP1 is maximum. This confirms early experiments performed with BAT mitochondria [10,11] which suggested that UCP1 possessed a native proton transport activity that did not require fatty acids and questions recent claims made in the literature [6]. The second issue concerns the ability of alkylsulfonates to activate UCP1. We show that undecanesulfonate (C11S) can activate UCP1 just like fatty acids. Since liposome studies have shown that alkylsulfonates cannot flip-flop [12], these results imply that the fatty acid cycling mechanism cannot be the underlying transport mechanism in UCP1.

2. Materials and methods

2.1. Mitochondrial preparation

Mitochondria were prepared from the brown adipose tissue of male adult Syrian hamsters which had been cold-adapted at 6 °C for at least 10 days prior to sacrifice. Animals were exposed to a 12-h light/12-h dark cycle with food and water ad libitum. The excised tissue was placed in ice-cold buffer containing 250 mM sucrose, 1 mM EDTA, 10 mM Tes (Na salt) pH 7.4 and carefully cleaned of extraneous tissue. When the effect of endogenous fatty acids was investigated, the tissue dissected from the left half of the animal was placed on the above buffer while the tissue collected from the right side was placed on buffer supplemented either with 10 or 50 mg/ml of bovine serum albumin (BSA) (fatty acid content less than 0.02%). The tissue samples were homogenized in their respective buffers at 4 °C and then passed through gauze. The final volume of the homogenate was 25–30 ml. First centrifugation was at $8000 \times g$ for 10 min, the supernatant removed and the fat layer deposited on the tube wall carefully eliminated. The pellet was resuspended in 30 ml of 250 mM sucrose, 5 or 10 mg/ml BSA,10 mM Tes (Na salt) pH 7.4 and spun at $800 \times g$. The supernatant was collected and centrifuged at $8000 \times g$ for 10 min and pellet resuspended in the same buffer. This step was repeated twice and the resulting pellet was resuspended in 1 ml of 50 mM KCl, 10 mM Tes pH 7.0 and 5 or 10 mg/ml BSA when required and kept on ice. Protein concentration was determined with the modified Lowry method in the presence of Triton X-100 and SDS using BSA as standard.

2.2. Measurement of mitochondrial respiration

The rates of oxygen consumption were determined with a Hansa-Tech oxygen electrode. Most experiments were performed in our standard buffer containing 50 mM KCl, 1 mM EGTA, 2 mM potassium phosphate, 2 mM $MgCl_2$, 10 mM KTes pH 7.0, BSA 1 mg/ml with 5 mM pyruvate plus 5 mM malate as substrates. Other additions are described in the figure legends. Protein concentration was 0.3 mg/ml and temperature 25 °C. Experiments designed to test the activation of UCP1 by alkylsulfonates were performed either in our standard buffer for respiration supplemented with 1 μM cyclosporin A and 0.3 mM GDP or in a buffer similar to the one described in Ref. [3]: 50 mM KCl, 4 mM potassium phosphate, 20 mM KTes pH 7.2, 1 mM EDTA, 2 mM $MgCl_2$, 1 μM cyclosporin A, 10 μM CoA, 1 mM carnitine, 0.3 mM GDP, 0.2 mM ATP and 1mg/ml BSA. Pyruvate and malate were also used as substrates. Addition of the alkylsulfonate was performed once the rate of respiration had come to a stable rate after the oxidation of the endogenous fatty acids. Undecanesulfonate was from Lancaster (Morecambe, England) and pentanesulfonate was from Aldrich. The rest of the reagents were from Sigma and of the highest purity available.

3. Results and discussion

3.1. The activity of UCP1 in the absence of fatty acids

The transport activity of the UCP1 in the absence of fatty acids is of striking importance since it has deep mechanistic implications. If the uncoupling observed in BAT mitochondria relies on the fatty acid transport activity of the UCP1, in the absence of fatty acids there would be no uncoupling. On the other hand, if UCP1 is a proton carrier and fatty acids were merely activators, they would not be essential. The unique fatty acid sensitivity of BAT mitochondria was recognized some 40 years ago (reviewed in Refs. [13,14]). It was described that in order to observe energy conservation it was necessary not only for the complete removal of free fatty acids but also the addition of purine nucleotides [15]. In 1998, Garlid et al. [6] argued that the observation of partially uncoupled BAT mitochondria, in the absence of nucleotides but in the presence of albumin, was an indication of the existence of residual fatty acids bound to the mitochondrial

membranes. These fatty acids presumably bind to mitochondria during homogenization and they assumed that, due to a hysteresis effect, albumin was unable to remove them afterward. The authors stated that if 2–5 mg/ml albumin were present during homogenization and the rest of the isolation process, the uncoupling was reduced to undetectable levels. Data were not presented and a previously published article used as reference [16] does not contain such data. Interestingly, this article does present a comparison between proteoliposome preparations made either with BSA-treated BAT mitochondria or without the albumin treatment. The proton fluxes measured revealed that BSA only caused a modest reduction (35%) in proton conductance [16]. In any case, experiments with BAT mitochondria were designed in the 1960s precisely to address the problem of endogenous fatty acids and thus 6 mg/ml albumin were used during homogenization [10], and there were also claims that the use of up to 25 mg/ml yielded no significant improvement in oxidative phosphorylation [14].

The availability of high-quality BSA that is essentially fatty acid-free prompted us to re-examine these claims. We have performed the suggested experiments initially homogenizing the tissue in the presence of 10 mg/ml albumin, but since results did not demonstrate any improvement in coupling, we increased the albumin concentration during homogenization up to 50 mg/ml (Table 1 and Fig. 1). Furthermore, to minimize the build up of endogenous fatty acids due to phospholipase activity, experiments were carried out immediately after isolation of mitochondria, i.e. they were completed less than 1 h after the isolation. Table 1 presents the results of such experiments and even with the highest concentrations of albumin (50 mg/ml during homogenization and 10 mg/ml during the rest of the isolation process), the initial rates of respiration approximate those observed in the presence of FCCP. Addition of GDP lowers the rate of respiration almost tenfold. The initial rate of respiration will depend on the concentration of UCP1. We have used hamsters that had been maintained at 6 °C for 10–14 days prior to sacrifice and the UCP1 content should be around 1 nmol/mg mitochondrial protein [17]. The low levels of uncoupling claimed by Garlid et al. could be explained if, as it appears, they use hamsters maintained at room temperature (reference to the housing of animals only found in Ref. [18]).

Table 1
Rate of respiration of BAT mitochondria isolated in the presence of varying amounts of fatty acid-free BSA

Homogenization	NO BSA	10 mg/ml	50 mg/ml
Isolation	5 mg/ml	5 mg/ml	10 mg/ml
Initial rate	345 ± 10	380 ± 10	271 ± 43
GDP inhibited rate	37.8 ± 5.1	33.2 ± 3.0	35.4 ± 2.1
FCCP rate	303 ± 17	314 ± 13	302 ± 35

Measurements were performed less than 15 min after isolation. Respiration buffer in all cases contained 1 mg/ml albumin. Values are the mean ± S.E. of three to five independent determinations performed in duplicate and are expressed in nmol 'O' consumed per minute and milligram of mitochondrial protein. GDP was 3 mM and FCCP 10 μM.

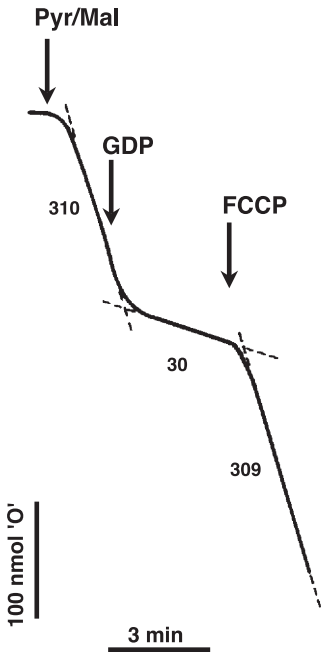

Fig. 1. Representative oxygen electrode trace of the respiration of BAT mitochondria in the absence of fatty acids. BAT was homogenized in 50 mg/ml BSA and mitochondria isolated in the presence of 10 mg/ml BSA. Immediately after isolation, they were placed in the electrode chamber in a medium containing 50 mM KCl, 1 mM EGTA, 2 mM potassium phosphate, 2 mM $MgCl_2$, 10 mM KTes pH 7.0, BSA 1 mg/ml. Respiration was initiated with the addition of 5 mM pyruvate plus 5 mM malate. GDP (3 mM) and FCCP (10 μM) were added as indicated by the arrows. Figures represent the respiratory rates in nmol 'O' min^{-1} mg^{-1}.

These experiments reveal one critical point that should be fully understood. The respiratory rates observed in the absence of nucleotide are, as stated above, almost identical to the rates observed in the presence of an uncoupler. It could be possible that, even after these thorough albumin treatments, we are unable to remove the last traces of fatty acids. However, the important question is why the respiratory activity is maximum. There are two possibilities. First, we could consider that these residual fatty acids still represent a saturating concentration of fatty acids that bring UCP1 to its maximum activity. This possibility raises two questions: what is the K_m for the activation of UCP1 and why these fatty acids do not equilibrate with albumin. The answer could be that the affinity is so high that the residual fatty acids remain bound to UCP1 and do not equilibrate with the lipid bilayer since otherwise they would eventually be removed by albumin. This explanation would make the acute control of the protein's activity difficult and would certainly contradict the postulates of the fatty acid cycling hypothesis that requires the equilibrium with the lipid bilayer for the flip-flop to occur. Additionally, this extremely low K_m would not be easily accommodated to the available experimental data. The alternative could be that in the absence of added nucleotides, there is a high and

ohmic proton conductance that is independent of the presence of fatty acids [11]. We have long favoured this second possibility [8] and early experiments pointed in this direction. We must emphasize that since in the cytosol of the brown adipocyte, there will be always purine nucleotides, this fatty acid-independent activity, while being mechanistically important, is of little physiological relevance.

The magnitude of the GDP-sensitive component of the respiratory rate can be used to assess the activity of the protein. Therefore, if in the cold-adapted hamster there exists 1 nmol UCP1 dimer per mg of mitochondrial protein that is responsible for a respiratory activity of around 300 nmol O min^{-1} mg^{-1} (mean of the values presented in Table 1) the estimated proton transport activity would be of 40 μmol H^+ min^{-1} mg $UCP1^{-1}$ at 30 °C. This is probably a low estimate of the activity of UCP1 because the respiratory rate is limited by the respiratory chain activity. Indeed, FCCP yields the same final rate. The absence of nucleotide will also result in the development of a low membrane potential, i.e. lower driving force [19,20]. In line with these considerations, we have previously reported that cold adaptation of the guinea-pig, raises the UCP1 content (determined from the GDP-binding capacity) from 0.097 ± 0.014 (animals reared at 28 °C) to 0.73 ± 0.10 nmol/mg after 18 days in at 6 °C [20]. The corresponding activity for UCP1 from the cold-adapted animal is around 70 μmol H^+ min^{-1} mg $UCP1^{-1}$ and goes up to 120 μmol H^+ min^{-1} mg $UCP1^{-1}$ in the warm-adapted animal where the UCP1 content is low, membrane potential reaches higher values and substrate oxidation should not be limiting. The basal activity of UCP1 expressed recombinantly in *S. cerevisiae* has been questioned [6]. The concentration of UCP1 present in mitochondria from the recombinant yeast strains that we have routinely used is around 0.17 nmol/mg [21]. When GDP is added to mitochondria isolated from these yeasts, the decrease in the respiratory rate is around 60 nmol 'O' min^{-1} mg^{-1} and would represent an activity of 32 μmol H^+ min^{-1} mg $UCP1^{-1}$ at 20 °C [8]. Therefore, the activity of UCP1 in yeast mitochondria is lower but not far from the activity found in animal mitochondria when the temperature difference is taken into account. We must emphasize that in this section, we are always referring to the activity of the protein in the absence of fatty acids and under respiring conditions, i.e. in the presence of a proton electrochemical potential gradient that acts as driving force.

3.2. The influence of albumin on the activity of UCP1

The ability of fatty acids to uncouple oxidative phosphorylation in all mitochondria types has been shown to be mediated by the mitochondrial transporters, ADP/ATP carrier (AAC) being the most significant probably due to its high abundance [7,22]. Most of the studies performed to investigate the effect of fatty acids on respiring mitochondria are done in the absence of albumin (see Table 2 and references therein). It could be thought that the high capacity of the lipid bilayer to adsorb fatty acids could make the contribution of albumin not too significant, but this is not the case. Fig. 2A presents the effect of palmitate on the respiration of brown fat mitochondria in the presence of varying concentrations of BSA (0.4, 1 and 2 mg/ml). It is clear that the lack of correlation between the total palmitate and the respiratory rate while the correlation appears when the molar ratio to albumin is taken into account (Fig. 2B). The vertical displacement of the curves in Fig. 2B points to the presence of endogenous fatty acids that can be better buffered when higher albumin is present. We can estimate the concentration of endogenous fatty acids in these experiments by fitting the curves to an expression that considers that the proton fluxes

Table 2
Carrier-mediated fatty acid uncoupling

Mitochondria source	Parameter determined	Albumin (μM)	Fatty acid	Range μM	Proposed carrier mediating FA effect	Reference
Brown fat	respiration	44	palmitic	6–128	UCP1	[23]
S. cerevisiae	respiration	16	palmitic	16–80	UCP1	[8]
Muscle	respiration	32	palmitic	10–40	AAC	[24]
Liver	respiration/ΔΨ	0	oleic	5–43	AAC and bilayer	[25]
Liver	respiration	0	palmitic	10–60	AAC	[26]
Heart	respiration	0	myristic	25–55	AAC	[27]
Potato tuber	ΔΨ	0	lauric	20–60	AAC	[28]
S. cerevisiae	respiration/ΔΨ	0	oleic	10–50	AAC	[29]
Heart	ΔΨ	32	palmitic	5–100	AAC and UCP2 (?)	[30]
Chicken muscle	respiration	0	palmitic	4–18	AAC and avUCP	[31]
Liver	respiration	0	palmitic	10–60	AAC and AGC	[32]
Liver	malonate uptake	0	myristic	250–2000	DiC	[33]
Liver	Pi transport	0	lauric	10–500	PiC	[34]
Tomato	respiration	0	linoleic	1.2–20	plant UCP	[35]
Candida	respiration	0	linoleic	1–30	CpUCP	[36]
Protozoa	respiration	0	linoleic	1.3–24	AcUCP	[37]

Summary of the conditions used by different laboratories to characterize the effect of fatty acids on isolated mitochondria. In each case, the fatty acid added and the concentration range employed are indicated.

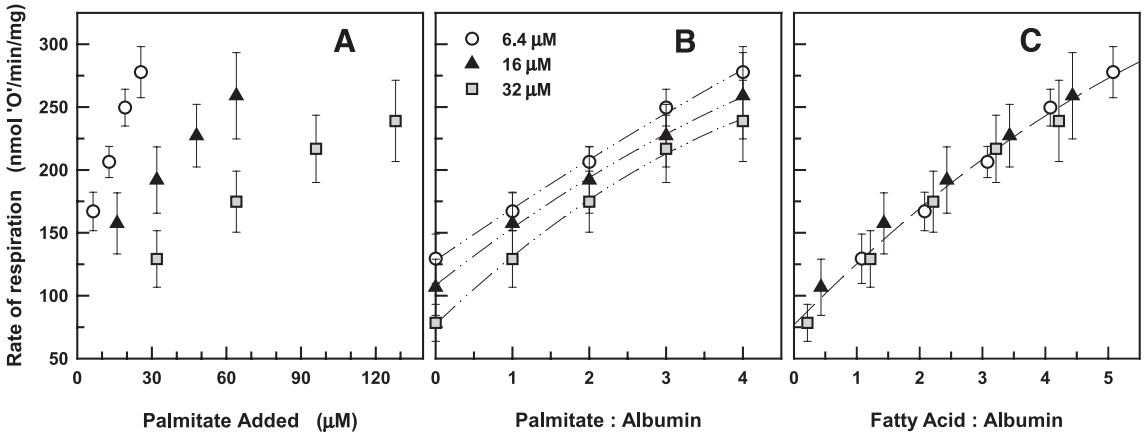

Fig. 2. Influence of albumin on the respiratory rate of BAT mitochondria. Mitochondria were isolated under our conventional protocol (see Materials and methods) and the rates of respiration measured in the presence of increasing concentrations of palmitate. Respiration buffer was the same as in Fig. 1 but with varying BSA concentrations. Panel A shows the lack of correlation between the total palmitate added and the rate of respiration. Panel B shows the relation between the rate of respiration and the molar ratio palmitate to albumin. Panel C shows that unique correlation between the rate of respiration and the molar ratio fatty acid to albumin considering the presence of 6.9 μM endogenous fatty acids (see text for further details).

have two components: a basal rate (UCP1-independent) and a UCP1-dependent proton flux. We also assume that the fatty acid activation of proton transport in UCP1 follows Michaelis–Menten kinetics [8]. For these calculations, the relevant fatty acids are those bound to the mitochondrial membranes and are estimated from previously published values taking into account the fatty acid to albumin ratios [8,23]. The fatty acid concentration will be the sum of the endogenous fatty acids plus the exogenously added palmitate. With the data presented in Fig. 2B, the estimated concentration of endogenous fatty acids would be 6.9 ± 1.1 μM. When the ratios fatty acid to albumin are recalculated to take into account the presence of the endogenous fatty acids, as expected, there is a single correlation between the molar ratio and the rate of respiration (Fig. 2C).

The concentration of endogenous fatty acids in these preparations may seem to contradict the considerations being made in the previous section. However, the preparation conditions for this set of experiments were not designed to minimize the levels of endogenous fatty acids: mitochondria were isolated under our standard conditions (no albumin during homogenization and 5 mg/ml during isolation) and experiments were not done immediately after isolation. The generation of endogenous fatty acids is strikingly rapid and their effects can be clearly detected. Fig. 3 shows a comparison of the same preparation of BAT mitochondria 15 min and 3 h after isolation. The experiments test the ability of GDP to inhibit UCP1 in the absence of albumin. When mitochondria are isolated in the presence of albumin and placed upon isolation on the electrode chamber in the absence of albumin, respiration is fast but can be inhibited by GDP (Fig. 3A). However, after 3 h of storage at 4 °C endogenous fatty acids build up and now GDP hardly inhibits respiration. Addition of 1 mg/ml BSA quickly binds fatty acids and restores respiratory control (Fig. 3B). It is interesting to note, that initial rates in Fig. 3B are lower than those observed in trace Fig. 3A. This observation is not new [19] and can be interpreted as the presence of toxic lysoderivatives that inhibit respiration but can nevertheless be removed by albumin.

The first studies on BAT mitochondria emphasized the high sensitivity to fatty acid uncoupling and the need for the presence of albumin in the respiration buffers (reviewed in Refs. [13,14]). The high fatty acid sensitivity is also reflected in the need of low fatty acid to albumin ratios to stimulate

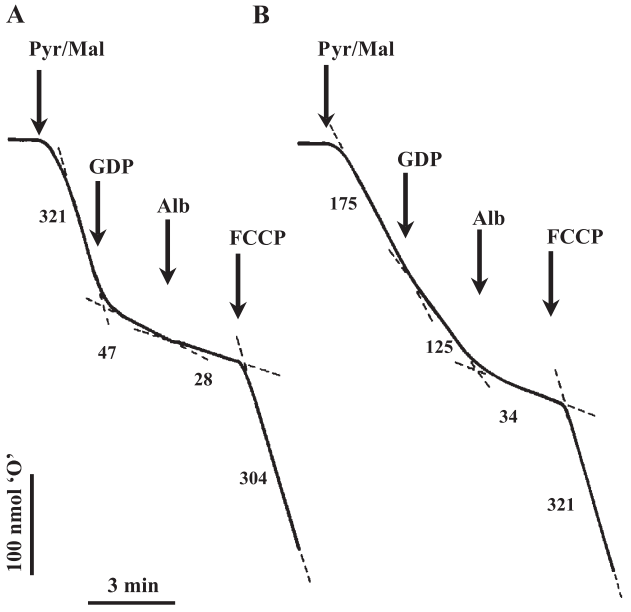

Fig. 3. Effect of endogenous fatty acids on the respiration of BAT mitochondria. Mitochondria were isolated as described in Fig. 1 and incubated in the same medium but without albumin. Trace A, mitochondria 15 min after isolation. Trace B, mitochondria 3 h after isolation. GDP (3 mM), BSA (1 mg/ml) and FCCP (10 μM) were added as indicated by the arrows. Figures represent the respiratory rates in nmol 'O' min^{-1} mg^{-1}.

respiration in these mitochondria [20,23,38]. This high fatty acid sensitivity can be transferred to yeast mitochondria when UCP1 is expressed recombinantly [8]. Ablation of UCP1 in transgenic animals led initially to the conclusion that UCP1 was not required for the fatty acid response of brown fat cells [39]. However, a more careful selection of the genetic background of the mice demonstrated that in the UCP1-knockout mice there was a significant lower uncoupling effect of fatty acids [40]. The uncoupling elicited on other types of mitochondria requires either the absence of albumin or the addition of high fatty acid concentrations as exemplified in Table 2. Recent works have placed all UCPs and many mitochondrial carriers on similar grounds with respect to their ability to act as fatty acid anion carriers [41]. We have long emphasized that while this is probably true for most members of the mitochondrial carrier family including some UCPs, the uncoupling protein from brown adipose tissue, UCP1, is still a unique case and this is consistent with the physiology of the brown adipocyte and the regulation of thermogenesis [13,42].

3.3. Sulfonates as probes for uncoupling protein mechanism

Experiments with brown fat mitochondria in the early 1970s indicated that these mitochondria had an atypical permeability to anions at neutral pH [4]. This anion permeability was soon related to the nucleotide-sensitive uncoupling pathway that was responsible for the thermogenic capacity of the tissue (reviewed in Ref. [42]). Jezek and Garlid [5] demonstrated years later that the reconstituted UCP1 could catalyze a GDP-sensitive transport of a wider variety of anions. These observations have ended up in the proposal that fatty acids are also UCP1 substrates. Therefore, their hypothetical mechanism for the fatty acid activation of thermogenesis would be a protonophoric cycle: UCP1 would catalyze the translocation of the anionic form of the fatty acid. Once in the cytosolic side of the membrane, the carboxylate group would pick up a proton and the protonated fatty acid would flip-flop back to the matrix side where the proton would be released and the protonophoric cycle completed [6,7].

The requirements of the transported substrates do not appear to be very stringent when analyzed with UCP1 reconstituted in liposomes [5,43]: they must be monovalent anions and a second polar group cannot be present unless it is close to the carboxylate. It has also been described that increasing hydrophobicity also increases the rate of permeation. One intriguing limitation to the transmembrane flip-flop is the presence of the bulky-planar structure of the benzene ring at the end of the tail and thus a compound like phenyl-hexanoic acid does not permeate [44]. However, *all-trans* retinoic acid or TTNPB are strong UCP1 activators [45]. Alkylsulfonates constitute an interesting group of substrates because of their resemblance to fatty acids but with a pK_a for the sulfonic group much lower than that of the carboxylate. Long-chain alkylsulfonates have been considered as fundamental proofs of the fatty acid cycling mechanism. Long-chain alkylsulfonates are accumulated in proteoliposomes incorporating UCP1 in a nucleotide-sensitive manner [12]. However, since the pK_a of the sulfonic group is too acidic, it cannot be protonated at physiological pH and therefore it cannot flip-flop to allow the transbilayer movement of the proton [12]. A recent publication showed that, in proteoliposomes, undecanosulfonate (C11S) caused an increase in the GDP-sensitive proton permeability if propranolol was present [3]. Propranolol and C11S would cross the bilayer forming a neutral ion pair and carrying a proton. C11S would return through UCP1 and propranolol through the lipid bilayer. In this article, it was also shown for the first time, that in respiring BAT mitochondria, the activation of respiration by C11S. Again, authors claimed that uncoupling was only observed if propranolol was present. The experiments showed the uncoupling effect of C11S in the presence of propranolol but not the effect of C11S alone [3]. Since these experiments are absolutely critical for the unravelling of the role of fatty acids in the molecular mechanism of UCP1, we have performed the experiments under conditions close to the ones described in Ref. [3]. Fig. 4 shows representative oxygen electrode traces for the effect of the two agents separately and in combination. Clearly, C11S promotes a

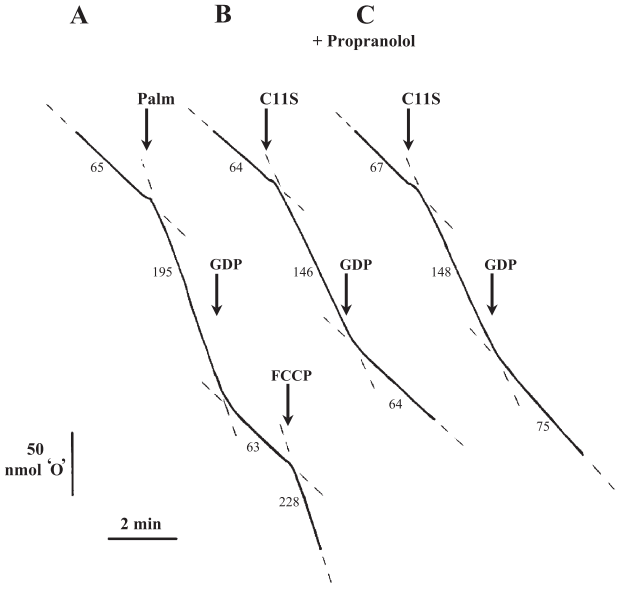

Fig. 4. Representative traces of the effect of palmitate (Palm) and undecanesulfonate (C11S) on the respiration of BAT mitochondria. Respiration medium contained 50 mM KCl, 4 mM potassium phosphate, 20 mM KTes pH 7.2, 1 mM EDTA, 2 mM $MgCl_2$, 1 μM cyclosporin A, 10 μM CoA, 1 mM carnitine, 0.3 mM GDP, 0.2 mM ATP and 1 mg/ml BSA. Experiments were initiated with the addition of 5 mM pyruvate plus 5 mM malate. Once endogenous fatty acids had been oxidized and a stable respiratory rate obtained, 32 μM palmitate (trace A) or 200 μM undecanosulfonate (trace B) were added. In trace C, 200 μM propranolol was present in the incubation medium. GDP (3 mM) and FCCP (10 μM) were added as indicated. Figures represent the respiratory rates in nmol 'O' min^{-1} mg^{-1}.

nucleotide-sensitive respiratory increase in the absence of propranolol (Fig. 4B) although the activation is much lower than that of palmitate (Fig. 4A). Propranolol does not significantly enhance the effect of C11S and the only apparent difference is an increase in the GDP-insensitive rate (Fig. 4C). Fig. 5 shows a comparison of the activation of respiration by C11S and pentanesulfonate (C5S) that is used as control, in the absence of propranolol. Fig. 6 provides a full statistical account of all the observations. The same results were obtained when we used our conventional respiratory medium (where CoA, ATP, carnitine are omitted and thus endogenous fatty acids are not oxidized) but including 0.3 mM GDP and 1 μM cyclosporin A (data not shown).

The results of these experiments have a clear interpretation. If C11S is unable to flip-flop [12] and it can activate respiration in a GDP-sensitive manner, then the protonophoric cycle cannot be the underlying mechanism of the uncoupling mediated by UCP1. There is another hypothetical mechanism, the "proton buffering model" that proposes that the fatty acid acts as a prosthetic group [9]. The carboxylate would bind protons and deliver them to a site from which they are translocated to the other side of the membrane. Long-chain alkylsulfonates and fatty acids would provide a protonable group that would be part of the translocation pathway. Although the pK_a of an acid group can change significantly depending on the protein environment, it is possible that the pK_a of the sulfonic group may be too low to be readily protonable at physiological pH. If this were to be the case, these anionic molecules could act by altering the carrier conformation and transport properties so that protons could overcome the nucleotide inhibition [17]. None of these two possibilities can be dismissed at present. In any case, the structural requirements for the activating ligands would be related to the nature of their binding site and not to their ability to flip-flop.

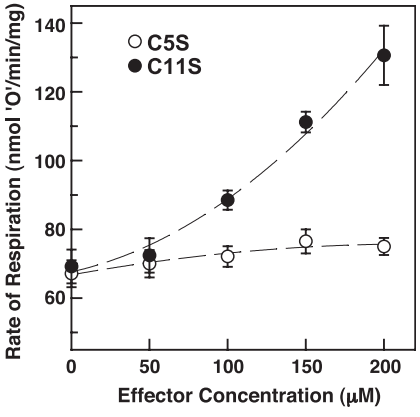

Fig. 5. Effect of undecanosulfonate (C11S) and pentanosulfonate (C5S) on the rate of respiration of BAT mitochondria. Respiration buffer and assay conditions were the same as in Fig. 4. Data points are the mean ± S.E. of three to four independent experiments performed in duplicate.

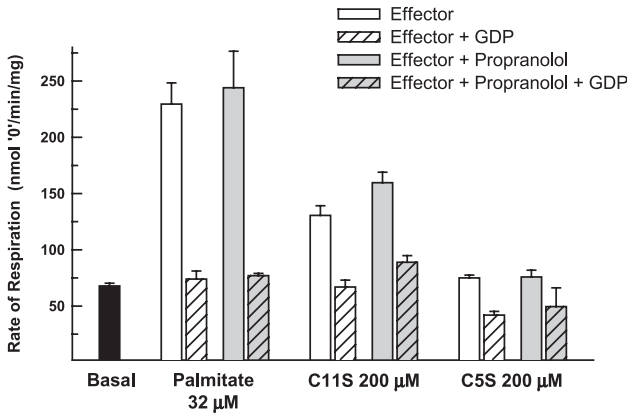

Fig. 6. Effects of palmitate, undecanosulfonate and pentanosulfonate on the respiration of BAT mitochondria. Bars represent the mean ± S.E. of three to six independent experiments performed at least in duplicate. "Basal" is the mean value of the respiratory rates prior to the addition of the effectors. Propranolol (200 μM) was present where indicated. To obtain the GDP-inhibited rates, 3 mM nucleotide was added as in Fig. 4.

3.4. Concluding remarks

Fatty acids can uncouple mitochondria from all sources. The demonstration that their action could be partially blocked with carboxyatractylate, a specific inhibitor of the AAC, led to the proposal that this carrier was facilitating the translocation of the fatty acid anion [7,24,26]. Subsequently, other mitochondrial carriers have been shown to allow the permeation of the fatty acid anion (see Table 2 and references therein) and the model extended to the rest of members of the UCP family [6]. Additionally, these mitochondrial transporters are known to switch from their specific carrier mechanism to a channel/pore mode (reviewed in Ref. [46]). The altered carrier function has probably pathophysiological significance. Thus, for example, the permeability transition appears to be due to the switch of the AAC to a channel/pore mode. The AAC (and probably other mitochondrial carriers) would act as sensor for the presence of abnormally high levels of dangerous species (ROS, NO, etc.) or critical energetic conditions (low membrane potential, ATP/ADP depletion, matrix alkalinization, high Ca^{2+}, etc.). The result is the opening of the permeability transition pore and the initiation of the apoptotic cascade. Fatty acids have also been shown to induce the classical cyclosporin-sensitive permeability transition [47–49]. The assay conditions, passive swelling, do not require the protonophoric action of fatty acids and thus it must be interpreted as due to the switch of the AAC to the pore mode. We have hypothesized that the carrier-mediated fatty acid uncoupling does not involve the specific translocation of the fatty acid anion but rather the switch to the channel/pore mode [13].

The physiological context of the fatty acid effects on UCP1 should place this protein on a different ground. Experiments presented here also point to a different molec-

ular mechanism of action. The hormonally induced increase in fatty acids in the brown adipocyte was soon related to the signalling cascade for the initiation of thermogenesis and the high fatty acid sensitivity of their mitochondria [15,50]. As we have already stated, this high fatty acid sensitivity correlates precisely with the presence of UCP1 [8,20,40]. In fact, the concentration of fatty acids required to observe the uncoupling mediated by the rest of mitochondrial carriers are much higher (Table 2), although those levels may be found in pathological conditions. Indeed, high fatty acids levels are a common complication of diseases like obesity, diabetes, generalized lipodystrophy, etc. The lipotoxicity causes the disruption of cell function and can eventually lead to cell death (“lipoapoptosis”) [49,51]. The mechanism of activation of lipoapoptosis has been related in many cases to a ceramide-dependent pathway. This would not be the only route since fatty acid oxidation can also cause an elevation of ROS production that could trigger apoptosis (reviewed in Ref. [52]). However, since fatty acids are known to uncouple oxidative phosphorylation, their direct action on the mitochondrial carriers could also lead to the initiation of apoptosis.

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