Biochimica et Biophysica Acta, 1059 (1991) 55–62

ADONIS 0005272891001798

BBABIO 43431

On the nature of the mitochondrial proton leak

Guy C. Brown and Martin D. Brand

Department of Biochemistry, University of Cambridge, Cambridge (U.K.)

(Received 1 October 1990)

Key words: Mitochondrion; Proton permeability; Cation permeability; Fatty acid; Respiratory control

Respiring mitochondria have a significant passive permeability to protons; the mechanism of this proton leak is unknown. Several putative mechanisms were tested. Mitochondrial permeability to small sugars was unaffected by energization, suggesting that there is no significant dielectric breakdown at high membrane potential. Mitochondria are argued to have a proton permeability that is 6 to 8 orders of magnitude higher than the permeability to other cations, suggesting that the proton leak is probably not via a simple pore or membrane defect. 15–30% of the proton leak of freshly prepared mitochondria was extractable with bovine serum albumin and is probably due to fatty acids. Little if any of the proton leak appears to be due to cycling of ions other than protons, or to be associated with the functional activity of the proton pumps. The mitochondrial proton leak shares several properties with the proton permeability of pure phospholid bilayers, suggesting that they share the same mechanism, although the leak through the bilayer in mitochondria may be modified by the presence of proteins.

Introduction

It has previously been shown that the leak of protons across the inner membrane of isolated mammalian mitochondria is significant and that the rate of this proton leak increases disproportionately with increased mitochondrial membrane potential $\Delta\psi$ (Refs. 1–3, reviewed in Refs. 4–6). (The flux of protons, hydroxonium and hydroxyl ions will not be distinguished here and all will be referred to as proton flux or proton leak.) In the absence of oxidative phosphorylation and transport of other ions, all the protons pumped by the respiratory chain out of the mitochondria return into the mitochondria by this proton leak (or leaks). The first evidence that the proton leak increased disproportionately with $\Delta\psi$ was the finding that respiration (in the absence of phosphorylation or ion transport) could be substantially inhibited with only a small decline in $\Delta\psi$ [1]. This was interpreted to mean either (a) there was a large drop in proton leak rate with a small drop in $\Delta\psi$ [1], or (b) there was a large increase in proton/electron stoichiometry of the respiratory chain with a small drop in $\Delta\psi$ [7]. There is now evidence against a large change in proton/electron stoichiometry [8–10], but it is still possible that there may be a small change with $\Delta\psi$ [11], (see Ref. 5 for review). More direct measurements of the mitochondrial proton leak have shown that it does indeed increase disproportionately with $\Delta\psi$ [2,3,11].

A significant proton leak (measured indirectly by electron flux rate) which rises disproportionately with $\Delta\psi$ has been found in isolated rat liver mitochondria [1], guinea-pig brown adipose tissue mitochondria [12], Jerusalem artichoke mitochondria [13], bovine heart submitochondrial particles [14], vesicles of *Paracoccus denitrificans* [15], intact cells and chromatophores of *Rhodopseudomons capsulata* [16,17], and in mitochondria in intact lymphocytes [18]. The proton leak accounts for roughly 30% of mitochondrial respiration in resting intact rat liver cells [18,19] and has significant control over cellular respiration rate [19]. The amount of mitochondrial proton leak is controlled by thyroid hormone [18,20–22]. Thus the proton leak may have significant control over energy metabolism and heat production in a wide range of organisms (see Refs. 6, 23).

Abbreviations and symbols used: $\Delta\psi$, mitochondrial membrane potential; BSA, bovine serum albumin; DCCD, *N*,*N*′-dicyclohexylcarbodiimide, FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TMA, tetramethylammonium, TPMP, methyltriphenylphosphonium; State 4, state of mitochondria respiring with substrate, phosphate and ATP present; Static head, state of mitochondria with substrate present, but not ATP/ADP or uncoupler.

Correspondence (present address): G.C. Brown, Department of Physiology, University College London, Gower Street, London, WC1E 6BT, U.K.

The mechanism of proton leak is unknown in mitochondria. Possible pathways include: (a) a membrane protein or proteins, (b) the phospholipid bilayer, or (c) protein/phospholipid interfaces. The disproportionate rise in proton leak rate in mitochondria at high $\Delta\psi$ has led some authors to suggest that the pathway or energy barrier for protons leaking back across the membrane changes at high $\Delta\psi$ [1], while others have suggested that a disproportionate rise in ion leak rate with $\Delta\psi$ is what we should expect for no change in pathway or energy barrier [2], (see Ref. 4 for review). However, the fact that a disproportionate relationship is found in mitochondria does not in itself indicate whether or not there is a change in pathway or energy barrier for protons at high $\Delta\psi$. Several monovalent cations other than protons also enter mitochondria with a rate which rises disproportionately with increasing $\Delta\psi$ [3,4,21], and this has lead us to suggest that protons and other cations may have a common mechanism of entry [3]. However, this need not be so, as many different mechanisms of transport may lead to a disproportionate relationship between transport and $\Delta\psi$ [4]. Mitochondria contain carriers for various ions, and cation (or anion) uniport together with electroneutral exchange for protons (or hydroxyl ions) will lead to ion cycling with net proton transport down its electrochemical gradient. Cation cycling may contribute to the net proton leak flux in mitochondria.

More information about the mechanisms of proton leaks is available from model systems. Phospholipid bilayers show the same disproportionate rise in proton leak with $\Delta\psi$ as mitochondria [2,24–28]. Phospholipid bilayers have a considerable higher permeability (i.e., flux per unit concentration gradient) to protons than to other ions [28]. Several different mechanisms of proton leak in phospholipid bilayers have been considered: (a) lipid protonophores [26,27]; (b) reversible dielectric breakdown [29]; (c) hydrated bilayer defects [28]; or (d) transient hydrogen-bonded water chains [28].

Gutknecht [26,27] has proposed that most proton conductance in planar bilayers is due to anionic lipid contaminant protonophores, and this conductance can be inhibited either by extracting the protonophores with bovine serum albumin (BSA) or by decreasing the membrane dipole potential by adding phloretin to the bilayer. Application of voltages above 150 mV to pure phospholipid bilayers or biological membranes generates transiently stable pores in the bilayer (a process termed reversible dielectric breakdown), the permeability of which is relatively unselective [29,30]. Thermal fluctuations in the packing of the bilayer phospholipids have been suggested to lead to transient bilayer defects [31] which might mediate the leak to ions. In order to explain the higher permeability of bilayers to protons compared to other ions it has been suggested that transient bilayer defects may be lined with hydrogen-bonded water molecules forming a water wire along which protons may migrate as they do in ice [28].

There are reports that many proteins can increase bilayer permeability without acting as channels [32], although there are conflicting papers that suggest no change in conductance when proteins are inserted [24,33]. Proteins could increase permeability at the protein/bilayer interface by: (a) introducing defects at the interface; (b) reducing the activation energy for previously occurring pathways by increasing the membrane dielectric constant; or (c) introducing pathways at the interface due to hydrogen bonds or charges.

In the present paper we have sought to define the pathways of proton and other ion leaks in isolated mitochondria, by testing several different putative mechanisms.

Materials and Methods

Rat liver mitochondria were isolated as in Ref. 34. Mitochondrial swelling was followed by mitochondrial light scattering as in Ref. 3. Respiration rate was measured with a Clark-type oxygen electrode. Mitochondrial membrane potential ($\Delta\psi$) was measured simultaneously with a TPMP electrode as in Refs. 34, 35. Distribution of radiolabels between mitochondrial matrix and incubation medium was determined as in Ref. 35. Materials were from the sources indicated in Ref. 3.

Results and Discussion

Does the mitochondrial inner membrane undergo reversible dielectric breakdown in the coupled state?

Liposomes and biological membranes undergo reversible dielectric breakdown when subjected to external electric fields equivalent to a membrane potential of above 150 mV [29,30,36]. This treatment results in a greatly increased permeability to small molecules, and this permeability is relatively unselective [29,30]. The mitochondrial $\Delta\psi$ is above 150 mV in state 4 or when respiring in the presence of excess respiratory substrate and the absence of adenine nucleotides or uncouplers. If the inner mitochondrial membrane undergoes reversible dielectric breakdown at high $\Delta\psi$ we would expect the permeability of mitochondria to small neutral molecules to increase at high $\Delta\psi$. We tested this possibility by assessing whether the swelling of mitochondria incubated in media consisting of neutral sugars was dependent on energization of the mitochondria. The rate of mitochondrial swelling in different osmolytes is proportional to the rate of osmolyte entry and to the rate of absorbance decrease of the mitochondria (at any given absolute absorbance) [3]. Mitochondria were added to a cuvette containing media whose main osmotic support was either sucrose,

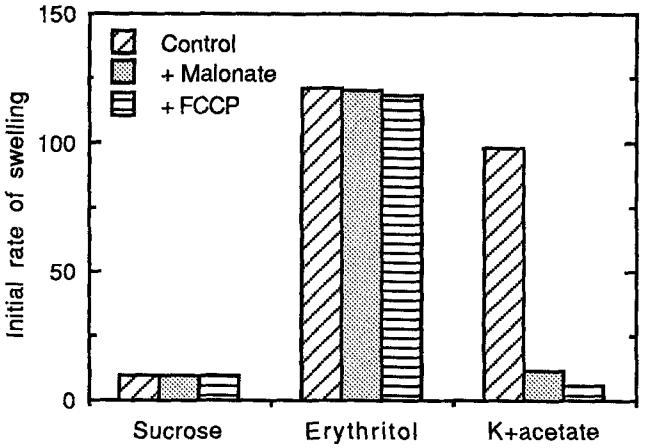

Fig. 1. Effect of deenergization on the rate of mitochondrial swelling in sucrose, erythritol and potassium acetate. The initial rate of swelling was measured by the initial rate of absorbance decrease at 600 nm, on addition of mitochondria (0.5 mg/ml) to a medium containing 240 mosmol/l of either sucrose, erythritol or potassium acetate, plus 2 mM succinate, 0.5 mM EGTA, 5 μM rotenone, brought to pH 7.2 with TMA·OH at 25°C, plus either no additions, 100 nM FCCP or 1.8 mM TMA·malonate. Rates are reported in units of 0.001 absorbance units per min. In all cases the initial A_{600} was 1.2. The column heights represent the mean initial rate of swelling of two replicates. The experiment was repeated on another preparation of mitochondria with similar results, i.e., no detectable effect of deenergization (+ FCCP or malonate) on swelling in sugars.

erythritol or potassium acetate. All the media also contained succinate to energize the mitochondria, while the uncoupler FCCP or the respiratory inhibitor malonate were added to some cuvettes in sufficient quantity to inhibit generation of a detectable $\Delta\psi$. Fig. 1 shows that the rate of swelling of mitochondria in the sugars sucrose and erythritol was completely unaffected by energization, therefore the permeability of mitochondria to these sugars is unaffected by $\Delta\psi$. For comparison the energy dependence of swelling in potassium acetate was tested. Fig. 1 shows that swelling in potassium acetate was almost completely dependent on energization, indicating that K^+ permeation is greatly enhanced by $\Delta\psi$, as shown previously [3].

The energy dependence of mitochondrial permeability to sugars was also tested by following the uptake of [^{14}C]mannitol and [^{14}C]sucrose into mitochondria (measured as in Ref. 34) in the presence and absence of the respiratory inhibitor myxothiazol. We have previously shown that mannitol and sucrose slowly permeate into respiring mitochondria [34]. Respiratory inhibition had no detectable effect on the rate of mannitol or sucrose permeation. For example, mitochondria incubated with [^{14}C]mannitol in energized conditions had a mannitol-inaccessible volume of 0.57 μl/mg protein (0.04 standard error) after 2 min with mannitol, and 0.30 (0.04) after 20 min, while mitochondria in the presence of excess myxothiazol had a mannitol-inaccessible volume of 0.51 (0.03) after 2 min and 0.27 (0.06) after 20 min.

Since dielectric breakdown of biological membranes induced by external fields does considerably enhance permeability to sucrose, mannitol and erythritol [30], it appears to follow from the above results that energization of mitochondria does not result in significant dielectric breakdown. Patch-clamped erythrocyte membranes undergo reversible breakdown above 150 mV [36], the mitochondrial inner membrane appears to be resistant to breakdown at this $\Delta\psi$, presumably due to some difference in structure.

Can we infer from the fact that dielectric breakdown is not a significant pathway in sugar permeability that dielectric breakdown is not a significant pathway for cation permeability at high $\Delta\psi$? This inference is not strictly legitimate, as an electric field may enhance the apparent permeability of a pore to ions relative to neutral molecules.

Is it possible that dielectric breakdown is a significant pathway for protons at high $\Delta\psi$? This possibility seems to be ruled out by the relative permeabilities of mitochondria to protons and other cations at high $\Delta\psi$. K^+ uniport permeability is the highest of the cation permeabilities induced by energization of mitochondria [37], and has an apparent K_m of about 5 mM and V_{max} of 3.5 nmol K^+/mg mitochondrial protein per min for liver mitochondria at pH 8 [38] and a K_m of about 12 mM and V_{max} of 3–7 nmol K^+/mg per min for heart mitochondria at pH 7 [39]. These K_m values are apparent only, because the permeabilities are very sensitive to $\Delta\psi$, and the higher K^+ flux at higher K^+ concentration may lower the steady-state $\Delta\psi$. Diwan [40] estimated the unidirectional K^+ flux into energized liver mitochondria at pH 7 and 20°C to be about 1 nmol K^+/mg per min at 5 mM external K^+. This provides an upper-limit estimate of the steady-state K^+ influx rate. The respiration rate in similar conditions is about 10 nmol O/mg per min, equivalent to steady-state proton influx of about 60 nmol H^+/mg per min (assuming $H^+/O = 6$) with an external proton concentration of 10^{-7} M. The relative H^+/K^+ permeabilities at static head are thus at least 10^6 at 5 mM K^+ and pH 7. In heart mitochondria the coupled respiration rate is 50–100 nmol O/mg per min [37] at pH 7, 25°C, in conditions in which the maximal K^+ influx is 3–7 nmol/mg per min. Thus the relative H^+/K^+ permeability is at least 10^8. It is exceedingly unlikely that a simple aqueous pore would give rise to relative H^+/K^+ permeabilities of 6–8 orders of magnitude and it is therefore unlikely that the mitochondrial proton flux at high $\Delta\psi$ is significantly mediated by dielectric breakdown.

Are weak acid protonophores such as fatty acids involved in the proton permeability?

Gutknecht [26] has proposed that the proton permeability of black lipid membranes is mediated by weak acid lipid contaminants acting as protonophores. This

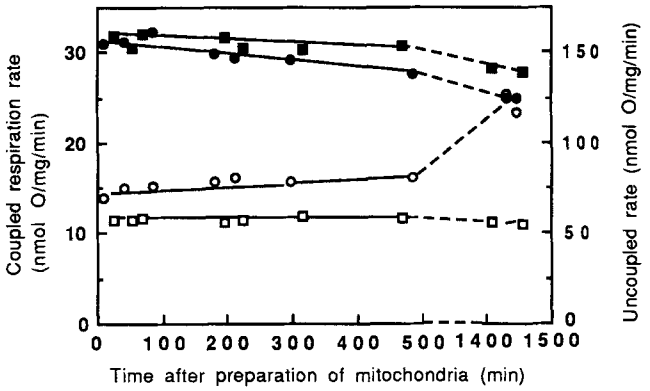

Fig. 2. Effect of BSA on coupled and uncoupled respiration rate as a function of time after preparation of the mitochondria. Mitochondria (1.35 mg/ml) were incubated in 125 mM KCl, 5 mM Hepes, 1 mM EGTA, 5 μM rotenone (brought to pH 7.4 with KOH) either in the presence (□, ■) or absence (○, ●) of BSA (3 mg/ml) for 5 min in an oxygen electrode chamber at 25°C. Then 4.2 mM TMA succinate was added to establish the steady-state coupled respiration rate (○, □), and after a further 5 min sufficient FCCP was added to reach the maximal steady-state uncoupled respiration rate (●, ■) (0.2 μM FCCP in the absence of BSA, 2 μM FCCP in the presence of BSA). The experiment was repeated on another preparation of mitochondria with very similar results, i.e., coupled and uncoupled respiration rates were almost constant with the age of the mitochondria in the presence of BSA, but in the absence of BSA the uncoupled rate slowly increased and the uncoupled rate slowly decreased.

permeability is removed by incubating the membrane with BSA [26]. BSA is known to increase the coupling of isolated mitochondria [41], probably by binding fatty acids. Fig. 2 shows the measured respiration rate at static head (in the presence of succinate and no adenine nucleotides) and after the addition of the uncoupler FCCP, in the presence and absence of BSA, as a function of the age of the mitochondria (i.e., the time between preparing the mitochondria and assaying respiration, during which the mitochondria are at 4°C and essentially anaerobic). The uncoupled rate is initially unaffected by the presence of BSA and declines slowly with time. In the absence of BSA the coupled respiration rate rises slowly with time. The presence of BSA reduces this rate and the BSA uninhibitable rate is constant with time. This indicates that in isolated mitochondria there are probably at least two different pathways of proton leak: (a) a pathway that is inhibited or extracted by BSA, and probably due to fatty acids, which slowly increases with age of the mitochondria, and (b) a quantitatively more important pathway not inhibitable by BSA, and relatively constant with age of the mitochondria.

To get a better estimate of the relative contributions of these two pathways, the BSA inhibition of respiration was assayed on three separate preparations of mitochondria within 1 h of preparation. The average inhibition of static head respiration was 16% (standard deviation 5%). This does not, however, indicate that 16% of proton leak flux is via the pathway extractable by BSA, as reducing the leak raises $\Delta\psi$ (see Fig. 3a) and thus stimulates the BSA-insensitive leak. The relation between the inhibition of respiration and the relative flux through the BSA extractable pathway is dependent on the relative elasticities of the two leak

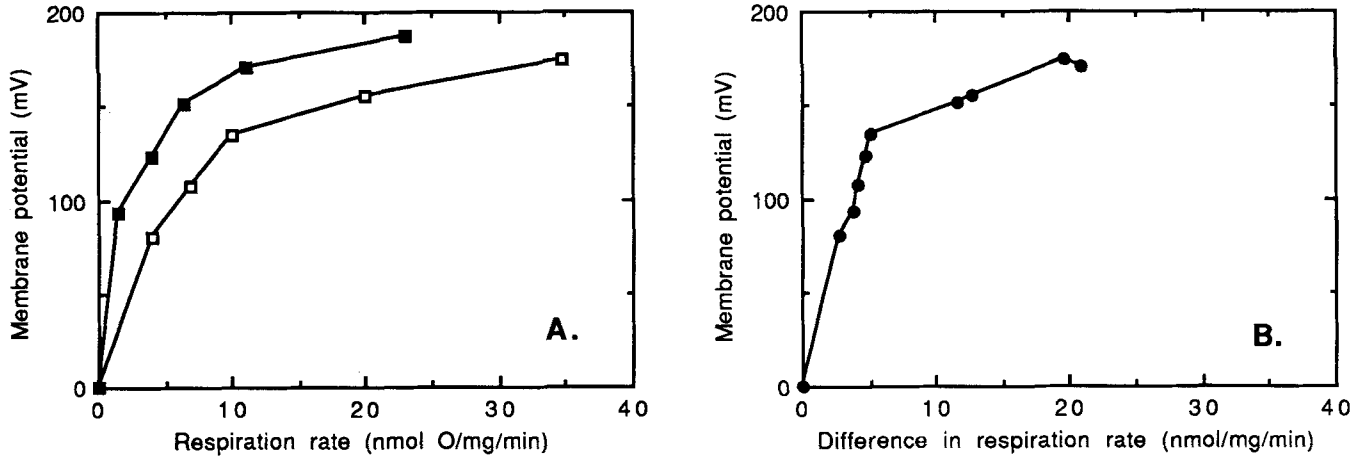

Fig. 3. Effect of BSA on the mitochondrial proton leak and its dependence on $\Delta\psi$. 2 ml of isolated mitochondria (60 mg/ml) was mixed either with 0.5 ml of the isolation medium (□) or with 10% (w/w) BSA in 0.5 ml of the isolation medium (■). Both preparations were left at 4°C for 20 h, and then added (at 2 mg/ml) to 120 mM KCl, 5 mM Hepes, 1 mM EGTA, 5 μM rotenone, 0.5 μg nigericin/ml (pH 7.0 with KOH), and incubated in an oxygen electrode chamber at 30°C, with a TPMP electrode. The TPMP electrode was calibrated in situ with 5 μM TPMP, and the binding correction due to TPMP binding to BSA was negligible with the small amount of BSA present. 2 mM potassium succinate was added and different steady states of respiration and TPMP uptake were established by titrating with malonate. (A) shows the relationship between the steady-state respiration rate and $\Delta\psi$ with (■) and without (□) BSA. The difference between the two curves is plotted in (B), i.e., the difference between the interpolated respiration rate ± BSA at any $\Delta\psi$ is plotted against $\Delta\psi$, to give the dependence of the BSA-extractable leak on $\Delta\psi$. The experiment was repeated on a separate preparation of mitochondria with a similar result, i.e., the $\Delta\psi$ dependence of the BSA-extractable leak is non-proportional.

pathways and the respiratory chain to $\Delta\psi$ [42,43]. Assuming that the elasticities of the two leak pathways to $\Delta\psi$ are roughly equal (see below) and the flux control coefficient of the leak processes over respiration is about 0.7 at 30°C [44], then the proportion of the leak flux through the BSA extractable pathway is about 23% (16/0.7) immediately after mitochondrial preparation.

Doubling the concentration of BSA or doubling the time of incubation with BSA had no affect on the extent of BSA inhibition of respiration, indicating that inhibition was maximal.

Whether the BSA extractable pathway is present in vivo is not possible to say. It may have been generated during preparation of the mitochondria (which takes about 50 min from killing the rat to use of the mitochondria).

The response of the BSA-extractable pathway to $\Delta\psi$ was estimated by examining the relationship between coupled respiration rate and $\Delta\psi$ in the presence and absence of BSA in 1-day-old mitochondria. Respiration was varied by adding graded amounts of the respiratory inhibitor malonate. Fig. 3a shows the resultant curves, and Fig. 3b shows the difference between the respiration rates with and without BSA at any given $\Delta\psi$, which should reflect the $\Delta\psi$ dependence of the flux through the BSA extractable pathway. This dependence (Fig. 3b) is roughly similar to that of the BSA-independent pathway (indicated by the curve in Fig. 3a in the presence of BSA).

The proton leak component extracted by BSA is probably free fatty acids [41]. Fatty acids uncouple mitochondrial respiration; however, the mechanism by which they do so is controversial [45,46]. We examined the dependence of the fatty-acid-mediated proton leak by determining the relationship between static head respiration rate and $\Delta\psi$ in the presence and absence of added palmitate. The proton leak mediated by palmitate had a disproportionate dependence on $\Delta\psi$ (not shown), as previously demonstrated for oleic acid [46]. This is not unexpected, as the proton conductance induced in pure black lipid bilayers also has a disproportionate dependence on $\Delta\psi$ [25].

The uncoupling mediated by fatty acids is partially inhibitable by carboxyatractyloside (an inhibitor of the adenine nucleotide carrier) [47]. To examine whether the BSA-extractable proton leak is mediated by fatty acids, we determined whether carboxyatractyloside could inhibit the coupled respiration rate in the presence and absence of BSA. We found that 3 μM carboxyatractyloside inhibited coupled respiration by 10% (standard deviation 4% for six determinations) in the absence of BSA and 1.5% (standard deviation 2% for six determinations) in the presence of BSA (3 mg/ml); the presence of BSA itself inhibited respiration by 22%. Oligomycin (an inhibitor of the H^+-ATPase) also inhibited coupled respiration by 2%. The carboxyatractyloside inhibition of the BSA extractable uncoupling indicates that at least a large proportion of the BSA extractable uncoupling is mediated by fatty acids.

Fatty acids might promote proton leak either by acting as protonophores, or by stimulating the already existing leak pathway. They might do the latter by increasing the negative surface charge of the mitochondrial inner membrane, and thus increasing the proton concentration at the surface of the membrane. The observation that carboxyatractyloside partially inhibits fatty acid uncoupling led Andreyev et al. [47] to suggest that fatty acid uncoupling is mediated by the adenine nucleotide carrier. An alternative explanation would be that carboxyatractyloside binding to the membrane increases the negative surface charge to the extent that partitioning of fatty acids into the membrane is lowered. Rottenberg [48] has suggested that fatty acids act to decouple oxidative phosphorlation.

Gutknecht [27] has shown that the proton leak mediated by weak acid protonophores in black lipid membranes is inhibited by phloretin (an agent which reduces the dipole potential of the membrane). We tested whether phloretin addition could inhibit coupled respiration. We found that concentrations of phloretin up to 5 μM (a concentration that significantly decreases fatty acid mediated proton leak [27]) had no effect on respiration (Fig. 4), but higher concentrations stimulated coupled respiration and inhibited uncoupled respira-

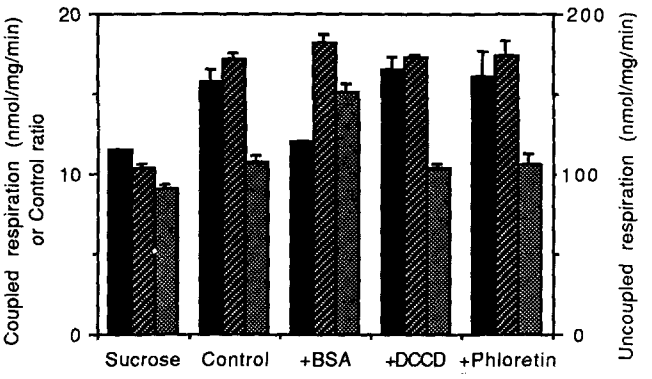

Fig. 4. Effect of various incubation conditions on static head and uncoupled rates of respiration and the coupling ratio. Mitochondria were isolated in sucrose and incubated (at 2 mg/ml) in an oxygen electrode vessel at 25°C in a basic medium containing 125 mM KCl, 5 mM Hepes, 1 mM EGTA, 5 μM rotenone (pH 7.4 with TMA·OH). After 5 min preincubation 4.2 mM TMA·succinate was added followed by 0.3 μM FCCP (3 μM FCCP in the presence of BSA). In the figure the three columns of each set represent the static head rate (black), the uncoupled rate (stripes) and coupling ratio (stippled). The first set of columns represents the rates when the 125 mM KCl of the medium was replaced by 250 mM sucrose. The second set was just the basic medium. In the third set 3 mg BSA/ml was added to the basic medium. In the fourth set 2.5 nmol DCCD/mg was added. In the fifth set, 5 μM phloretin was added. The column heights and error bars represent the means and range of two determinations for each treatment.

tion, presumably reflecting disruption of the membrane. This suggests that the dipole potential has no significant effect on mitochondrial proton leak, perhaps because of the high protein content of the mitochondrial inner membrane.

Wojtczak [49] has provided evidence that fatty acids stimulate K^+ uniport in mitochondria and has suggested that endogenously generated fatty acids may account for energised K^+ uptake in the absence of added fatty acids. We tested this by determining the effect of preincubating mitochondria with BSA on the rate of swelling in K^+ acetate containing BSA. The initial rate of swelling in K^+ acetate after addition of succinate was limited by K^+ uniport and was followed as the initial rate of absorbance decrease as in Ref. 3. Mitochondria (0.5 mg/ml) were preincubated for 5 min in either the presence or absence of 3 mg/ml BSA. With the initial rate of absorbance decrease on energization equal to 100% (±4%) the rate in the presence of BSA was 105% (±5%). Thus, there was no significant difference in K^+ uniport for mitochondria preincubated with BSA, indicating that K^+ uniport is not mediated by endogenous fatty acids (at least in freshly prepared mitochondria).

Does ion cycling contribute to the proton leak?

If cations enter mitochondria by a uniport pathway and exit via a cation/proton electroneutral exchange pathway, then in a steady-state for cations, the cations cycle across the membrane while there is net transport of protons into the mitochondria. Similarly, if anions leak out of the mitochondria electrophoretically at high $\Delta\psi$ and re-enter by electroneutral pathways, this cycling would also contribute to a net proton leak.

To test whether the cycling of K^+ and Cl^- contributes significantly to net proton leak, coupled and uncoupled respiration rates were compared in a sucrose and KCl medium. The coupled respiration was reduced in the sucrose medium (as compared to the KCl medium) by 27% (range of two replicates 2%), but the uncoupled rate was also reduced by 39% (±3%) so that the coupling ratio was reduced by 17% (±1%) in the sucrose medium (see Fig. 4). This indicates that the contribution of K^+ and Cl^- cycling to the proton leak is small or non-existent. Coupled and uncoupled rates were also compared, with the KCl in the incubation medium replaced by isoosmotic (and same pH) potassium gluconate, potassium sulphate, or potassium Hepes. These media slightly increased the coupled rates (+16%, +32% and +11%, respectively, relative to the KCl medium), while slightly decreasing the uncoupled rates (−16%, −9% and −9%). Since the coupled rate is lower and the respiratory control higher in the chloride medium relative to the gluconate, sulphate and Hepes media (ions which are very unlikely to cycle across the inner membrane), it follows that Cl^- cycling in the KCl medium must make a negligible contribution to the proton leak in the coupled state.

We noted above that Diwan [40] had estimated the unidirectional K^+ uniport flux to be about 1 nmol K^+/mg per min at pH 7 in the coupled state for liver mitochondria, when the proton influx rate should be roughly 60 nmol H^+/mg per min. Thus, according to these figures, K^+ cycling would contribute up to 2% to the proton leak. In heart mitochondria Jung et al. [39] have estimated the maximal unidirectional K^+ uniport in isolated heart to be 3–7 nmol K^+/mg per min when respiration rate is between 50–100 nmol O/mg per min [37], equivalent to 300–600 H^+/mg per min, i.e., the contribution of K^+ cycling to H^+ leak is up to 1% in isolated heart mitochondria.

In vivo, several inorganic ions other than K^+ or Cl^- are present in the cell at significant amounts. To test whether the cycling of these ions may contribute to the proton leak, we examined how the addition of 20 mM NaCl, $KHPO_4$ or $KHCO_3$ affected coupled and uncoupled respiration of mitochondria incubated in 120 mM KCl. The addition of NaCl had little or no effect on respiration (coupled respiration was decreased by 2% (range 6%) and uncoupled respiration was decreased by 1% (range 4%)). Addition of phosphate or bicarbonate, however, stimulated the coupled rate to a small extent (+8% ±5% for phosphate, +7% ±2% for bicarbonate) and inhibited the uncoupled rate −25% ±3% for phosphate, −36% ±3% for bicarbonate). The stimulation of the coupled rate and decrease of the coupling ratio is consistent with phosphate and bicarbonate cycling, making a significant but small contribution to the net proton leak in vivo (as suggested in Ref. 50). However, the coupled rates in potassium phosphate and bicarbonate media were similar to those in potassium gluconate and Hepes (ions which are unlikely to cycle, see above); thus, any anion cycling must be small.

Calcium cycling can make a significant contribution to the proton leak in vitro with high external calcium present. However it is thought to make an insignificant contribution in vivo, due to the low free calcium concentration in the cytosol. For example, in heart, mitochondrial calcium cycling has been estimated to account for less than 0.2% of tissue respiration [51].

We conclude that the contribution of ion cycling to the proton leak in isolated mitochondria is small.

Do proteins mediate the proton leak?

There is some evidence that even in the absence of phosphorylation the H^+-ATPase may contribute to the proton leak in submitochondrial particles [52], chromatophores of *Rhodopseudomonas capsulata* [53] and intact chloroplasts, and this conductance can be inhibited by *N,N'*-dicyclohexylcarbodiimide (DCCD). To test the possibility that part of the mitochondrial pro-

ton leak is mediated by the H^+-ATPase we determined the effect of DCCD on coupled (static head) and uncoupled respiration. Fig. 4 shows that it had no significant effect on either rate at 2.5 nmol DCCD/mg, a concentration sufficient to completely inhibit oxidative phosphorylation. A similar conclusion can be drawn from the lack of effect of oligomycin on static head respiration. We conclude that the H^+-ATPase does not significantly contribute to the proton leak in mitochondria. Higher concentrations of DCCD increased the coupled respiration rate, possibly due to disruption of the membrane (see also Ref. 54).

We noted the finding above that in the presence of BSA, the inhibition of coupled respiration by carboxyatractyloside was the same as that by oligomycin, i.e., about 2%. Thus, the adenine nucleotide carrier does not contribute to proton leak. If the proton leak was via the H^+ pumps of the respiratory chain, we might expect the leak rate to depend on the redox state of the pumps or inhibition of the pumps, but we have previously shown that this is not so [8].

We conclude that the mitochondrial proton pumps do not contribute to the proton leak (unless the leak routes through these proteins are unrelated to the routes taken by pumped protons). Other proteins may of course contribute to the proton leak.

What does mediate the proton and other cation leaks?

To summarise: the finding above that the entry of small sugars into mitochondria is not enhanced at high $\Delta\psi$ indicates that mitochondria do not undergo significant dielectric breakdown. The six to eight orders of magnitude higher permeability of mitochondria for protons over K^+ indicates that the proton leak mechanism is very selective and rules out simple dielectric breakdown or bilayer defects; however, the same selective permeability is shown by phospholipid bilayers, so the selectivity does not rule out some bilayer mechanism such as transient hydrogen bonded chains. Between 15–30% of the proton leak in isolated mitochondria is extractable by BSA and is probably due to fatty acids. No detectable proportion of the K^+ leak is BSA-extractable. Little if any of the proton leak is mediated by K^+, Na^+ or Cl cycling, but it is possible that phosphate or bicarbonate cycling might contribute to the net proton leak in vivo to a small extent. The mitochondrial proton pumps are not significantly involved in the proton leak.

What can we conclude from the above findings? A small and variable proportion of the leak can be mediated by fatty acids, but most of the leak is mediated by either an unknown protein (or proteins) or/and by a bilayer mechanism specific for protons. Dilger et al. [32,55] have found that the mitochondrial membrane is 2 to 3 orders of magnitude more permeable to thiocyanate, perchlorate and the protonophore 5,6-dichloro-2-trifluoromethylbenzimidazole than pure phospholipid bilayers, even when made from mitochondrial lipids. This increase in permeability was mimicked by raising the dielectric constant of the pure phospholipid bilayer, and Dilger et al. [32,55] proposed that the permeability of mitochondria to all ions passing through the bilayer would be increased by the effect of proteins raising the dielectric constant of the membrane. The findings of Krishnamoorthy and Hinkle [2] that isolated mitochondria are 1 to 2 orders of magnitude more permeable to protons than liposomes made from mitochondrial phospholipids is therefore compatible with the proposal that most of the mitochondrial proton leak is via the bilayer, modified by presence of proteins.

The proton leaks of pure phospholipid bilayers and of mitochondria share several common properties: (a) high specificity relative to other ions, (b) non-proportional dependence on $\Delta\psi$, and (c) relative insensitivity to pH [28,56]. These similarities give support to the proposal that the mitochondrial leak is via the phospholipid bilayer (modified by the presence of proteins).

Acknowledgements

Many thanks to Mark Leach and Mary George for expert technical assistance. This work was supported by a research fellowship from St. Catharine's College, Cambridge, to G.C.B. and a grant from the Science and Engineering Research Council, U.K., to M.D.B.

References

1 Nicholls, D.G. (1974) Eur. J. Biochem. 50, 305–315.
2 Krishnamoorthy, G. and Hinkle, P.C. (1984) Biochemistry 23, 1640–1645.
3 Brown, G.C. and Brand, M.D. (1986) Biochem. J. 234, 75–81.
4 Garlid, K.D., Beavis, A.D. and Ratkje, S.K. (1989) Biochim. Biophys. Acta 976, 109–120.
5 Murphy, M.P. (1989) Biochim. Biophys. Acta 977, 123–141.
6 Brand, M.D. (1990) Biochim. Biophys. Acta 1080, 128–133.
7 Pietrobon, D., Azzone, G.F. and Walz, D. (1981) Eur. J. Biochem. 117, 387–394.
8 Brown, G.C. (1989) J. Biol. Chem. 264, 14704–14709.
9 Hafner, R.P. and Brand, M.D. (1991) Biochem. J. 275, 75–80.
10 Zolkiewska, A., Zablocka, B., Duszynski, J. and Wojtczak, L. (1989) Arch. Biochem. Biophys. 275, 580–590.
11 Murphy, M.P. and Brand, M.D. (1987) Nature 329, 170–172.
12 Nicholls, D.G. (1977) Eur. J. Biochem. 77, 349–356.
13 Mandolino, G., Desantis, A. and Melandri, B.A. (1983) Biochim. Biophys. Acta 723, 428–439.
14 Sorgato, M.C. and Ferguson, S.J. (1979) Biochemistry 18, 5737–5742.
15 Kell, D.B., John, P. and Ferguson, S.J. (1978) Biochem. Soc. Trans. 6, 1292–1295.
16 Clark, A.J., Cotton, N.P.J. and Jackson, J.B. (1983) Eur. J. Biochem. 130, 575–580.
17 Cotton, N.P.J., Clark, A.J. and Jackson, J.B. (1984) Eur. J. Biochem. 142, 193–19.

18 Nobes, C.D., Brown, G.C., Olive, P.N. and Brand, M.D. (1990) J. Biol. Chem. 265, 12903–12909.
19 Brown, G.C., Lakin-Thomas, P.L. and Brand, M.D. (1990) Eur. J. Biochem. 192, 355–362.
20 Hafner, R.P., Nobes, C.D., McGown, A.D., and Brand, M.D. (1988) Eur. J. Biochem. 178, 511–518.
21 Hafner, R.P., Leake, M.J. and Brand, M.D. (1989) FEBS Lett. 248, 175–178.
22 Horrum, M.A., Tobin, R.B. and Ecklund, R.E. (1990) Mol. Cell. Endocrinol. 68, 137–141.
23 Brand, M.D. (1990) J. Theor. Biol. 145, 267–286.
24 O'Shea, P.S., Petrone, G., Casey, R.P. and Azzi, A. (1984) Biochem. J. 219, 719–726.
25 Gutknecht, J. (1984) J. Membr. Biol. 82, 105–112.
26 Gutknecht, J. (1987) J. Bioenerg. Biomembr. 19, 427–442.
27 Gutknecht, J. (1988) J. Membr. Biol. 106, 83–93.
28 Deamer, D.W. and Nicholls, J.W. (1989) J. Membr. Biol. 107, 91–103.
29 Zimmermann, U. (1982) Biochim, Biophys. Acta 694, 227–277.
30 Schwister, K. and Deuticke, B. (1985) Biochim. Biophys. Acta 816, 332–348.
31 Owenson, B. and Pratt, L.R. (1984) J. Phys. Chem. 88, 6048–6052.
32 Dilger, J. and McLaughlin, S. (1979) J. Membr. Biol. 46, 359–384.
33 Mimms, L.T., Zampighi, G., Nozaki, Y., Tanford, C. and Reynolds, J.A. (1981) Biochemistry 20, 833–840.
34 Brown, G.C. and Brand, M.D. (1988) Biochem. J. 252, 473–479.
35 Brown, G.C. and Brand, M.D. (1985) Biochem. J. 225, 399–405.
36 Chernomordik, L.V., Sukharev, S.I., Popov, S.V., Pastushenko, V.F., Sokivko, A.V., Abidov, I.G. and Chizmadzhev, Y.A. (1987) Biochim. Biophys. Acta 902, 360–373.
37 Brierley, G.P., Jurkowitz, M., Scott, K.M. and Merola, A.J. (1971) Arch. Biochem. Biophys. 147, 545–556.
38 Diwan, J.J. and Harrington, P. (1977) Biochem. Soc. Trans. 7, 203–205.
39 Jung, D.W., Chavez, E. and Brierley, G.P. (1977) Arch. Biochem. Biophys. 183, 452–459.
40 Diwan, J.J. (1973) Biochem. Biophys. Res. Commun. 50, 384–391.
41 Helsinki, D.R. and Cooper, C. (1960) J. Biol. Chem. 235, 3573–3579.
42 Brown, G.C., Hafner, R.P. and Brand, M.D. (1990) Eur. J. Biochem. 188, 321–325.
43 Hafner, R.P., Brown, G.C. and Brand, M.D. (1990) Eur. J. Biochem. 188, 313–319.
44 Brand, M.D., Hafner, R.P. and Brown, G.C. (1989) Biochem. J. 255, 535–539.
45 Luvisetto, S., Pietrobon, D. and Azzone, G.F. (1987) Biochemistry 26, 7332–7338.
46 Pietrobon, D., Luvisetto, S. and Azzone, G.F. (1987) Biochemistry 26, 7339–7347.
47 Andreyev, A.Y., Bondareva, T.O., Dedukhova, V.I., Mokhova, E.N., Skulachev, V.P. and Volkov, N.I. (1988) FEBS Lett. 226, 265–269.
48 Rottenberg, H. (1983) Proc. Natl. Acad. Sci. USA 80, 3313–3317.
49 Wojtczak, L. (1974) FEBS Lett. 44, 25–30.
50 Garlid, K.D. and Beavis, A.D. (1986) Biochim. Biophys. Acta 853, 187–204.
51 Crompton, M. (1985) Curr. Top. Membr. Trans. 25, 231–276.
52 Seren, S., Caporin, G., Galiazzo, F., Lippe, G., Ferguson, S.J. and Sorgato, M.C. (1985) Eur. J. Biochem. 152, 373–379.
53 Clark, A.J., Cotton, N.P.J. and Jackson, J.B. (1983) Biochim. Biophys. Acta 723, 440–453.
54 Brand, M.D., Al-Shawi, M.K., Brown, G.C. and Price, B.D. (1985) Biochem. J. 225, 407–411.
55 Dilger, J.P., McLaughlin, S.G.A., McIntosh, T.J., and Simon, S.A. (1979) Science 206, 1196–1198.
56 Heaton, G.M. and Nicholls, D.G. (1976) Biochem. J. 156, 635–646.