Mitochondrial proton leak in muscle is responsible for approx. 15% of the standard metabolic rate, so its modulation could be important in regulating metabolic efficiency. We report in the present paper that physiological concentrations of AMP (K \text{app} = 80 \mu M) increase the resting respiration rate and double the proton conductance of rat skeletal-muscle mitochondria. This effect is specific for AMP. AMP also doubles proton conductance in skeletal-muscle mitochondria from an ectotherm (the frog \textit{Rana temporaria}), suggesting that AMP activation is not primarily for thermogenesis. AMP activation in rat muscle mitochondria is unchanged when uncoupling protein-3 is doubled by starvation, indicating that this protein is not involved in the AMP effect. AMP activation is, however, abolished by inhibitors and substrates of the adenine nucleotide translocase (ANT), suggesting that this carrier (possibly the ANT1 isoform) mediates AMP activation. AMP activation of ANT could be important for physiological regulation of metabolic rate.

Key words: adenine nucleotide translocase, nucleotides, proton leak, uncoupling, uncoupling proteins.

INTRODUCTION

The electrochemical proton gradient created by the activity of the respiratory chain is coupled to ATP synthesis, so that most protons pumped out from mitochondria during oxidative phosphorylation cycle through the ATP synthase, producing ATP. However, some leak back through other pathways producing heat [1,2].

Uncoupling protein (UCP) 1 in brown adipose tissue dissipates the electrochemical gradient generating heat instead of ATP [3]. This protein is activated by fatty acids and is inhibited by purine nucleotides. UCP2 and UCP3 are highly similar to UCP1 at the amino acid level, and are expressed in various tissues or predominantly in skeletal muscle respectively [4–6]. Their uncoupling activity is a matter of debate [2]. Another mitochondrial inner-membrane protein is the adenine nucleotide translocase (ANT), which exchanges cytosolic ADP for mitochondrial ATP. This carrier and the aspartate/glutamate antiporter are involved in fatty acid-induced uncoupling [7].

Mitochondrial proton leak in muscle is responsible for approx. 15% of the standard metabolic rate in rats [8], so its modulation could be important in regulating metabolic efficiency. We have recently reported that nucleoside di- and tri-phosphates have no effect on rat skeletal-muscle mitochondrial proton conductance [9]. However, we report in the present paper that 1 mM AMP increases the resting respiration rate and doubles the proton conductance.

EXPERIMENTAL

Isolation of skeletal-muscle mitochondria

Female Wistar rats (4–8 weeks old) were killed by stunning followed by cervical dislocation. The skeletal muscle was immediately dissected from the hindlimbs, weighed and placed in ice-cold medium, containing 50 mM Tris/HCl (pH 7.4), 100 mM KCl and 2 mM EGTA. Mitochondria were isolated as previously described [9–11], with all steps carried out at 4 °C. Protein concentration was determined by the biuret method.

Measurement of oxygen consumption

Oxygen consumption was measured using a Clark-type oxygen electrode (Hansatech, King’s Lynn, Norfolk, U.K.) maintained at 37 °C and calibrated with air-saturated assay medium [120 mM KCl, 5 mM KH\textsubscript{2}PO\textsubscript{4}, 3 mM Hepes, 1 mM EGTA and 0.3% (w/v) defatted BSA, pH 7.2], which was assumed to contain 406 nmol O/ml at 37 °C [12]. Electrode linearity was routinely checked by following the uncoupled respiration rate in the presence of 0.2 mM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) from 100 to 0% air saturation. Respiratory control ratios (the State 3 respiration rate with 250 \mu M ADP divided by the State 4 rate after ADP phosphorylation) with succinate as substrate were 3.8 ± 0.2 (mean ± S.D.; n = 15). We routinely measured the respiration rate in the absence of ADP and in the presence of oligomycin (to inhibit any ATP synthesis) as a crude indicator of mitochondrial proton conductance. Mitochondria (0.5 mg of protein/ml) were incubated in assay medium containing 5 \mu M rotenone, 1 \mu g/ml oligomycin and 4 mM succinate. AMP was brought to pH 6–7 and added at a concentration of 1 mM.

Measurement of proton conductance

The respiration rate of mitochondria in the presence of oligomycin, to inhibit the ATP synthase, is proportional to the rate at which protons leak across the mitochondrial inner membrane. The kinetic response of the proton-conductance pathway to its driving force (membrane potential) can therefore be measured as the relationship between respiration rate and mitochondrial membrane potential when the potential is varied by titration with electron-transport-chain inhibitors [1,13–15]. We determined respiration rate and membrane potential simultaneously using electrodes sensitive to oxygen and to the potential-dependent...
probe triphenylmethyl phosphonium cation (TPMP⁺) [1,16]. Mitochondria (0.5 mg of protein/ml) were incubated in assay medium containing 5 μM rotenone, 1 μg/ml oligomycin and 80 ng/ml nigericin (to collapse the difference in pH across the inner membrane). The electrode was calibrated with sequential additions of TPMP⁺ up to 2 μM, then 4 mM succinate was added to start the reaction. Respiration and potential were progressively inhibited through successive steady states by additions of malonate up to 2.5 mM. At the end of each run, 0.2 μM FCCP was added to dissipate the membrane potential and release all TPMP⁺ back into the medium, allowing correction for any small electrode drift. The TPMP⁺-binding correction for skeletal muscle was taken to be 0.35 (μl/mg of protein)⁻¹ [17].

RESULTS AND DISCUSSION

In the presence of oligomycin (to inhibit the ATP synthase), 1 mM AMP stimulated the respiration of rat skeletal-muscle mitochondria by 55 ± 1.9 % (mean ± S.E.M.; n = 15; P < 0.001). AMP activation showed simple saturation (Figure 1a), with the half-maximal effect at 80 μM AMP (Figure 1b), which is in the physiological range of approx. 100 μM [18,19]. Other experiments (results not shown) indicated that stimulation by AMP was unaffected by the addition of 2 mM MgCl₂, 1 mM EDTA, or 50 μM diadenosine pentaphosphate (to prevent AMP metabolism through adenylyl kinase), or by pH values between 6.5 and 7.3. There was no effect on respiration of ADP, ATP or the nucleoside monophosphates or diphosphates of guanine, cytosine, thymine or uracil at 1 mM. In the absence of added MgCl₂ or EDTA the nucleoside triphosphates each stimulated respiration by 60 %, but this was entirely explained by chelation of endogenous contaminating Mg₂⁺, which is a potent inhibitor of the basal proton conductance in muscle mitochondria [9].

To test whether AMP directly activated mitochondrial proton conductance, we assayed its kinetics by measuring the response of the proton-leak rate to its driving force, i.e. the mitochondrial membrane potential. An increased proton-leak rate (measured as the oligomycin-insensitive oxygen-consumption rate) at any given potential indicates increased proton conductance of the membrane. See references [1,2,20] for a full discussion of this assay. The proton-leak rate was increased by 1 mM AMP at all membrane potential values; it increased the proton conductance of the mitochondria approx. 2-fold across the whole range of driving forces (Figure 2a). AMP had no effect on the overall kinetics of the reactions of substrate oxidation (results not shown). AMP also doubled the proton conductance of skeletal-muscle mitochondria isolated from the frog Rana temporaria (Figure 2b). Since the frog is an ectotherm, this indicates that the primary physiological function of AMP activation is not thermogenesis.

Other nucleotide effects on mitochondrial proton conductance have been reported. The classic example is purine nucleotide inhibition of UCP1 from brown adipose tissue [21]. UCP1 has a high affinity for GDP, ATP and ADP, and is also inhibited by AMP with a Kᵣₒ of 110 μM [22]. The AMP effect on UCP1 appears to reflect the relatively broad specificity of inhibition by purine nucleotides, unlike the AMP activation in skeletal muscle, which is highly AMP specific. The UCP1 homologues UCP2 and UCP3 have been proposed to act as uncouplers [23], although this is controversial [24,25], and there is some evidence that they are regulated by nucleotide binding [26–28]. CMP stimulates liver mitochondrial proton conductance, but at higher concentrations than tested in the present study; Kᵣₒ was approx. 4 mM [29]. Purine nucleotides and carboxyatractylate inhibit the high proton conductance of foetal liver mitochondria [30]. ATP stimulates a proton-conductance pathway in yeast mitochondria, but AMP has no effect at the concentrations used in the present study [31].

We tested whether the AMP activation of proton conductance was mediated by homologues of UCP1. We recently showed that starvation of rats for 24 h led to increased amounts of UCP2 and UCP3 mRNA in skeletal muscle, and of UCP3 protein in skeletal-muscle mitochondria, but did not alter proton conductance [25]. Figure 2(c) shows that the stimulatory effect of 1 mM AMP was not increased, despite the 2-fold increase in UCP3 protein measured by Western-blot analysis [25] in the same mitochondrial preparations. This observation suggests that AMP does not activate proton conductance by effects on UCP3.

We also tested whether the AMP activation of proton conductance was mediated by another member of the mitochondrial inner-membrane transporter family, ANT, which exchanges cytosolic ADP for mitochondrial ATP across the mitochondrial inner membrane [32,33]. This carrier has a single binding site which alternately faces the cytosol and the matrix. The binding site is described as specific for the substrates ADP and ATP [33], but it may also bind [34] or transport [35] AMP. The diagnostic feature of the carrier is its highly specific inhibition by atractylate.

Figure 1 Effect of AMP on resting respiration of rat skeletal-muscle mitochondria

(a) Respiration rate as a function of AMP concentration. Assays, in a Clark oxygen electrode, contained 0.5 mg of mitochondrial protein/ml, 120 mM KCl, 5 mM KH₂PO₄, 3 mM HEPES, 2 mM MgCl₂, 1 mM EGTA, 0.3% defatted BSA, 5 μM rotenone, 1 μg/ml oligomycin and 4 mM succinate, pH 7.2. Assays were performed at 37 °C. Mitochondria had respiratory-control ratios of 3.6 ± 0.2 (mean ± S.D.; n = 15). Data are presented as: means ± S.E.M. (n = 6–12, except at 50 μM AMP where n = 2). (b) Hanes plot of the data in (a). r² = 0.98. The intercept on the x-axis gives the Kᵣₒ value for AMP, and corresponds to 80 μM.
carboxyatractylate and bongkrekate [32,33]. These inhibitors did not change the State 4 rate, but they prevented the increase in muscle mitochondrial State 4 respiration caused by 1 mM AMP and 1 mM ADP (Table 1), strongly implicating ANT in the effect of AMP. These inhibitor concentrations prevented stimulation of State 4 respiration by ADP showing that they fully inhibited ANT. Moreover, the amounts of carboxyatractylate that were just sufficient to reverse the AMP stimulation of State 4 and to inhibit ANT over, the amounts of carboxyatractylate and bongkrekate [32,33]. These inhibitors did not change the State 4 rate, but they prevented the increase in muscle mitochondrial State 4 respiration caused by 1 mM AMP and 1 mM ADP (Table 1), strongly implicating ANT in the effect of AMP. These inhibitor concentrations prevented stimulation of State 4 respiration by ADP showing that they fully inhibited ANT. Moreover, the amounts of carboxyatractylate that were just sufficient to reverse the AMP stimulation of State 4 and to inhibit ANT and return the State 3 rate to the State 4 rate were not significantly different (P = 0.28; n = 4; see Figure 3). Stimulation by 200 μM AMP was abolished by 1 mM ATP or 1 mM ADP (Table 1) but not by the other purine or pyrimidine nucleotides listed above (which are not good substrates for ANT), supporting this conclusion. The fact that at least 1 mM ATP is always present in muscle in vivo does not necessarily mean that the AMP stimulation is not physiologically relevant. It is well known, for example, that UCP1 is inhibited by ATP, but this does not imply that the protein is always inactive, as β-agonists are able to stimulate UCP1 in brown adipose tissue even in the presence of high ATP. There was no time dependence of the activation or

**Table 1** Inhibition of AMP-stimulated respiration rate by substrates or inhibitors of ANT

<table>
<thead>
<tr>
<th>Addition</th>
<th>+ BSA</th>
<th>— BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>State 4</td>
<td>141 ± 4.3</td>
<td>ND</td>
</tr>
<tr>
<td>AMP (200 μM)</td>
<td>177 ± 11</td>
<td>ND</td>
</tr>
<tr>
<td>AMP + ADP</td>
<td>145 ± 26</td>
<td>ND</td>
</tr>
<tr>
<td>AMP + ATP</td>
<td>135 ± 6.4</td>
<td>ND</td>
</tr>
<tr>
<td>State 4</td>
<td>123 ± 9.7</td>
<td>168 ± 23</td>
</tr>
<tr>
<td>AMP (1 mM)</td>
<td>188 ± 12</td>
<td>215 ± 21</td>
</tr>
<tr>
<td>AMP + carboxyatractylate</td>
<td>112 ± 20</td>
<td>ND</td>
</tr>
<tr>
<td>AMP + atraclylate</td>
<td>114 ± 19</td>
<td>ND</td>
</tr>
<tr>
<td>AMP + bongkrekate</td>
<td>ND</td>
<td>169 ± 11</td>
</tr>
</tbody>
</table>

**Figure 2** Effect of AMP on mitochondrial proton conductance

Mitochondria were from (a) skeletal muscle from rats fed ad libitum, (b) skeletal muscle from frog (Rana temporaria), (c) skeletal muscle from rats starved for 24 h and (d) rat liver. AMP (1 mM) was either absent (closed circles) or present (open circles). Respiration rate and membrane potential were measured simultaneously using electrodes sensitive to oxygen and to the potential-dependent probe TPMP**. For (a) and (c), rat skeletal-muscle mitochondria were incubated as described in the legend of Figure 1 but with 80 ng/ml nigericin and 50 μM diadenosine pentaphosphate. Nigericin addition increases basal respiration slightly, so lowers the percentage stimulation by AMP compared with Figure 1. The TPMP** electrode was calibrated with sequential additions of TPMP** up to 2 μM. Malonate was added sequentially added up to 2 mM to change the mitochondrial potential. After each run, 0.2 μM FCCP was added to release TPMP** for baseline correction. The TPMP-binding correction was 0.35 (μl/mg of protein)**. Data are presented as means ± S.E.M. of seven (a) or three (c) experiments performed in triplicate. For (b), frog mitochondria (preparation based on that described in [40]) were incubated as in (a) but at 1 mg of protein/ml and 5 mM succinate, pH 7.4, without diadenosine pentaphosphate, at 25 °C. TPMP was added up to 2.55 μM, and malonate up to 5.3 mM. FCCP (2.9 μM) was used, and assumed TPMP binding was 0.4 (μl/mg protein)**. Data are presented as means and ranges of two experiments performed in duplicate. For (d) liver mitochondria were incubated as in (a) but at 1 mg of protein/ml. TPMP was added up to 5 μM, and malonate up to 5 mM. FCCP (2 μM) was used, and assumed TPMP binding was 0.4 (μl/mg protein)**. Data are presented as means and ranges of two experiments performed in triplicate.

**Figure 3** Carboxyatractylloside sensitivity of AMP-stimulated respiration and State 3 respiration in rat skeletal-muscle mitochondria

(a) State 4 respiration in the presence of 1 mM AMP and 1 μg/ml oligomycin. (b) State 3 respiration rate in the presence of 250 μM ADP. Maximal inhibition was found at 2.02 ± 0.2 and 1.76 ± 0.2 nmol carboxyatractylloside/mg of protein respectively (P = 0.28; n = 4). Details are as for Figure 1.
of the carboxyatractylate inhibition, suggesting that AMP activation was directly on the translocase and not a result of AMP uptake followed by activation of a different target within the mitochondria. Fatty acids activate proton leak through ANT [36] but two lines of evidence suggest that the AMP activation is different from activation by fatty acids; AMP activation occurred in the presence of albumin (which chelates fatty acids) and, unlike fatty acid activation, was much less in liver mitochondria (Figure 2d). In agreement with [17] Figure 2 shows that proton conductance is higher in skeletal-muscle mitochondria than in liver mitochondria. The respiratory control ratio of the muscle mitochondria was 3.8 (see the Experimental section), indicating good quality preparations. Thus the difference in proton conductance between liver and muscle mitochondria is intrinsic, and not due to differences in damage during isolation.

An interesting correlation emerged from the tissue distribution of the AMP activation of proton conductance by ANT. Unlike skeletal muscle, there was little or no effect of 1 mM AMP on proton conductance in rat liver mitochondria (Figure 2d). The stimulation of State 4 respiration by 1 mM AMP in mitochondria from four rat tissues was 55% in skeletal muscle, 41% in heart, 27% in kidney and 7% in liver. Two isoforms of ANT (ANT1 and ANT2) with 98% amino acid sequence similarity have been identified in rats [37]. ANT1 as a proportion of total ANT mRNA is 81% in skeletal muscle, 63% in heart, 35% in kidney and 25% in liver [38]. The identical ranking of AMP potency and relative ANT1 mRNA abundance in these four tissues raises the hypothesis that it is ANT1 that is involved in the AMP stimulation of mitochondrial proton conductance.

The activity of the AMP-activated proton-conductance pathway of ANT was high: it doubled the basal proton cycling rate over a range of potentials (Figure 2a). In State 4 it consumed 25–50 nmol O₂/min per mg of protein, which (since 6 H⁺ are cycled per O atom consumed) was equivalent to 150–300 nmol H⁺/min per mg of mitochondrial protein. The content of ANT in skeletal-muscle mitochondria is approx. 1.5–2.0 nmol dimer/mg of protein ([38]; see Figure 3), so the turnover number is approx. 2 H⁺ s⁻¹. In brown adipose tissue mitochondria, UCP1 increases basal proton conductance up to 25-fold [14]. The content of UCP1 in cold-adapted rat brown adipose tissue mitochondria (0.5–0.9 nmol dimer/mg of protein) [4,39] is approx. half the content of ANT in skeletal-muscle mitochondria, so proton transport through the AMP-activated pathway is approx. 2/(2×5) or 4% of the rate of proton transport by UCP1 at the same potential. The membrane potential of muscle mitochondria in situ [8] is much higher than in activated brown adipose tissue [14], so the actual rate of proton transport by ANT could approach that of UCP1. In addition, the total body complement of ANT is much higher than that of UCP1, so the AMP-activated proton-transport rate has the potential for significant thermogenesis.

Since proton leak in muscle is a significant contributor to standard metabolic rate [8], AMP-stimulated proton conductance in muscle might form part of a physiological mechanism of acute regulation of energy dissipation and standard metabolic rate, potentially changing it by 5–10%. Sustained changes in metabolic rate of this magnitude could have significant effects on body weight, so the AMP-stimulated proton conductance of skeletal-muscle mitochondria is a potential target for anti-obesity and anti-cachexia pharmaceuticals.

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AMP decreases the efficiency of skeletal-muscle mitochondria


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