Effects of magnesium and nucleotides on the proton conductance of rat skeletal-muscle mitochondria

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During oxidative phosphorylation most of the protons pumped out to the cytosol across the mitochondrial inner membrane return to the matrix through the ATP synthase, driving ATP synthesis. However, some of them leak back to the matrix through a proton-conductance pathway in the membrane. When the ATP synthase is inhibited with oligomycin and ATP is not being synthesized, all of the respiration is used to drive the proton leak. We report here that Mg^{2+} inhibits the proton conductance in rat skeletal-muscle mitochondria. Addition of Mg^{2+} inhibited both oligomycin-inhibited respiration and the proton conductance, while removal of Mg^{2+} using EDTA activated these processes. The proton conductance was inhibited by more than 80 % as free Mg^{2+} was raised from 25 nM to 220 μ M. Half-maximal inhibition occurred at about 1 μ M free Mg^{2+} ,

which is close to the contaminating free Mg^{2+} concentration in our incubations in the absence of added magnesium chelators. ATP, GTP, CTP, TTP or UTP at a concentration of 1 mM increased the oligomycin-inhibited respiration rate by about 50 %. However, these NTP effects were abolished by addition of 2 mM Mg^{2+} and any NTP-stimulated proton conductance was explained completely by chelation of endogenous free Mg^{2+} . The corresponding nucleoside diphosphates (ADP, GDP, CDP, TDP or UDP) at 1 mM had no effect on oligomycin-inhibited respiration. We conclude that proton conductance in rat skeletal-muscle mitochondria is very sensitive to free Mg^{2+} concentration but is insensitive to NTPs or NDPs at 1 mM.

Key words: ADP, ATP, GDP, proton leak, uncoupling.

INTRODUCTION

The transfer of electrons down the respiratory chain in the mitochondrial inner membrane is coupled to transport of protons from the matrix to the cytosol. The resulting electrochemical proton gradient performs useful work when protons re-enter the matrix through the F_0F_1 -ATPase (causing synthesis of ATP from ADP and P_i [1]) or through other proteins to drive ion and substrate transport. However, the electrochemical proton gradient can also be dissipated by leak reactions catalysed by endogenous mitochondrial proton-conductance pathways [2–5]. In the absence of oxidative phosphorylation, all the protons pumped by the respiratory chain out of the mitochondria return by this proton leak, and there is oxygen consumption without coupled ATP synthesis.

There are two types of proton conductance: basal and inducible [4,5]. Both can be assayed reliably by measuring their kinetics [2,3]. Basal proton conductance is found in all mitochondria. Its mechanism and regulation are unclear, but the evidence that it is catalysed by specific uncoupling proteins is weak [4–6]. It is responsible for 50 % of the respiration of resting perfused rat skeletal muscle [7], and for 35 % of the respiration of a maximally stimulated hindlimb muscle model [8]. The basal proton conductance in all rat tissues causes up to 20–25 % of standard metabolic rate [3,7,8]. Inducible proton conductance occurs through specific uncoupling proteins, and is tightly regulated. The inducible proton leak in brown-adipose-tissue mitochondria is catalysed by uncoupling protein (UCP) 1 [9]. UCP1 is inhibited by purine nucleotides such as ATP and GDP, and is activated by

fatty acids. The existence and mechanisms of inducible proton leak in other tissues are not yet established, but it is widely hypothesized that UCP3 in skeletal muscle, and UCP2 in a range of tissues, may catalyse proton leak and may be regulated by fatty acids and nucleotides (see [4,5,10–12]).

In the present paper we report that micromolar Mg^{2+} strongly inhibits the basal proton conductance of mitochondria isolated from rat skeletal muscle. Apparent stimulation of the proton conductance by ATP, GTP and other nucleoside triphosphates is shown to be a secondary effect that can be explained entirely by chelation of endogenous contaminating Mg^{2+} .

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 dissected immediately from the hindlimbs, weighed and placed in C-P 1 medium (100 mM KCl, 50 mM Tris/HCl and 2 mM EGTA, pH 7.4) on ice. Mitochondria were isolated as described previously [13,14], with all steps at 4 °C. Tissue was shredded with a sharp blade, then minced with sharp scissors, rinsed with C-P 1 medium four or five times, stirred for 4 min in C-P 2 medium [100 mM KCl, 50 mM Tris/HCl, 2 mM EGTA, 1 mM ATP, 5 mM $MgCl_2$, 0.5 % (w/v) BSA and 18.7 units of protease (nagarse)/g of tissue, pH 7.4] and homogenized in C-P 2 using a Polytron tissue homogenizer. The homogenate was stirred in

Abbreviations used: UCP, uncoupling protein; TPMP⁺, triphenylmethyl phosphonium cation; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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C-P 2 for 6 min, then centrifuged at 490 *g* for 10 min. The supernatant was filtered through muslin and centrifuged at 10368 *g* for 10 min. Mitochondrial pellets were resuspended in C-P 1, combined and centrifuged at 10368 *g* for 10 min then at 3841 *g* for 10 min, and resuspended in C-P 1. 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_2PO_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 [15]. Electrode linearity was checked routinely by following the uncoupled respiration rate in the presence of 0.2 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) from 100 to 0% air saturation. Respiratory control ratios (state-3 respiration rate with 250 μM ADP divided by state-4 rate after ADP phosphorylation) with succinate as substrate were 4.2 ± 0.4 ($\pm$ S.D.). We routinely measured respiration rate in the absence of ADP and 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 μM rotenone, 1 $\mu\text{g/ml}$ oligomycin and 4 mM succinate. Nucleoside diphosphates and triphosphates were brought to pH 6–7 and added at concentrations 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 [2,3,16,17]. We determined respiration rate and membrane potential simultaneously using electrodes sensitive to oxygen and to the potential-dependent probe triphenylmethyl phosphonium cation (TPMP⁺) [3,18]. Mitochondria (0.5 mg of protein/ml) were incubated in assay medium containing 5 μM rotenone, 1 $\mu\text{g/ml}$ oligomycin and 80 ng/ml nigericin (to collapse the difference in pH across the inner membrane). The electrode was calibrated with sequential additions up to 2 μM TPMP⁺, then 4 mM succinate was added to start the reaction. Respiration and potential were inhibited progressively 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 to the medium, allowing correction for any small electrode drift. The TPMP⁺ binding correction for skeletal muscle was taken to be $0.35 (\mu\text{l/mg of protein})^{-1}$ [19].

Determination of free Mg^{2+} concentration

Free Mg^{2+} concentration was calculated from total Mg^{2+} in the assay medium, assuming that endogenous Mg^{2+} and Mg^{2+} binding to mitochondria were negligible compared with the large concentrations of added Mg^{2+} and Mg^{2+} chelator. Apparent stability constants were calculated from absolute stability constants at the appropriate pH, temperature and ionic strength [20].

RESULTS AND DISCUSSION

Effects of free Mg^{2+} on the proton conductance of mitochondria isolated from rat skeletal muscle

Non-phosphorylating mitochondrial respiration is used entirely to drive the proton leak across the inner membrane [2,3], and its rate is controlled mainly by the proton conductance [21], so we first measured respiration rates of rat skeletal-muscle mitochondria in the presence of oligomycin as a quick but crude indicator of mitochondrial proton conductance.

Addition of 1 mM EDTA, which chelates Mg^{2+} very effectively (apparent dissociation constant 2.5 μM at pH 7.2), caused a large increase in the respiration rate. Respiration was then decreased by sequential additions of Mg^{2+} from 0.25 to 3 mM (Figure 1). These observations suggest strongly that Mg^{2+} inhibits the proton conductance of skeletal-muscle mitochondria. We have also observed a stimulation of state-4 succinate oxidation by EDTA and its reversal by Mg^{2+} in rat liver mitochondria under the same conditions used in muscle (results not shown). These effects had been described previously in rat liver mitochondria [22], but ascribed to activation of succinate dehydrogenase [23]. Since state-4 respiration is controlled by both proton conductance and substrate oxidation [21], an increase in respiration rate could be due to an increase in substrate oxidation (the membrane potential would increase) or to an increase in proton conductance (the membrane potential would decrease). To distinguish between these possibilities, it is necessary to measure the mitochondrial membrane potential, and the best way is to determine the kinetics of the proton-conductance pathway with and without Mg^{2+} by titrating respiration rate and potential with an inhibitor of electron transport.

Figure 2 shows such a titration of the kinetic response of proton leak rate (measured as respiration rate in the presence

Figure 1 Effects of EDTA and MgCl_2 on oligomycin-inhibited respiration in rat skeletal-muscle mitochondria

Oxygen consumption by skeletal-muscle mitochondria (0.5 mg of protein/ml) in assay medium [120 mM KCl, 5 mM KH_2PO_4 , 3 mM Hepes, 1 mM EGTA and 0.3% (w/v) defatted BSA, pH 7.2] containing 5 μM rotenone, 1 $\mu\text{g/ml}$ oligomycin and 4 mM succinate was measured at 37 °C as described in the Experimental section. EDTA or MgCl_2 was added sequentially as indicated. Values are means $\pm$ S.E.M. from three independent experiments with three replicates per experiment.

Figure 2 Kinetics of proton leak in the presence of 2 mM MgCl_2 or 1 mM EDTA

Oxygen consumption and mitochondrial membrane potential of skeletal-muscle mitochondria (0.5 mg of protein/ml) in assay medium (see Figure 1) containing 5 μM rotenone, 1 $\mu\text{g/ml}$ oligomycin, 80 ng/ml nigericin, 2 μM TPMP⁺ and 4 mM succinate were measured at 37 °C. Rate and potential were titrated through successive steady states by additions of malonate up to 2.5 mM in the presence of 2 mM MgCl_2 (●) or 1 mM EDTA (○). See the Experimental section for details. Values are means $\pm$ S.E.M. from three independent experiments with three replicates per experiment.

of oligomycin) to membrane potential in an assay medium containing either 2 mM MgCl_2 or 1 mM EDTA. The curve in the presence of Mg^{2+} was displaced strongly compared with the curve with EDTA, showing that the proton conductance was inhibited strongly by Mg^{2+} . At comparable values of the membrane potential, Mg^{2+} depressed the proton leak rate (and hence the proton conductance) by more than 80 % compared with the rate with EDTA present. These results show that the depression of oligomycin-inhibited respiration by Mg^{2+} in Figure 1 is caused primarily by an inhibition of proton conductance, not by a direct inhibition of substrate-oxidation reactions. Mg^{2+} is unlikely to have its effects by raising the mitochondrial H^+/O ratio, since, if anything, it has been shown to lower this ratio [24]. On the other hand, addition of bongkreikic acid to the medium at 4 μM (which prevents mitochondria from entering into state 3 after ADP addition) does not block the increase in the oligomycin-inhibited respiration rate caused by 1 mM EDTA and therefore it is unlikely that the permeability transition pore contributes to this effect.

Figure 3 shows the effect of free Mg^{2+} concentration on oligomycin-inhibited respiration rate. Half-maximal inhibition occurred at about 1 μM free Mg^{2+} , which is well below the physiological concentration. This concentration of Mg^{2+} gave rates that were close to the control rates obtained in the absence of added Mg^{2+} or EDTA, suggesting that the contaminating free Mg^{2+} concentration in our incubations in the absence of magnesium chelators was about 1 μM . The stimulation of respiration by EDTA in Figure 1 is then explained by chelation of this endogenous Mg^{2+} .

Others have also reported effects of Mg^{2+} on mitochondrial proton conductance. There are several reports that EDTA causes some uncoupling in rat liver mitochondria that is reversed partially by addition of Mg^{2+} (e.g. [22,23]). Jung and Brierley [25] found that high-affinity binding sites for Mg^{2+} and Ca^{2+} control

Figure 3 Effect of free Mg^{2+} concentration on oligomycin-inhibited respiration

For details see Figure 1. Total MgCl_2 added was varied, and free Mg^{2+} concentration was calculated from apparent stability constants (see the Experimental section). Values are means $\pm$ S.E.M. from three independent experiments with three replicates per experiment.

inner-membrane permeability to small ions. Goubert et al. [26] found that dietary magnesium deficiency led to increased UCP1-catalysed proton conductance in mitochondria isolated from rat brown adipose tissue. *In vitro*, 2 mM Mg^{2+} decreased the basal proton conductance in brown-adipose-tissue mitochondria from control or magnesium-deficient rats [26]. We cannot say from our results whether the effect of Mg^{2+} on proton conductance in rat skeletal-muscle mitochondria is caused by interactions with specific membrane proteins, or whether it reflects enhanced membrane integrity, particularly in relation to the lipid content of the mitochondrial inner membrane.

Effects of nucleotides on the proton conductance of mitochondria isolated from rat skeletal muscle

We studied the effects of ATP, GTP, CTP, TTP and UTP at 1 mM on the oligomycin-inhibited respiration rate of rat skeletal-muscle mitochondria. Figure 4 shows that all the NTPs increased oxygen consumption by about 50 %. There was no effect of any of the corresponding nucleoside diphosphates (e.g. ADP or GDP, Figure 4).

Effects of nucleotides on mitochondrial proton conductance are well known. The classic example is UCP1 [27], which catalyses an inducible proton leak in brown-adipose-tissue mitochondria that is inhibited by purine nucleotides, particularly GDP and ATP. There is also an inhibition of proton conductance in yeast mitochondria by ATP [28]. UCP3 is a UCP1 homologue that is highly skeletal-muscle-specific [29,30]. Its uncoupling activity is a matter of debate [4–6,10,12]. We first postulated that the increase in respiration rate was due to an activation of a UCP3 uncoupling activity. However, NTPs strongly chelate divalent cations. To test whether the effect of nucleotides worked through Mg^{2+} chelation, we retested the nucleotide effects at saturating Mg^{2+} concentration. At 2 mM Mg^{2+} , we found no effect of any of the NTPs or NDPs on oligomycin-inhibited respiration rate (e.g. ATP, GTP, ADP or GDP, Figure 4). We also removed Mg^{2+} using EDTA. With 1 mM EDTA, there was a residual stimulation of oligomycin-inhibited respiration by purine (e.g. ATP or GTP, Figure 4) but not by pyrimidine nucleoside triphosphates (e.g.

Figure 4 Effect of nucleotides on oligomycin-inhibited respiration

For details see Figure 1. Respiration was measured in the presence of 1 mM NTP, 1 mM NDP, 2 mM MgCl_2 or 1 mM EDTA, as indicated, and compared with controls with no additions. Diadenosine pentaphosphate (50 μM) was present for experiments with NTPs. Values are means $\pm$ S.E.M. from 2–13 independent experiments.

Figure 5 Effect of 1 mM ATP on the kinetics of the proton leak in the presence of 1 mM EDTA

For details see Figure 2. Mitochondria were incubated in the presence of 1 mM EDTA and 50 μM diadenosine pentaphosphate in the absence ($\square$) or presence ($\blacksquare$) of 1 mM ATP. Values are means $\pm$ S.E.M. from three independent experiments with three replicates per experiment.

UTP, Figure 4). However, the residual stimulation of respiration by ATP was not caused by stimulation of the proton conductance, since the proton leak titration curve was not significantly shifted by ATP in the presence of EDTA (Figure 5). Instead, it reflected an effect of purine nucleotides on the reactions of substrate oxidation. Thus except for this activation of substrate oxidation in the presence of EDTA, all of the effects of NTPs on proton conductance and respiration were caused by changes in free Mg^{2+} concentration. When measured in the presence of 1 mM

EDTA to clamp free Mg^{2+} , or in the presence of excess Mg^{2+} , the effects of NTPs on proton conductance were prevented. There was no evidence that the nucleotide effects were mediated directly by specific proteins such as UCP3 or other UCPs.

Our results suggest that proton conductance in rat skeletal-muscle mitochondria is insensitive to nucleoside triphosphates and nucleoside diphosphates. However, it is strongly inhibited by Mg^{2+} , with a half-maximal effect at about 1 μM free Mg^{2+} . Intracellular free Mg^{2+} will always be much higher than 1 μM , so the effect of Mg^{2+} on proton conductance is unlikely to be a physiological regulatory mechanism. We suggest that high Mg^{2+} should always be added in assays to investigate the physiological proton leak in isolated mitochondria.

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