Energization-dependent endogenous activation of proton conductance in skeletal muscle mitochondria

Nadeene PARKER*†, Charles AFFOURTIT*, Antonio VIDAL-PUIG† and Martin D. BRAND*

* MRC Dunn Human Nutrition Unit, Hills Road, Cambridge CB2 0XY, U.K., and † Metabolic Research Laboratories, Level 4, Institute of Metabolic Science, Box 289, Addenbrooke's Hospital, Cambridge CB2 0QQ, U.K.

Leak of protons into the mitochondrial matrix during substrate oxidation partially uncouples electron transport from phosphorylation of ADP, but the functions and source of basal and inducible proton leak in vivo remain controversial. In the present study we describe an endogenous activation of proton conductance in mitochondria isolated from rat and mouse skeletal muscle following addition of respiratory substrate. This endogenous activation increased with time, required a high membrane potential and was diminished by high concentrations of serum albumin. Inhibition of this endogenous activation by GDP [classically considered specific for UCPs (uncoupling proteins)], carboxyatractylate and bongkrekate (considered specific for the adenine nucleotide translocase) was examined in skeletal muscle mitochondria from wild-type and Ucp3-knockout mice. Proton conductance through endogenously activated UCP3 was calculated as the difference in leak between mitochondria from wild-type and Ucp3-knockout mice, and was found to be inhibited by carboxyatractylate and bongkrekate, but not GDP. Proton conductance in mitochondria from Ucp3-knockout mice was strongly inhibited by carboxyatractylate, bongkrekate and partially by GDP. We conclude the following: (i) at high protonmotive force, an endogenously generated activator stimulates proton conductance catalysed partly by UCP3 and partly by the adenine nucleotide translocase; (ii) GDP is not a specific inhibitor of UCP3, but also inhibits proton translocation by the adenine nucleotide translocase; and (iii) the inhibition of UCP3 by carboxyatractylate and bongkrekate is likely to be indirect, acting through the adenine nucleotide translocase.

Key words: adenine nucleotide translocase, carboxyatractylate, GDP, proton leak, uncoupling protein (UCP), UCP3.

INTRODUCTION

During substrate oxidation, protons are pumped out of the mitochondrial matrix, creating an electrochemical proton gradient. This protonmotive force is used to drive ATP synthesis. However, substrate oxidation is not fully coupled to ATP synthesis; some of the electrochemical gradient is dissipated by protons leaking back into the matrix through natural proton conductance pathways across the mitochondrial inner membrane [1,2]. Mitochondrial proton leak may cause up to 25% of the resting metabolic rate [3,4] and may still be significant when ATP turnover is high [5]. The prominence of mitochondrial proton leak suggests that it is physiologically important, and several roles have been suggested [4], including protection against mitochondrial free radical production [6] and regulation of insulin secretion in pancreatic β-cells [7].

Proton leak occurs through several pathways, some basal and some inducible. Membranes are themselves intrinsically leaky to protons [8] but proteins embedded in a bilayer can greatly increase permeability [4,9,10]. Half to two-thirds of the basal mitochondrial proton conductance is dependent upon the most abundant carrier protein in the mitochondrial inner membrane, the ANT (adenine nucleotide translocase) [11]. ANT-dependent basal proton conductance occurs even if the carrier is inhibited by carboxyatractylate, showing that this leak pathway does not require normal carrier function [11].

Unlike basal proton conductance, specific activators and inhibitors affect inducible proton conductance. Inducible conductance can be catalysed by ANT in the presence of fatty acids [12,13], AMP [14] or the lipid peroxidation product hydroxynonenal [15]. This pathway is inhibited by carboxyatractylate, bongkrekate and by the ANT substrates, ATP or ADP. It is not known to be sensitive to other nucleotides. Other mitochondrial carriers, such as the glutamate [16] and phosphate carriers [17], will catalyse proton conductance in the presence of fatty acids. Specific UCPs (uncoupling proteins) also catalyse inducible proton conductance [18–21]. The best understood is UCP1, found primarily in brown adipose tissue and specialized for adaptive thermogenesis. Proton conductance through UCP1 is activated by fatty acids [22], superoxide [23], derivatives such as hydroxynonenal [15,24] and hydroxynonenal analogues (such as retinoic acid [25]). UCP2, found in a variety of tissues [26], and UCP3, found primarily in skeletal muscle [27,28], can be activated by fatty acids [29,30], superoxide [23] and hydroxynonenal and its analogues [15].

A striking aspect of the UCPs is the inhibition of their proton conductance by a broad range of purine nucleotides. Unlike the proton conductance of the adenine nucleotide carrier, UCPs are strongly inhibited not only by ATP and ADP, but also by GTP and GDP [14]. This difference in specificity has led to widespread use of GDP as a diagnostic inhibitor of UCP function in vitro (e.g., [31–34]), particularly in systems that lack a UCP-ablated control [35–38].

In the present study we report an endogenous increase in the proton conductance of skeletal muscle mitochondria incubated at a high membrane potential. It is catalysed by UCP3 and ANT. Although it is inhibited by GDP and carboxyatractylate, these inhibitors do not show the expected classical specificity.
EXPERIMENTAL

Animals

Female Wistar rats (Charles River) and male or female mice were housed at 21 ± 2°C, 57 ± 5% humidity, 12/12 h light/dark, with standard laboratory chow and water ad libitum, following UK Home Office Guidelines for the Care and Use of Laboratory Animals. Ucp3 knockout (termed Ucp3KO) [39] and wild-type (termed WT) sibling-paired mice were used at age 12–15 weeks. Ucp3 ablation was confirmed by PCR analysis of genomic Ucp3 and Western blot analysis in skeletal muscle mitochondria.

Mitochondria

Animals (one rat or four mice per preparation) were killed by stunning and cervical dislocation. Mitochondria were isolated from total hind limb skeletal muscle [15]. Mitochondria from WT and Ucp3KO mice were prepared and assayed in parallel.

Proton-leak kinetics

Proton-leak rates across the inner membrane of isolated mitochondria were measured as the oxygen consumption required to pump the protons back out of the mitochondrial matrix (in the presence of oligomycin to prevent ATP synthesis) [40]. Proton-leak kinetics were measured as the dependence of proton-leak rate on its driving force, protonotive force (measured as membrane potential in the presence of nigericin to abolish pH gradients). Respiration rate and membrane potential were measured simultaneously using electrodes sensitive to oxygen or the membrane-potential-dependent probe, TPMP+ (triphenylmethylphosphonium). Assay medium contained 0.35 mg/ml mitochondrial protein, 120 mM KCl, 5 mM KH2PO4, 3 mM HEPES, 1 mM EGTA, 0.3% (w/v) defatted BSA (unless otherwise stated), 5 μM rotenone, 1 μM oligomycin and 0.11 μM nigericin. The TPMP electrode was calibrated with sequential additions of 0.5 μM TPMP up to 2.5 μM and the mitochondria were energized with 4 mM succinate. Leak kinetics were measured by varying oxygen consumption and membrane potential by sequential additions of malonate (approx. 0.1–2.3 mM). After each run, 0.2 μM FCCP (carbonyl cyanide -trifluoromethoxyphenylhydrazone) was added to release matrix TPMP, allowing correction for any small baseline drift. The TPMP-binding correction was taken to be 0.35 (μl/mg protein)−1 [41].

Measurement of ANT content by carboxyatractylate titre

Respiration was measured without oligomycin or nigericin. In order to establish the maximum phosphorylating rate 1 mM ADP was added, and then respiration was successively inhibited by additions of carboxyatractylate (Calbiochem) until the resting non-phosphorylating rate was re-established. Carboxyatractylate binds ANT very tightly, so ANT content was interpolated as the minimum amount of carboxyatractylate required to abolish phosphorylating respiration [11,42].

Statistics

To test for differences in proton conductance by ANOVA or paired Student’s t tests, we calculated respiration rates (by linear interpolation between flanking points) at the highest membrane potential common to all conditions on a given day. Means for each condition (± S.E.M.) are plotted in bar graphs, with the mean value of the highest common potential indicated.

RESULTS

Time-dependent endogenous activation of proton conductance in energized rat skeletal muscle mitochondria

Preliminary investigations of proton-leak kinetics in skeletal muscle mitochondria suggested that proton conductance increased as the time of energization with succinate was extended. Figure 1 characterizes this endogenous activation of proton conductance. Mitochondria were incubated in assay medium for a total of 8 min (Figure 1A). They were energized with succinate for the last 0.75, 2.5 or 5 min, then proton-leak kinetics were measured. Figure 1(B) shows that respiration driving proton...
leak increased as incubation with succinate was extended. To allow comparison of proton-leak rates at the same driving force, Figure 1(C) shows rates interpolated at 175 mV, the highest common membrane potential in the experiment, indicated by the vertical line. The proton-leak rate at 175 mV measured after 5 min with succinate was significantly higher than that measured after 0.75 min with succinate. The total time in the assay was always 8 min, therefore endogenous proton conductance increases as the time of incubation with succinate is extended. Leak kinetics are measured by titrating membrane potential and respiration rate with malonate over 3 min. If time with succinate is important in inducing proton leak, endogenous activation should continue during the malonate titration, causing later points to be more uncoupled than earlier points. To test this, Figure 1(D) shows a compilation of leak kinetics measured in five separate experiments, each with a different concentration of malonate added at $t = 0$. Time with succinate was the same for all titration points (broken line). This was overlaid on the leak kinetics measured during one standard experiment using five sequential additions of malonate (as in Figure 1B), where time with succinate differed between titration points (solid line). There was no difference in leak kinetics between the two protocols. Therefore energization-dependent uncoupling does not increase over the 3 min taken to measure the proton-leak kinetics suggesting no further increases after the first malonate addition.

**Malonate attenuates the energization-dependent increase in proton conductance by lowering membrane potential in rat skeletal muscle mitochondria**

To test the ability of malonate to blunt energization-dependent, endogenous activation of proton leak, 430 µM malonate was added immediately before succinate. At $t = 0$, after incubation with succinate for 1.5 min, this concentration of malonate lowered the membrane potential by approx. 12 mV (X in Figure 2). Succinate was added 1.5 min or 5 min before the leak titration, which was completed as normal with sequential additions of malonate (Figure 2B). Figure 2(A) shows the control without malonate pre-addition.

To allow comparison of proton-leak rates at the same driving force, Figure 2(C) shows the time-dependent increases in rates with succinate, interpolated at the highest common membrane potential, 169 mV. The leak induced between 1.5 and 5 min was significantly lower when 430 µM malonate was present, therefore malonate does indeed attenuate the energization-dependent increase in proton conductance.

Pre-addition of malonate could inhibit energization-dependent induced leak directly, or by lowering protonmotive force, or by oxidizing the electron transport chain. To test these possibilities, the experiment in Figure 2(C) was repeated using the complex IV inhibitor, KCN, at a concentration that lowers the membrane potential to the same extent as 430 µM malonate. Leak kinetics were measured in the absence (Figure 2D) or presence (Figure 2E) of 43 µM KCN. At the highest common membrane potential, proton conductance induced by succinate between 1.5 and 5 min in the presence of 43 µM KCN (Figure 2F) was significantly lower than in its absence. Unlike malonate, KCN reduces electron transport chain intermediates. The two inhibitors decreased energization-dependent proton conductance to a similar extent, suggesting that it is high membrane potential, not a particular redox state, that is required for energization-dependent induction of proton conductance.

The increase in proton conductance between 1.5 and 5 min incubation with succinate was also measured at intermediate concentrations of pre-added malonate or KCN. This produced intermediate drops in membrane potential at $t = 0$ following 1.5 min with succinate (equivalent to X in Figure 2B and Figure 2E). To characterize the ability of decreased membrane potential to blunt energization-dependent induction of proton conductance, Figure 2(G) shows the increase in leak rate (e.g. Figures 2C and 2F) plotted against the drop (X) in membrane potential caused by different concentrations of pre-added malonate or KCN. There was a linear decrease in activation as membrane potential during the activatory period was decreased.

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**Figure 2 Sensitivity of energization-dependent proton conductance in rat skeletal muscle mitochondria to membrane potential**

(A–C) Effect of malonate. (D–F) Effect of KCN. (A, B, D and E) Proton-leak kinetics. Succinate at 4 mM was added at $t = -1.5$ min (open symbols) or $t = -5$ min (closed symbols) (see Figure 1A). Proton-leak kinetics were measured between $t = 0$ and $t = 3$ min after no treatment (A and D) or addition of 430 µM malonate (B) or 43 µM KCN (E) immediately prior to addition of succinate. (C and F) Inhibition of energization-dependent proton leak by malonate and KCN. The increase in proton conductance at $t = 0$ caused by incubation with succinate between −5.0 min and −1.5 min was interpolated at the highest common membrane potential, 169 mV [solid vertical line in (A and B) in (C) and 170 mV [solid vertical line in (D and E) in (F)]. X indicates the drop in membrane potential measured at $t = 0$ caused by addition of malonate or KCN prior to addition of succinate at $t = -1.5$ min. (G) Effect of this drop in membrane potential on energization-dependent increase in proton conductance. The increases in proton-leak rate from (C) and additional experiments (results not shown) using 140 µM and 290 µM malonate (circles), and from (F) and additional experiments (results not shown) using 14 µM and 29 µM KCN (triangles) are plotted against the drop (X) in membrane potential at $t = 0$ after succinate addition at $t = -1.5$ min. Values are means ± S.E.M. of five (A–C) or six (D–F) experiments each performed in duplicate. *P < 0.03, significantly different from no addition as measured by paired Student’s t test. Lines in (G) are linear regressions.
The linear regressions for malonate and KCN overlap, suggesting a conserved mechanism of inhibition through the lowering of membrane potential. Extrapolation to zero effect suggests that a decrease of 20 mV is sufficient to prevent activation of proton conductance.

Therefore the endogenous energization-dependent activation of proton conductance following addition of succinate requires a high membrane potential. Activation increases over 5 min with succinate but during subsequent titters with malonate the membrane potential falls by more than 20 mV and further activation is prevented.

**What is the activator of the energization-dependent increase in proton conductance in rat skeletal muscle mitochondria?**

BSA removes putative endogenous activators, including fatty acids [43] and reactive oxygen species [6] and their derivatives such as reactive alkenals [15]. To test whether these endogenous activators were involved in the observed energization-dependent proton leak, the concentration of BSA was increased in the standard assay medium from 0.3 % (Figure 3A) or 1 % (w/v) BSA (B, G and H), after no pre-treatment or pre-treatment with 0.1 mM (triangles) or 1 mM phenylbutylnitrone (diamonds) or 600 μM palmitate (H). (C, F and I) Effect on energization-dependent proton leak of 1 % BSA, phenylbutylnitrone or palmitate. The increase in proton conductance at f = 0 caused by incubation with succinate between −5 and −1.5 min was interpolated at the highest common membrane potential, 172 mV (solid vertical line in (A, B, G and H)) in (C) and (I) and 169 mV (solid vertical line in (D and E)) in (F). Values are means ± S.E.M. of four (A–C), three (D–F) or three (G–I) experiments, each performed in duplicate. *P < 0.01, significantly different from 0.3 % BSA as measured by paired Student’s t test.

To test the possibility that the increase in energization-dependent leak is due to lipid peroxidation products, phenylbutylnitrone, a chain-breaking spin trap that quenches carbon-centred radical intermediates, was pre-added to the assay medium at concentrations known to inhibit lipid peroxidation [44]. Proton-leak kinetics were measured after energization with succinate for 1.5 or 5 min in the absence (Figure 3D) or presence (Figure 3E) of phenylbutylnitrone. At 169 mV, there was no significant effect of phenylbutylnitrone on leak induced between 1.5 and 5 min (Figure 3F), suggesting that pathways that generate lipid peroxidation products through a carbon-centred radical sensitive to phenylbutylnitrone are not involved in the energization-dependent uncoupling. However, other reactive oxygen species-related activators remain candidates.

Energization might either generate a pool of activator, or allow activation of uncoupling using a pre-existing activator pool (e.g. through an energization-dependent change in conformation of an UCP). To distinguish between these alternatives, we tested the effect of pre-addition of fatty acids in medium containing 1 % BSA. Proton-leak kinetics were measured in 1 % BSA in the absence (Figure 3G) or presence (Figure 3H) of 600 μM palmitate after energization with succinate for 1.5 or 5 min. Figure 3(I) replicates the observation in Figure 3(C) that 1 % BSA alone prevented time-dependent activation. Pre-addition of palmitate did not overcome the inhibitory effect of 1 % BSA, showing that time-dependent activation did not occur by potential-dependent changes in protein conformation that then allowed activation by a pre-existing pool of fatty acids. Fatty acids remain, however, one candidate for the endogenously generated activator, since...
proton-leak kinetics in the presence of palmitate (Figure 3H) were the same after 1.5 min incubation with succinate as those seen in 0.3% BSA after 5 min with succinate (Figure 3A).

These experiments are consistent with at least three different interpretations. The endogenous activator may be (i) fatty acids, (ii) some other molecule, generated during 5 min incubation at high membrane potential, or (iii) some molecule (e.g. hydroxynonenal) that is already present in the mitochondria before addition of succinate, but requires the membrane potential to be high for several minutes to allow the interaction that causes uncoupling.

**Which proteins catalyse the energization-dependent increase in proton conductance in rat skeletal muscle mitochondria?**

Uncoupling through the ANT is inhibited by carboxyatractylate [13], whereas uncoupling through UCPs is classically inhibited by GDP [14,45]. Proton-leak kinetics were measured after energization with succinate for 2.5 min in the absence and presence of carboxyatractylate and GDP to determine whether ANT and UCP3 were involved (Figure 4A). Figure 4(B) shows that at the highest common membrane potential, 180 mV, GDP and carboxyatractylate significantly inhibited proton conductance separately and in combination. The extent of the carboxyatractylate inhibition of proton conductance suggests that the majority is attributable to ANT. As observed previously [15], inhibition of proton conductance by GDP and carboxyatractylate was non-additive; characterization of the relative contributions of ANT and UCP3 therefore required further experiments.

**UCP3 and ANT each catalyse part of the energization-dependent increase in proton conductance in mouse skeletal muscle mitochondria**

The contribution of UCP3 to energization-dependent proton conductance was assessed using skeletal muscle mitochondria prepared from WT and *Ucp3*KO mice. Proton-leak kinetics were measured after energization with succinate for 1.5 or 5 min. Figure 5(A) shows that skeletal muscle mitochondria from WT mice showed a similar time-dependent increase in proton leak after addition of succinate to mitochondria from rat (Figure 1B). Figure 5(B) shows the same experiment performed using skeletal muscle mitochondria from *Ucp3*KO mice. There was still a difference between the curves, but it was smaller than in WT. The difference is quantified in Figure 5(C), which shows proton leak due to UCP3 measured at 169 mV [solid vertical line in (A and B)] as (A) minus (B). Values are means ± S.E.M. of seven experiments, each performed in duplicate. **P < 0.01, significantly different by paired Student's t test (5 min succinate addition compared with 1.5 min).
Specificity and lack of additivity of inhibition by GDP and carboxyatractylate in mouse skeletal muscle mitochondria

To investigate the lack of additivity between the inhibitors GDP and carboxyatractylate in rat skeletal muscle (Figure 4), and also seen in other studies [15], inhibition was measured in skeletal muscle mitochondria from WT and Ucp3KO mice. The same lack of additivity was seen in skeletal muscle mitochondria from WT mice (Figure 6A) as in rat: GDP partially inhibited proton conductance in the absence of carboxyatractylate but caused no further inhibition in the presence of carboxyatractylate. Figure 6(B) shows the same effect in mitochondria from Ucp3KO mice.

Figures 6(C) and 6(D) dissect out the effects of the inhibitors. Figure 6(C) represents the contribution of UCP3 (Figure 6A minus 6B) at the highest common membrane potential, 161 mV, after 5 min incubation with succinate. Surprisingly (since GDP is the classical inhibitor of UCPs), the proton conductance through UCP3 under these conditions was insensitive to GDP. More surprisingly (since carboxyatractylate is thought to be a highly specific inhibitor of ANT), it was almost fully sensitive to carboxyatractylate. This sensitivity of UCP3 to carboxyatractylate partially explains the lack of additivity of the two inhibitors: once UCP3 is inhibited by carboxyatractylate, any inhibition by GDP would have no further effect.

Figure 6(D) represents the contribution of ANT and other pathways (results from Figure 6B). The energization-dependent proton conductance of mitochondria from Ucp3KO mice was significantly lower in the presence of GDP, indicating that an endogenously activated pathway other than UCP3 was inhibited by GDP. Since this conductance was also strongly inhibited by carboxyatractylate, it was catalysed by ANT. This experiment shows that GDP is not specific for UCPs, but can also inhibit proton conductance through other pathways, specifically ANT. It completes the explanation of the lack of additivity of GDP and carboxyatractylate: once ANT is inhibited by carboxyatractylate, inhibition by GDP would have no further effect. Together, these results highlight potential promiscuity of the inhibitors GDP and carboxyatractylate: neither is specific for its classical target in our experiments.

A possible alternative explanation of the sensitivity of mitochondria from Ucp3KO mice to GDP is that the Ucp3KO targeting vector upregulates Ucp2 expression, due to proximity of the Ucp2 and Ucp3 genes [46], and that this UCP2 that is inhibited by GDP. We detected Ucp2 transcript in total hind limb skeletal muscle using real-time PCR, but at levels 30 times lower than in spleen or lung, with no significant difference between WT and Ucp3KO (results not shown). UCP2 was undetectable by Western blot analysis using skeletal muscle mitochondria (20 μg of protein) from either WT or Ucp3KO mice. The antibody can detect 125 pg of affinity-purified recombinant UCP2, so UCP2 content is below 6 ng/mg of mitochondrial protein, less than 5% of the UCP3 content of 140 ng/mg of protein in mouse skeletal muscle mitochondria [47]. UCP1 is not expressed in skeletal muscle of WT or Ucp3KO mice [48]. Therefore the putative presence of UCP1 or UCP2 does not explain the sensitivity of proton conductance in skeletal muscle mitochondria from Ucp3KO mice to GDP.

A possible alternative explanation of the decreased effect of carboxyatractylate in mitochondria from Ucp3KO mice is that ablation of UCP3 somehow decreased ANT content. We tested this possibility by titrating ANT activity with carboxyatractylate in skeletal muscle mitochondria from WT and Ucp3KO mice. UCP3 (4 pmol/mg of mitochondrial protein [47]) is approx. 0.2% as abundant as ANT (2 nmol/mg of mitochondrial protein [11]), so any sequestering of carboxyatractylate by UCP3 would be negligible. ANT content was not significantly different (WT, 1.52 nmol/mg of protein and Ucp3KO, 1.56 nmol/mg of protein; Figure 6E), eliminating this explanation.

Inhibition of UCP3 by carboxyatractylate could be direct or through ANT. To examine this, we tested the effects of an alternative ANT inhibitor, bongkrekate. Unlike carboxyatractylate, which binds to the C conformation of the carrier, bongkrekate binds to the M conformation [49]. BSA was omitted from the assay medium because it binds bongkrekate. Proton-leak kinetics were measured in skeletal muscle mitochondria isolated from WT (Figure 7A) and Ucp3KO (Figure 7B) mice after energization with succinate for 5 min in the absence or presence
performed in duplicate. One-way ANOVA was performed for (C) carboxyatractylate (light grey). (∗) multiple comparison test used post-hoc comparing other bars with control: mice. Succinate at 4 mM was added at t = −5 min (see Figure 1A) in the absence (closed symbols) or presence of 2.0 µM bongkrekate (dark grey) or 2.5 µM carboxyatractylate (light grey). (Proton leak due to UCP3 measured at 158 mV (solid vertical line in (A and B) as (A) minus (B)). Values are means ± S.E.M. of ten experiments, each performed in duplicate. One-way ANOVA was performed for (C); P = 0.015, with Dunnett's multiple comparison test used post-hoc comparing other bars with control. ∗ P < 0.05.

of bongkrekate or carboxyatractylate. Bongkrekate partially inhibited the energization-dependent proton conductance in both cases. Figure 7(C) shows the effects that were dependent on UCP3 (Figure 7A minus 7B). At the highest common membrane potential, 158 mV, both carboxyatractylate and bongkrekate inhibited proton conductance through UCP3. It is unlikely that UCP3 and ANT share two different high-affinity inhibitor sites, therefore both carboxyatractylate and bongkrekate act on the UCP3-associated leak indirectly through primary effects on ANT.

DISCUSSION
In the present study we describe an endogenous activation of the proton conductance of skeletal muscle mitochondria isolated from both rats and mice that increases with time after energization. This effect is not dependent upon redox state but requires a high membrane potential upon succinate addition. The effect of membrane potential may be to increase the production of endogenous fatty acids or other molecules that progressively activate uncoupling, or to sensitize target proteins to the effects of generated or pre-existing activators such as products of reactive oxygen species. The mechanism remains unclear. Membrane potential might change the conformation of membrane lipids to facilitate access by phospholipases, or might change the conformations of UCP3 and ANT to expose activator-binding sites. Mobilization of fatty acids could explain the lack of GDP sensitivity of the UCP3-dependent proton conductance, by analogy with UCP1, where fatty acids functionally compete with inhibitory nucleotides [50]. Whatever the activator and mechanism, energization-dependent uncoupling of skeletal muscle mitochondria may be important in limiting membrane potential during rest, to limit reactive oxygen species production [21] or prevent dielectric breakdown of the membrane [4].

Two catalysts of energization-dependent proton conductance were found to be UCP3 and ANT. In WT mouse skeletal muscle mitochondria after 5 min incubation with succinate, the relative contributions of UCP3, ANT and other pathways to proton conductance were 15–20%, 50–55% and 25–30% respectively. Since UCP3 content is only approx. 0.2% of ANT content (see above), this result demonstrates that the specific proton flux through UCP3 is 100–200 times greater than that through ANT. The greater specific activity of UCP3 may allow finer control of high membrane potential in tissues that contain this protein in addition to ANT.

We found that GDP partially inhibited the contribution of ANT to energization-dependent proton conductance. Khailova et al. [51] also concluded that GDP inhibits proton translocation by ANT, although they did not use Ucp knock out controls, which provide our most compelling evidence that GDP can inhibit proton conductance in the absence of UCP3. GDP and GTP bind weakly to ANT [52], but compete poorly with ADP and ATP, so they are not regarded as inhibitors of adenine nucleotide exchange. However, adenosine nucleotides are absent in assays of proton conductance, which may allow guanine nucleotides to bind and partially inhibit ANT-catalysed proton leak.

We found that carboxyatractylate inhibited the contribution of UCP3 to proton leak. Carboxyatractylate is a high-affinity inhibitor of ANT, and has not been shown previously to act on other proteins. However, most other studies have not looked for carboxyatractylate inhibition of UCP3 function. The observation that bongkrekate also inhibits UCP3-dependent proton conductance suggests that inhibition of UCP3 is indirect, via primary effects on ANT. It is unclear what these indirect effects might be, but they might involve protein–protein interactions between ANT and UCP3, or interactions through some other protein, lipid or small molecule. More exploration of the interaction is required to uncover the mechanism. However, the (indirect) inhibition of UCP3 by carboxyatractylate fully explains the hitherto puzzling low additivity between inhibition of proton conductance by GDP and carboxyatractylate.

Since inhibition by GDP and carboxyatractylate is also non-additive in mitochondria from kidney [15,44], carboxyatractylate may also inhibit UCP2 by a similar indirect mechanism. However, it appears that carboxyatractylate does not inhibit potato UCP [53] or mammalian UCP1, since guanine nucleotides are still inhibitory in the presence of carboxyatractylate in mitochondria from potato or mammalian brown adipose tissue. Thus caution should be shown when using sensitivity to GDP or carboxyatractylate as diagnostic of the involvement of a UCP or ANT in proton conductance. Observations of GDP or carboxyatractylate sensitivity should be verified by independent assays of the involvement of UCPs and ANT, such as sensitivity to genetic ablation or overexpression.

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REFERENCES


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