Contribution of mitochondrial proton leak to respiration rate in working skeletal muscle and liver and to SMR

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Rolfe, David F. S., John M. B. Newman, Julie A. Buckingham, Michael G. Clark, and Martin D. Brand. Contribution of mitochondrial proton leak to respiration rate in working skeletal muscle and liver and to SMR. Am. J. Physiol. 276 (Cell Physiol. 45): C692–C699, 1999.—Proton pumping across the mitochondrial inner membrane and proton leak back through the natural proton conductance pathway make up a futile cycle that dissipates redox energy. We measured respiration and average mitochondrial membrane potential in perfused rat hindquarter with maximal tetanic contraction of the left gastrocnemius-soleus-plantaris muscle group, and we estimate that the mitochondrial proton cycle accounted for 34% of the respiration rate of the preparation. Similar measurements in rat hepatocytes given substrates to cause a high rate of gluconeogenesis and ureagenesis showed that the proton cycle accounted for 22% of the respiration rate of these cells. Combining these in vitro values with literature values for the contribution of skeletal muscle and liver to standard metabolic rate (SMR), we calculate that the proton cycle in working muscle and liver may account for 15% of SMR in vivo. Although this value is less than the 20% of SMR we calculated previously using data from resting skeletal muscle and hepatocytes, it is still large, and we conclude that the futile proton cycle is a major contributor to SMR.

standard metabolic rate; proton pumping; redox energy; contracting skeletal muscle; working liver cells

STANDARD METABOLIC RATE (SMR; also termed basal metabolic rate) is the steady-state rate of heat production by a whole organism under a set of standard conditions. For mammals, these conditions are that the individual is awake but resting, stress free, not digesting food (prior food intake being at or around maintenance level) and maintained at a thermoneutral temperature. SMR is either measured directly as heat production or indirectly as oxygen consumption, from which it can be accurately predicted.

Mammals oxidize substrates at a considerable rate in the standard state, when no net work is done and all the free energy is dissipated. The question of which futile cycles are driven by respiration has been the subject of much research effort. Recent attempts to quantify the contribution of the cellular processes that underlie SMR indicate that the cycles catalyzed by the Na+-K+-ATPase and plasma membrane monovalent cation conductance pathways, Ca²⁺-ATPase and Ca²⁺ conductance pathways, and protein synthesis and degradation together account for ~40% of SMR in adult rats (see Refs. 8, 25, 29 for review). In addition, we recently showed (4, 24) that a futile cycle of proton extrusion across the mitochondrial inner membrane and the subsequent proton leak back to the matrix via endogenous proton conductance pathways accounted for about one-half of the oxygen consumption rate of resting, perfused rat skeletal muscle (24) and one-fourth of the oxygen consumption rate of resting isolated liver cells (4). Using these data, we calculated that, in vivo, the proton leak pathway in liver and skeletal muscle alone could account for one-fifth of rat SMR. When other tissues are included in this calculation, the proton cycle could account for 25% of SMR (24).

However, there are difficulties in extrapolating the data in Rolfe and Brand (24) to whole-animal SMR. Resting perfused skeletal muscle and isolated liver cells may have a lower ATP demand than under standard conditions in vivo. For example, skeletal muscle in a resting but awake rat will contract to maintain muscle tone and posture, and the liver may have some ATP demand for pathways such as the synthesis of urea and glucose, but these processes may have been partially or completely absent in our resting preparations. An unphysiologically low ATP demand might cause the contribution of proton leak to metabolic rate to be overestimated. There are two reasons for this. First, if ATP demand is low, then the contribution of proton leak will be proportionately increased. Second, if ATP demand is low, its driving force, the mitochondrial proton-motive force, will increase and drive the proton leak faster.

In the present paper, we set the ATP demand of our model systems to give double the resting respiration rates by inducing contraction in perfused skeletal muscle and by incubating the liver cells in a medium that stimulates gluconeogenesis and ureagenesis. We have measured the rate of the proton cycle under these new conditions and estimated its contribution to the oxygen consumption rate of skeletal muscle and liver under standard conditions in vivo.

EXPERIMENTAL PROCEDURES

Isolation and perfusion of the rat hindquarter. Male Hooded Wistar rats (~2 mo old, 180–220 g body wt) were maintained at a thermoneutral temperature (25°C) and had access ad libitum to a complete diet (Gibsons, Hobart, Australia) and drinking water. They were anesthetized with sodium pentobarbitone (60 mg/kg body mass ip), and the hindquarter was isolated from the rest of the vascular system of the rat.
essentially according to the method of Ruderman et al. (26), with additional modifications as described by Colquhoun et al. (9), the procedure taking ~20 min up to the cannulation of the vena cava and aorta. The resting hindquarter was perfused at a constant flow rate (0.5 ml·g muscle−1·min−1) with buffer containing (in mM) 118 NaCl, 5 KCl, 1 K2HPO4, 1.18 MgSO4, 25 NaHCO3, 8.3 glucose, 2.5 CaCl2, and 1.2 sodium pyruvate, together with 4.6% (wt/vol) BSA, 0.46 U/ml sodium heparin, and 35% (vol/vol) bovine red blood cells [isolated essentially as described by Dora et al. (10)]. All perfusions were carried out in a temperature-controlled cabinet maintained at 32°C. The perfusate was gassed with 95% air-5% CO2 (vol/vol) via a Silastic tube oxygenator, and the temperature was brought to 32°C by passage through a heat exchanger coil. About 200 ml of perfusate were allowed to flow to waste, and then the effluent was connected to a reservoir of 300 ml of perfusate containing 1 nM (45 nCi/ml) [3H]methyl-triphenylphosphonium cation ([3H]TPMP). The hindquarter was then perfused using this closed, recirculating system for the remainder of the experiment. Perfusion pressure was monitored via a side-arm proximal to the aorta.

Induction of contraction in the isolated hindquarter. The skin was removed from the thigh of the left limb, and the sciatic nerve was exposed in the flank and cut to allow the positioning of the distal cut end in a suction electrode. The nerve was secured by the tibiotarsal ligament, and the Achilles tendon was attached to a Harvard Apparatus isometric transducer, thereby allowing transmission of tension from the gastrocnemius-plantaris-platys muscle group. The perfusion rate was then increased to 1 ml·min−1·g muscle−1, and, 10 min after this flow rate increase, stimulation of the sciatic nerve was initiated and the resting length of the muscle was adjusted to attain maximal active tension on stimulation (to get maximum active tension, twitch stimulations of 10 ms in duration were used). The oxygen consumption rate of the hindquarter was monitored as described below to assess the effect of the flow rate changes on the respiration rate of the preparation. We found no significant difference in the rate of hindquarter oxygen consumption before and after the flow rate changes (0.358 ± 0.07 and 0.362 ± 0.04 µmol O2·min−1·g tissue−1, respectively; means ± SE; n = 5). Tension development was recorded during contraction by a recording oscilloscope (Telequipment model DM64) and by a Yokagawa 3056 chart recorder. Tetanic stimulation involved 200-ms trains of 100 Hz with 0.1-ms pulses applied every 2 s, adjusted (3–5 V) to attain full fiber recruitment (22). Figure 1 shows typical graphs of tension and oxygen consumption and of tension and membrane potential. Measurement of hindquarter oxygen consumption and electrochemical potentials. Arterial and venous perfusate samples were taken using gas-tight 1-ml syringes and stored on ice until they were analyzed for total oxygen content using a galvanic cell oxygen analyzer (TasCon oxygen content analyzer, manufactured by the Physiology Department, University of Tasmania). Two arterial samples were taken (one at 0.5 ml·min−1·g tissue−1 flow rate and one at 1 ml·min−1·g tissue−1) and up to eight venous samples (two at 0.5 ml·min−1·g tissue−1, one or two 5–10 min after increase of flow to 1 ml·min−1·g tissue−1) before contraction was induced, two after contraction was initiated, two after oligomycin was added, and two after cyanide addition). Duplicate analysis of each collected sample took an average of 15 min (20). Measurement of tissue membrane potential was achieved using [3H]TPMP as described previously (24). Samples (1 ml) were taken from the reservoir before and at various stages during the perfusion. Each sample was spun at 16,000 g in an Andermel bench centrifuge to pellet the erythrocytes, and duplicate supernatant samples (200 µl) were taken. Each sample was dispersed in 3.5 ml of scintillant (BCS, Amersham International, Amersham, Buckinghamshire, UK), and radioactivity was determined by counting in a liquid scintillation counter (LS 3801, Beckman Instruments, Irvine, CA) with corrections for quench. The amount of radioisotope taken up by the tissue was calculated as the total activity of the isotope added to the perfusate (measured by sampling the perfusate at the time the recirculating perfusion was started) less the total activity remaining in the perfusate once a steady-state value for the TPMP was established (~100 min). The average mitochondrial membrane potential (∆Ψ) was calculated as described previously (24), except that the plasma membrane potential was assumed to drop by 20 mV (15) in 15% of the preparation during contraction.

Experiments using liver cells. Female Wistar rats (~220-260 g) were starved overnight. Hepatocytes were prepared in the absence of glucose as described in Refs. 18 and 27 and kept in a conical flask on ice for up to 3 h before use. Measurements of viability (94.9 ± 1.2%), dry cell mass, respiration, and ∆Ψ in situ (using [3H]TPMP) and incubation protocols were as described in Refs. 3 and 12. Cells were taken from the stock solution on ice and diluted fivefold to 27.9 ± 1.0 mg dry mass/ml to 2 ml of medium in 20-ml glass vials maintained at 37°C in a shaking water bath and then gassed with 95% air-5% CO2. The medium contained (in mM) 106 NaCl, 5 KCl, 25 NaHCO3, 0.41 MgSO4, 10 NaH2PO4, 25 CaCl2, 10 sodium d-lactate, 1 sodium pyruvate, 10 L-glutamine, and 2 L-ornithine, together with 2.25% (wt/vol) defatted BSA, 1 µM TPMP, and 100 µg/ml inulin (pH 7.2). After 10 min of preincubation, appropriate radioisotopes and inhibitors were added (oligomycin, myxothiazol, 0.8 µCi 3H2O, and 0.04 µCi [14C]methoxyinulin or 0.08 µCi [3H]TPMP); 20 min later, duplicate incubations were transferred to oxygen electrodes for measurements of respiration rate and triplicate samples from one incubation were transferred to microcentrifuge tubes and centrifuged, for radioactivity measurements for cell volume (1.39 ± 0.06 µl/mg dry mass) and ∆Ψ (3). Potentials were calculated from measured TPMP uptake and cell volume as in Ref. 3, under the assumption that 19% of the volume of rat hepatocytes is occupied by mitochondria (12, 17, 18), with TPMP binding corrections of 0.44 in the mitochondrial matrix (19, 20), 0.20 in the cytoplasm (12, 17), and 0.71 in the medium (17) and a value for plasma membrane potential of ~32 mV (12, 17, 18). Wet weight of cells was calculated by multiplying dry mass by 4.77 (1).

Materials. Phentolamine was from Sigma Chemical (St. Louis, MO). BSA was from Boehringer Mannheim Biochemicals (Indianapolis, IN or Lewes, East Sussex, UK). Radiochemicals were from DuPont-NEN Radiochemicals (Boston, MA or Hounslow, Middlesex, UK). All other chemicals were analytical grade from Ajax Chemicals (Auburn, NSW, Australia) or Sigma Chemical (Poole, Dorset, UK).

RESULTS

The contribution of the mitochondrial proton cycle to the respiration rate of the contracting hindquarter was determined as described by Rolfe and Brand (24), and the results are shown in Fig. 2. Each individual experiment to determine the contribution of proton leak (plus nonmitochondrial oxygen consumption) and ATP turnover to the respiration rate of the contracting hindquarter lasted ~3 h. Steady-state values for resting oxygen

PROTON LEAK IN WORKING MUSCLE AND LIVER

C693
consumption and average ΔΨ were reached in ~20 and ~90 min, respectively. Hence the resting values for respiration rate and ΔΨ were measured after 1.5 h of perfusion. Contraction was then induced in the gastrocnemius-plantaris-soleus group of the left hindquarter, and the rates of hindquarter oxygen consumption and average membrane potential were measured after 15 min of contraction when a new steady state for these parameters had been attained. Contraction increased the respiration rate of the hindquarter by ~80% on average but did not alter the value for total tissue membrane potential as measured by the TPMP signal, although, since a drop in plasma membrane potential of 20 mV was assumed to occur in the contracting tissue, this meant that the calculated total tissue mitochondrial ΔΨ actually increased (by ~4 mV) during contraction.

After 45 min of contraction, during which the stability of the steady state was verified by constant measurement of ΔΨ and oxygen consumption, sufficient oligomycin to completely inhibit oxidative ATP synthesis (~50 µg/g perfused tissue) was added, and, 15 min after this addition, the new steady-state values of ΔΨ and oxygen consumption were measured. Oligomycin was shown to be in excess by demonstrating no effect on respiration of further additions. Note also that all contraction was abolished 5–10 min after addition of excess oligomycin. Addition of oligomycin reduced the hindquarter respiration rate by 51% and increased the ΔΨ by ~20 mV. As discussed previously (24), to measure the contribution of proton leak (plus nonmitochondrial oxygen consumption) to tissue respiration rate, it is necessary to return the tissue ΔΨ to its contracting, pre-oligomycin inhibition value. This was achieved by titrating the oligomycin...
The contributions of proton leak, ATP turnover, and nonmitochondrial oxygen consumption to respiration in noncontracting muscle can also be calculated from Fig. 2; they were 57, 27, and 16%, respectively, which agrees well with our previous estimates (52, 34, and 14%, respectively; Ref. 24), despite differences in experimental conditions (rat strain, perfusion with red blood cells, temperature 32 compared with 35°C).

Addition of lactate, pyruvate, glutamate, and ornithine to the basal incubation medium increased hepatocyte respiration from 1.01 ± 0.05 to 2.22 ± 0.33 μmol O₂·min⁻¹·g wet weight⁻¹ (means ± SE; data not shown). The contribution of proton leak to the oxygen consumption rate of isolated liver cells in this supplemented medium is shown in Fig. 3 and was determined in essentially the same way as described above for perfused muscle except that myxothiazol (an inhibitor of complex III) was used instead of cyanide. Figure 3 shows that the respiration rate of these cells was accounted for by mitochondrial proton leak (22 ± 2%, mean ± SE), ATP turnover (69 ± 2%, mean ± SE), and nonmitochondrial oxygen consumption (9 ± 1%, mean ± SE). Thus proton leak remains a significant contributor to the respiratory rate of hepatocytes in

![Graph showing mitochondrial proton leak and oxygen consumption](image)

**Fig. 2.** Measurement of contribution of mitochondrial proton leak to respiration rate of contracting, perfused rat skeletal muscle. Resting hindquarter was perfused using a recirculating system as described in EXPERIMENTAL PROCEDURES. Isometric contraction was induced in gastrocnemius-plantaris-soleus muscle group of left hindlimb via stimulation of sciatic nerve. Oxygen consumption was measured by taking arterial and venous perfusate samples that were then analyzed using a blood oxygen analyzer. Electrochemical potentials were assessed as tissue uptake of [3H]TPMP; a probe for ΔΨ. Contribution of proton leak to respiration rate of hindquarter was determined essentially as described by Rolfe and Brand (24). Steady-state values for resting oxygen consumption and ΔΨ were measured after 1.5 h of perfusion. Contraction was then induced in gastrocnemius-plantaris-soleus group of left hindquarter, and rates of hindquarter oxygen consumption and ΔΨ thus obtained were measured after 15 min of contraction when a new steady state had been attained. After 45 min of contraction, sufficient oligomycin to completely inhibit oxidative ATP synthesis (~50 μg/g perfused tissue) was added, and, 15 min after this addition, new steady state values of ΔΨ and oxygen consumption were measured. To measure contribution of proton leak [plus nonmitochondrial oxygen consumption] to tissue respiration rate, it is necessary to return tissue ΔΨ to its noncontracting, pre-oligomycin inhibition value. This was achieved by titrating oligomycin-inhibited hindquarter with cyanide (~100 μM) as outlined previously (24). Nonmitochondrial oxygen consumption rate (NM) was determined as outlined previously (24), using excess (1–2 mM) sodium cyanide. Values are means ± SE for 5 independent experiments.

![Graph showing mitochondrial oxygen consumption rate](image)

**Fig. 3.** Contributions of proton leak, ATP turnover, and nonmitochondrial oxygen consumption to respiration rate of hepatocytes. Hepatocytes from overnight-starved rats were incubated as described in text. Oxygen consumption was measured in an oxygen electrode, and ΔΨ was measured by uptake of radiolabeled TPMP as described in EXPERIMENTAL PROCEDURES. Contributions of proton leak, ATP turnover, and nonmitochondrial oxygen consumption to hepatocyte respiration rate were determined as described for muscle in Fig. 2, except that myxothiazol was used in place of cyanide. From right to left, points represent control (no additions to medium), 1 μg/ml oligomycin, and oligomycin plus 0.1, 0.2, 0.4, 0.8, and 8 μM myxothiazol. Note that 8 μM myxothiazol completely inhibited mitochondrial respiration and was therefore used to determine nonmitochondrial respiration rate. Respiration was accounted for by ATP turnover (68.5 ± 2.0%), mitochondrial proton leak (22.4 ± 1.7%), and nonmitochondrial oxygen consumption (9.2 ± 0.8%). Values are means ± SE for 6 independent experiments.
which ATP turnover has been stimulated to give double the respiration rate.

**DISCUSSION**

The data in Fig. 2 indicate that when the muscle is stimulated to contract the value of the average mitochondrial \( \Delta \Psi \) does not change and indeed may even increase slightly. This indicates that both the pathways involved in using the mitochondrial \( \Delta \Psi \) (ATP turnover) and those involved in producing the mitochondrial \( \Delta \Psi \) (by oxidation of substrates) are activated. The fact that the average value of mitochondrial \( \Delta \Psi \) remains constant during the rest-to-exercise transition means that the rate of proton leak across the mitochondrial inner membrane (which is controlled by \( \Delta \Psi \)) also remains constant, and this explains why the proton leak is still a major contributor to the respiration rate of the exercising hindquarter. However, perfused muscle is a complicated system, and, although it is clear that there is activation of the \( \Delta \Psi \) producers during contraction, the conclusion of equal activation of \( \Delta \Psi \) producers and consumers must be considered tentative.

In microsphere entrapment studies, it has been shown that, in the constant-flow-perfused hindlimb, perfusate flow is diverted from the nonworking to the working tissue during exercise (Newman and Clark, unpublished data), and such flow changes might lead us to misinterpret the data shown in Fig. 2. Diversion of flow from the nonworking tissue may cause the oxygen consumption rate of this part of the hindquarter to be limited by suboptimal substrate delivery, leading to a drop in the \( \Delta \Psi \) signal from this part of the hindquarter. However, the effect of this drop on the total tissue membrane potential signal would be either to accentuate any \( \Delta \Psi \) drop or to mask any \( \Delta \Psi \) increase in the contracting tissue. The data in Fig. 2 show no change or even an increase in the total mitochondrial \( \Delta \Psi \) on induction of exercise and thus, if anything, may underestimate a hypothetical \( \Delta \Psi \) increase in the contracting tissue.

Only 15% of the total muscle mass of the hindquarter was stimulated to contract, as estimated by dye infusion (10, 16, 28). This means that any changes in the mitochondrial \( \Delta \Psi \) in this portion of the hindquarter would have been difficult to detect against the background of the total tissue \( \Delta \Psi \). For example, a change of 40 mV in the mitochondria within the exercising tissue would only produce a 4-mV change in the total tissue membrane potential signal. Thus it is possible that the \( \Delta \Psi \) of the contracting tissue actually dropped but that we were not able to detect this drop, weakening our conclusion that substrate oxidation is activated during exercise induction. However, even a 40-mV drop in the total tissue mitochondrial \( \Delta \Psi \) would, from Fig. 2, only decrease the contribution of proton leak to the respiration rate of contracting hindquarter from 34 to 20%, thus reducing our estimate of the contribution of proton leak in liver and skeletal muscle to SMR from 15 to 10% and the total estimated contribution of leak in all tissues to 15%. Thus the difficulty of accurately measuring changes in mitochondrial \( \Delta \Psi \) does not affect the main conclusion of Fig. 2, that proton leak is a major contributor to the respiration rate of perfused, contracting skeletal muscle under conditions that may reflect those of standard metabolism in vivo.

How close are the conditions in our experiments to the standard state in vivo? As mentioned in the introduction, it is thought that a certain amount of muscular contraction occurs in standard metabolism (23, 25) and that the liver may have some gluconeogenic and ureagenic activity. We have increased by exercise induction the total respiration rate of the hindquarter to close to the maximum rate of oxygen consumption measured for resting skeletal muscle in vivo (0.787 \( \mu \)mol O\(_{2}\)·min\(^{-1}\)·g tissue\(^{-1}\) (21) compared with 0.65 \( \mu \)mol O\(_{2}\)·min\(^{-1}\)·g tissue\(^{-1}\) (Fig. 2)), and from this point of view our contracting hindquarter preparation is a better model of the standard state than our resting preparation (24). However, one of the main differences between perfused muscle and real life is that during SMR in vivo most of the muscle tissue is contracting a small amount, whereas in our system a small proportion of the total mass is contracting maximally and the remainder is resting. Thus the strength of our conclusion rests on the assumption that the mechanism by which muscle metabolism is stimulated when muscle tissue is exercising lightly is the same as when it undergoes maximal contraction. If, for example, only the ATP turnover reactions were stimulated during light exercise, leading to a drop in the mitochondrial \( \Delta \Psi \), then our model system would overestimate the contribution of proton leak to standard metabolism in skeletal muscle. However, as mentioned above, the drop in mitochondrial \( \Delta \Psi \) would have to be very large (>60 mV) to significantly affect our main conclusion, that mitochondrial proton leak is a significant contributor to SMR.

The experiments with liver cells were designed (as for muscle) to stimulate ATP-consuming pathways to somewhere near physiological levels. One crude assessment of whether we achieved this can be made by comparing the respiration rate of whole liver in vivo (2.84 \( \mu \)mol O\(_{2}\)·min\(^{-1}\)·g liver\(^{-1}\); Ref. 13) with the respiration rate of our liver cells, scaled in the appropriate way. Knowing the number of cells per whole liver (\( \sim 10^9 \)); Ref. 1) and the wet weight per cell (\( \sim 8 \) g/10\(^9\) cells; Ref. 1), we can calculate that the respiration rate of our experimental cell population was equivalent to 1.8 \( \mu \)mol O\(_{2}\)·min\(^{-1}\)·g liver\(^{-1}\) (64% of the in vivo rate). By this measure, our cell population was representative of liver cells in vivo.

The results in Figs. 2 and 3 show that the contribution of the mitochondrial proton cycle to respiration in stimulated skeletal muscle and liver cells is large, as is in resting muscle and hepatocytes (4, 24), although stimulation reduces the absolute magnitude of the contribution of proton leak from 52 ± 15 to 34 ± 14% (means ± SE; significantly different at the P < 0.10 level) in muscle and from 26 ± 7 to 22 ± 2% (means ± SE; significantly different at the P < 0.01 level) in liver cells. Rolfe and Brand (24) discussed the effect of the contribution of proton leak on the effective P/O ratio...
and compared with available data for protein synthesis in intact tissues and calculated that the effective P/O ratio in resting skeletal muscle would be 0.83. The effective P/O ratio in resting liver has been estimated as ~1.3–1.6 (4). The effective P/O ratio is obtained by multiplying the mechanistic P/O ratio of the mitochondria by the fraction of total tissue oxygen consumption used to drive mitochondrial ATP synthesis. The proportion of hindquarter oxygen consumption used to drive ATP synthesis was 57% in our contracting hindquarter system (Fig. 2) but was 34% in resting skeletal muscle (24). Thus the effective P/O ratio of skeletal muscle at almost double the resting respiration rate (conditions that may better reflect SMR) can be calculated, under the assumption that the muscle is oxidizing pyruvate, for which the mechanistic P/O ratio is 2.42 (2), to be ~1.4 (0.57 × 2.42). The same calculation for liver, with 69% of liver oxygen consumption used to drive mitochondrial ATP synthesis (Fig. 3), gives an effective P/O ratio of ~1.7. Thus the data presented in this paper support those of other workers (e.g., Refs. 5, 6) who consider that 31P-nuclear magnetic resonance saturation transfer measurements of P → ATP flux may significantly overestimate the effective P/O ratio of intact tissues. We therefore consider that the conclusion of others (e.g., Refs. 14, 19) that all of the oxygen consumption of intact tissues is used to drive ATP synthesis is incorrect even when respiration rate is doubled compared with the resting state (see Ref. 24 for a fuller discussion of this topic).

The contribution of proton leak, ATP turnover, and nonmitochondrial oxygen consumption to SMR may be calculated using the results shown in Figs. 2 and 3. The contributions of liver and skeletal muscle to rat SMR are 10–20 and 13–42%, respectively (see references cited in Ref. 24). Hence, calculation using the values for the contribution of proton leak to liver and skeletal muscle respiration [22% (Fig. 3) and 34% (Fig. 2), respectively] indicates that the contribution of liver and skeletal muscle proton leak to rat SMR is between 7% [(10% × 0.22) + (13% × 0.34)] and 19% [(20% × 0.22) + (42% × 0.34)], with an average of ~15%. If the contribution of proton leak in the other major oxygen-consuming tissues of the rat (which together account for 20% of rat SMR; Ref. 10) is similar to liver, then the contribution of proton leak to rat SMR may be as high as 23% [(20% × 0.22) + (42% × 0.34) + (20% × 0.22)]. With the same assumptions and the value of 9% (see Figs. 2 and 3) obtained for the contribution of nonmitochondrial oxygen consumption to the respiration rate of liver and skeletal muscle, the contribution of nonmitochondrial oxygen consumption to rat SMR could be as high as 7% [(20% × 0.09) + (42% × 0.09) + (20% × 0.09)]. Total ATP turnover, which accounts for 57% of skeletal muscle respiration (Fig. 2) and 69% of liver cell respiration (Fig. 3), would contribute a maximum of 52% [(20% × 0.69) + (42% × 0.57) + (20% × 0.69)] to rat SMR. The significance of the contribution of proton leak and nonmitochondrial oxygen consumption in liver and skeletal muscle to rat SMR is shown in Fig. 4 and compared with available data for protein synthesis, and Na+-K+-ATPase and Ca2+-ATPase activity in the rat, these being the only processes for which there is general agreement regarding their contribution to mammalian SMR.

What are the confidence limits of our estimate of the contribution of proton leak to SMR? These limits are difficult to quantify precisely, but the following points should be noted. First, we have (by stimulating the muscle ATP demand via contraction) pushed the oxygen consumption rate of the hindquarter close to the upper limit of estimates for the resting in vivo oxygen consumption rate of the rat hindlimb (see above), and this would, if anything, tend to underestimate the contribution of proton leak to skeletal muscle respiration. Second, we have not used free fatty acids as a substrate in this study, despite the fact that muscle may use lipids as the main fuel source for SMR. Many studies have shown that free fatty acids can uncouple mitochondria by increasing the activity of the proton cycle (e.g., Ref. 7), and so the use of pyruvate and glucose as the fuel source in this study would tend to underestimate the proton leak activity in muscle. These points indicate that the value for the contribution of proton leak to SMR derived from the data contained in this paper represents a lower-limit estimate.

In what ways could we have overestimated the contribution of proton leak to SMR? First, the estimate for nonmitochondrial oxygen consumption in the contracting tissue may have been misestimated, as contraction is necessarily abolished when cyanide is added to allow measurement of nonmitochondrial oxygen consumption rate. However, it is not clear whether this would lead to an overestimate or underestimate of nonmitochondrial oxygen consumption (and therefore of proton leak activity), or indeed whether nonmitochondrial oxygen consumption would be affected at all by...
muscle contraction. Second, the release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) as a result of sciatic nerve stimulation could cause mitochondrial uncoupling (and therefore an overestimate of proton leak activity) because mitochondrial ATP synthesis, which would normally drive the reuptake of Ca\(^{2+}\) by SR Ca\(^{2+}\)-ATPases, was abolished in our experiments (by addition of oligomycin) before the proton cycle activity was measured. However, as mentioned in RESULTS, the contribution of proton leak to the respiration rate of noncontracting muscle calculated from the data in Fig. 2 is essentially the same as in our previous paper (Ref. 24, in which Ca\(^{2+}\) release from the SR was blocked by dantrolene). In addition, preliminary experiments using dantrolene as described previously (24) showed that the proton leak activity in the presence of dantrolene-oligomycin (0.29 ± 0.07 μmol O\(_2\)·min\(^{-1}\)·g tissue\(^{-1}\); mean ± SE, n = 2) was not significantly different from that of subsequent experiments (0.33 ± 0.11 μmol O\(_2\)·min\(^{-1}\)·g tissue\(^{-1}\); mean ± SE, n = 5). Dantrolene was not used in the latter set of experiments because, in contrast to our previous work, the preliminary experiments showed that addition of oligomycin did not cause a drop in the average tissue membrane potential, a drop that dantrolene had previously been used to prevent (24). Note that, in the same preliminary experiments, tissue pH gradients were measured and were found to be unaffected by the addition of oligomycin after contraction was initiated (data not shown), in agreement with our previous studies. However, the plasma membrane potential was not monitored in the preliminary experiments (or in the subsequent ones), and this omission might have caused us to misestimate the proton leak activity; nevertheless, we have shown that oligomycin and cyanide have no effect on the plasma membrane potential in noncontracting muscle (24), and we feel that it is unlikely that this would be different in contracting muscle. Finally, the development of edema, which occurs toward the end of long perfusions, may artificially increase the proton leak activity. However, we have previously shown (24) that the rate of respiration of the perfused tissue in the presence of saturating amounts of oligomycin is constant despite development of edema in the hindlimbs similar to that seen in the experiments described in this paper. In general, therefore, we consider that the data in this paper represent a reasonably accurate underestimate of the contribution of proton leak to the oxygen consumption rate of lightly working skeletal muscle.

In conclusion, we have shown that proton leak accounts for ~34 and ~22% of the resting oxygen consumption rates in perfused skeletal muscle and isolated liver cells, respectively, under conditions in which respiration rate is double the resting value, which may be a reasonable approximation to the maximal resting rates of liver and skeletal muscle in vivo. However, in the case of the muscle preparation, although the whole preparation is more than doubling its respiration rate, that of the contracting tissue, which represents 15% of the whole preparation, is increasing >10-fold, and thus any extrapolation to the in vivo condition should be taken with caution. We have shown that proton leak in skeletal muscle and liver at double their resting respiration rates accounts for between 7 and 19% (mean 15%) of rat SMR. If proton leak activity is similar in other tissues to that seen in liver, the contribution of proton leak to rat SMR would be between 11 and 23% (mean 19%). We conclude that proton leak is a major contributor to rat SMR even when the respiration rates of tissues in the standard state are significantly above their resting rates.

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