Respiratory inhibitors and uncouplers prevent the aeration-induced increase in mitochondrial anion conductivity

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1. When mitochondria are stirred in air the rate of anion conductivity increases, this effect being enhanced by the addition of respiratory substrate. 2. This effect is reversible if the mitochondria are stored for a period of time under N₂. 3. The aeration-induced increase in mitochondrial anion conductivity can also be prevented by the addition of respiratory inhibitors rotenone and antimycin A, as well as by 30 μM-cyanide. 4. A decrease in this aeration-induced anion conductivity can also be observed upon the addition of the uncouplers carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (2 μM) and 2,4-dinitrophenol (100 μM). 5. Simultaneous measurements of mitochondrial anion conductivity and membrane potential show a relationship between the level of membrane potential and anion conductivity. 6. It is suggested that the level of membrane potential is either directly or indirectly responsible for the level of mitochondrial anion conductivity.

INTRODUCTION

The electrogenic transport of anions such as Cl[−] across the mitochondrial inner membrane observed under conditions of energization or at alkaline pH (Azzi & Azzone, 1967; Brierley, 1969) is recognized as being due to a pH-dependent pore or channel (Selwyn *et al.*, 1979; Beavis & Garlid, 1987) and has been given the acronym IMAC (Inner Membrane Anion-conducting Channel) by the latter authors, who have also postulated a role for matrix Mg²⁺ in the control of the anion conductivity. These groups have studied the channel by observations of the rate of passive osmotic mitochondrial swelling estimated by light-scattering changes. More direct evidence for the existence of anion-conducting channels in the mitochondrial inner membrane has been provided by Sorgato *et al.* (1987) by patch-clamping the inner membrane.

Although this channel is highly selective for anions against cations, it has low specificity for different anions, and in many ways it resembles the nucleotide-phosphate-controlled thermogenin or uncoupling protein channel in brown-adipose-tissue mitochondria (Nicholls, 1979; Cannon & Nedergaard, 1985) and might therefore be expected to uncouple oxidative phosphorylation. The role of the IMAC in energy-conserving liver or muscle mitochondria is at present obscure, although suggestions have been made that it could function as a safety-valve to prevent excessive alkalinization of the matrix, have a role in thyroid-controlled uncoupling or mitigate the effects of hyperoxia (Selwyn, 1987). The last suggestion is based on the observations of Andersson *et al.* (1987) on the properties of mitochondria isolated from ischaemic muscle and on our previous observations that the anion conductivity of isolated mitochondria is low when they are stored under anaerobic conditions, but high when the stock suspension is stirred in air (Selwyn *et al.*, 1986). The effect of aeration is enhanced by the addition of respiratory substrates to the stock suspension, and we

have therefore investigated whether the effect of O₂ is mediated via the respiratory chain rather than by some direct effect on the channel constituents or by some other redox-dependent process.

The present paper describes the results of these investigations, which show that the effect of O₂ is dependent on the proton-pumping capability of the respiratory chain.

EXPERIMENTAL

Materials

Tetraphenylphosphonium bromide, rotenone and antimycin A were obtained from Sigma. Carbonyl cyanide *p*-(fluoromethoxy)phenylhydrazone (FCCP) was obtained from BCL, Lewes, Sussex, U.K. All other chemicals were of analytical grade.

Methods

Rat liver mitochondria were prepared by the method of Selwyn *et al.* (1970) from adult Wistar rats. The final suspension of mitochondria was adjusted to 100 mg/ml in 0.25 M-sucrose containing 5 mM-Hepes/KOH, pH 7.5. The protein concentration of the mitochondrial suspension was assayed by using the biuret method of Gornall *et al.* (1949) with BSA as standard.

During all experiments, the mitochondria (1 ml), suspended in 0.25 M-sucrose/5 mM-Hepes/KOH were stirred on ice in the presence of 5 mM-potassium succinate, pH 7.5, under either air or N₂ (as detailed in the Figure legends). Any additions to this preincubation are specified in the Figure legends.

The standard assay for anion uniport was osmotic swelling of the mitochondria suspended in 4 ml of 100 mM-NH₄Cl containing 5 mM-Hepes (titrated to pH 8.0 with NH₃), with 12.5 μM-FCCP present to permit charge and pH balance of the Cl[−] uniport. The vessel was thermostatically controlled at 25 °C. A portion (4 mg) of

Abbreviations used: FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; IMAC, inner-membrane anion-conducting channel; TPP⁺, tetraphenylphosphonium cation.

mitochondrial protein was added to this from the preincubation. The initial rate of mitochondrial swelling was measured by a decrease in light-scattering (Selwyn *et al.*, 1970).

The membrane potential of the mitochondria was routinely estimated by monitoring the uptake of tetraphenylphosphonium cation (TPP^+) using a TPP^+ -selective electrode constructed by the method of Kamo *et al.* (1979). A standard incubation comprised (in a vessel thermostatically controlled at 25 °C) 2.5 ml of 100 mM-KCl containing 5 mM-Hepes, pH 7.5, with 10 μM - TPP^+ present. A 10 mg portion of mitochondrial protein was added to this from the preincubation at time intervals specified in the Figures.

RESULTS

The effect of repeated transitions between anaerobic and aerobic storage conditions on the rate of passive anion uniport across the mitochondrial inner membrane, as measured by uncoupler-stimulated swelling in iso-osmotic NH_4Cl medium at pH 8, is shown in Fig. 1. It can be seen that both the increase in the rate of anion uniport produced by aerobic, and the decrease produced by anaerobic, preincubation are reversible. This argues strongly against either effect being caused by damage to the mitochondrial inner membrane, since the capacity for resynthesis or repair of membrane components under the storage conditions must be limited. As reported previously (Selwyn *et al.*, 1986), addition of respiratory substrates such as pyruvate or succinate enhances the effect of keeping the suspension oxygenated, leading to very high rates of anion uniport. This suggests that the effect of aeration is mediated by the respiratory chain. In consequence, investigation was made of the role of the respiratory chain and ion movements in mediating the effects of presence or absence of O_2 .

Inhibiting the respiratory chain by adding rotenone and antimycin A to the preincubation prevents the increase in anion uniport produced by aeration, as shown

Fig. 1. Effect of aerobic and anaerobic storage on the rate of mitochondrial anion conductivity

Preincubation conditions were as described in the Experimental section. The lower curve represents the rate of anion conductivity of unstirred mitochondria. The upper curve shows the rate of anion conductivity of the mitochondria alternately exposed to air and O_2 -free nitrogen. These results are representative of four experiments.

Fig. 2. Effect of rotenone and antimycin A on the levels of mitochondrial swelling

Preincubation conditions were as described in the Experimental section. Rotenone (2 μM) and 2 μM -antimycin A (dissolved in ethanol) (■) were added to the preincubation at zero time. Ethanol (2 μl) (●) was added to a second preincubation at zero time as a control. These results are representative of four experiments.

in Fig. 2. Addition of another potent inhibitor of respiration, cyanide, at 30 μM , also blocks the increase in the rate of anion conduction (results not shown).

Simultaneous measurements of anion uniport by passive swelling in NH_4Cl and of membrane potential using TPP^+ as described in the Experimental section showed that, under aerobic preincubation conditions, the increase in the rate of anion uniport parallels an increase in the membrane potential, whereas under anaerobic conditions both remain at a low level (Fig. 3). After aerobic preincubation, prolonged (40 min) preincubation under N_2 produces a decrease in both the rate of anion uniport and the membrane potential.

Fig. 4 shows that rotenone plus antimycin A also causes a sharp decrease in the membrane potential. These observations conclusively demonstrate the role of the respiratory chain and suggest that the level of the membrane potential may be important. This hypothesis has been tested by adding uncouplers to the mitochondria during preincubation. Fig. 5 shows that the addition of FCCP to the preincubation is very effective in abolishing the effect of oxygenation on the rate of anion uniport, although it would be expected to increase the rate of respiration. FCCP caused a rapid decline in the membrane potential as well as a decrease in the rate of anion uniport (Fig. 6). It appears from both this Figure and Figs. 3 and 4 that the rate of anion uniport parallels the decrease of membrane potential after a short lag of at least 5 min. The lag may be even longer, because although the assay methods allow virtually instant measurement of the rate of anion uniport, the measurement of membrane potential takes longer. Thus it is impossible to obtain an estimate of membrane potential within less than 5 min of changing the preincubation conditions. A similar abolition of the effect of aerobic preincubation on the rate of anion uniport was found with the classic uncoupler 2,4-dinitrophenol at 0.1 mM.

An alternative way in which the increase in membrane potential could be limited is by the addition of ADP,

Fig. 3. Effect on the levels of mitochondrial swelling and membrane potential of switching from an aerobic to an anaerobic atmosphere

Preincubation conditions were as described in the Experimental section. The mitochondria were placed under N₂ at the times indicated. Sample sizes were 4 mg of protein for the light-scattering measurements (●) and 10 mg for the membrane-potential measurements (○). These results are representative of three experiments.

Fig. 5. Effect of FCCP on the levels of mitochondrial swelling

Preincubation conditions were as described in the Experimental section. FCCP (2 μM) (■) was added at zero time. Ethanol (2 μl) (●) was added to a second preincubation at zero time as a control. These results are representative of five experiments.

Fig. 4. Effect of rotenone and antimycin A on the levels of mitochondrial swelling and membrane potential

Preincubation conditions were as described in the Experimental section. Rotenone (2 μM) and 2 μM -antimycin A were added to the preincubation at the time indicated. Sample sizes were 4 mg of protein for the light-scattering measurements (●) and 10 mg for the membrane-potential measurements (○). Ethanol (2 μl) was added to a second preincubation as a control. These results are representative of at least four experiments.

Fig. 6. Effect of the FCCP on the levels of mitochondrial swelling and membrane potential

Preincubation conditions were as described in the Experimental section. FCCP (2 μM) was added to the preincubation at the time indicated. Sample sizes of 4 mg of protein were taken for the light-scattering measurements (●) and 10 mg for the membrane-potential measurements (○). These results are representative of five experiments.

Fig. 7. Effect of ADP on the levels of mitochondrial swelling and membrane potential

Preincubation conditions were as described in the Experimental section. 12.5 mM-ADP was added to the incubation at the time indicated. Sample sizes were 4 mg of protein for the light-scattering measurements (●) and 10 mg of protein for the membrane-potential measurements (○). These results are representative of three experiments.

thereby allowing phosphorylation with endogenous phosphate and proton flow through the F_1 -ATPase. It can be seen from Fig. 7 that, although the decrease in membrane potential produced by ADP is small, it is nevertheless accompanied by a small decrease in the rate of anion uniport.

DISCUSSION

The results reported here, namely that inhibitors, which block the respiratory chain, or uncouplers, which abolish the protonmotive force, both prevent the aeration-induced increase in anion conductivity, show (i) that the effects of storage under aerobic or anaerobic conditions are produced by the oxygen acting as the terminal electron acceptor of the respiratory chain, and (ii) that the proton-pumping function of the chain is important. Thus either respiratory inhibitors, which block both electron transfer and proton pumping, or uncouplers, which dissipate any protonmotive force but stimulate electron transfer, prevent or reverse the increase in anion conductivity produced by aeration.

When ADP (12.5 mM) is added to the preincubation, there is a small decrease in the membrane potential. This is consistent with small changes in the levels of protonmotive force observed under phosphorylating conditions (for a review, see Ferguson, 1985). It is noteworthy that the decrease in the level of anion conductivity is also small, thus reinforcing the idea of a relationship between the levels of anion conductivity and membrane potential.

One of the possible functions of the IMAC is that it could act as a 'safety valve' limiting the levels of protonmotive force and thus preventing dielectric breakdown of the mitochondrial inner membrane. It has also been suggested (Selwyn, 1987) that an anion channel in the inner membrane of mitochondria could protect against potentially harmful high concentrations of O_2 by uncoupling oxidative phosphorylation and increasing O_2 use. On the basis of the observations reported here it is

suggested that the level of membrane potential is one of the factors that control the IMAC, by either a direct or indirect method.

One of the indirect processes that could result from a build-up of membrane potential and control the level of anion conductivity is a redistribution of cations, for example Mg^{2+} , which has been implicated in the control of the IMAC (Beavis & Garlid, 1987). No change is observed in the levels of Mg^{2+} when the mitochondria undergo transitions from aerobic to anaerobic storage (results not shown).

It is unlikely that the controlling mechanism operating under these conditions is the matrix pH. Observations by Andersson *et al.* (1987) on mitochondria of hepatocytes and by ourselves in isolated mitochondria (results not shown) have shown there to be no change in the matrix pH under anaerobic conditions; also, under the preincubation conditions employed in our experiments, the ΔpH would only be a small component of the protonmotive force. It is more likely that the controlling process, if not the membrane potential itself, is a consequence of the changes in levels of membrane potential.

Our conclusions from the observations reported here are that (i) the effect of O_2 on the anion-conducting channel is mediated via the respiratory chain and membrane potential, (ii) the pH-dependent IMAC is regulated by the membrane potential, increasing in conductivity or opening at high membrane potential and (iii) this is either a direct effect or is produced by some as yet unidentified consequence of high membrane potential. This last conclusion is supported by the observations of Sorgato *et al.* (1987), who patch-clamped the mitochondrial inner membrane and observed an anion-selective channel that was sensitive to voltage but not to pH.

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