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EFFECTS OF Ca^{2+} ON OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA FROM THE THERMOGENIC ORGAN OF MARLIN

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Summary

Mitochondria from the muscle-derived thermogenic (heater) organ and oxidative red muscle of the blue marlin (*Makaira nigricans*) were studied in order to evaluate aspects of the mechanism of thermogenesis in heater tissue. We investigated whether short-term Ca^{2+}-induced uncoupling of mitochondria contributes to the thermogenic cycle of the heater organ by enhancing the respiration rate. Specific electrodes were used to obtain simultaneous measurements of oxygen consumption and Ca^{2+} fluxes on isolated mitochondria, and the effects of various concentrations of Ca^{2+} on respiration rates and the ADP phosphorylated/atomic oxygen consumed (P/O) ratio were examined. Addition of Ca^{2+} in excess of 10 $\mu mol\,l^{-1}$ to respiring heater organ or red muscle mitochondria partially inhibited state 3 respiration and reduced the P/O ratio, indicating that the mitochondria were partially uncoupled. These effects were blocked by 2 $\mu mol\,l^{-1}$ Ruthenium Red. In heater organ mitochondria, state 3 respiration rate and the P/O ratio were not significantly reduced by 1 $\mu mol\,l^{-1}$ free Ca^{2+}, a concentration likely to be near the maximum achieved in a stimulated cell. This indicates that transient increases in cytosolic Ca^{2+} concentration may not significantly reduce the P/O ratio of heater organ mitochondria. The activity of 2-oxoglutarate dehydrogenase in heater organ mitochondria was stimulated by approximately 15 % by Ca^{2+} concentrations between 0.2 and 1 $\mu mol\,l^{-1}$. These results suggest that heater organ mitochondria are able to maintain a normal P/O ratio and should maintain ATP output during transient increases in Ca^{2+} concentration, supporting a model in which an ATP-consuming process drives thermogenesis. Activation of mitochondrial dehydrogenases by low levels of Ca^{2+} may also enhance respiration and contribute to thermogenesis.

Key words: heater organ, non-shivering thermogenesis, muscle, respiratory coupling, 2-oxoglutarate dehydrogenase, blue marlin, *Makaira nigricans*.

Introduction

Several large pelagic fishes of the suborder Scombroidei (mackerels, tunas and billfishes) have independently evolved a unique form of endothermy. The billfishes (family Istiophoridae), swordfish (family Xiphiidae) and the butterfly mackerel (family Scombridae) heat the brain and eyes using a specialized thermogenic organ (the heater organ) that has evolved from muscle (Carey and Teal, 1966; Carey, 1982; Block, 1987). Telemetry studies have shown that swordfish diving in cold water are able to maintain their cranial temperature up to 14 °C above that of the surrounding water (Carey, 1990). By generating metabolic heat, the heater organ dampens the effects of rapid temperature changes on the central nervous system.

Biochemical studies show that heater tissue has exceptional aerobic metabolic capacity (Tullis *et al.* 1991; Ballantyne *et al.* 1992), however, a mechanism by which oxidative activity is stimulated needs to be described. In mammalian brown fat, the only other tissue described that functions primarily to provide heat, a high rate of respiration is maintained by a unique uncoupling protein in the mitochondrial inner membrane. The uncoupling protein is a proton pore that collapses the mitochondrial proton gradient, thus driving respiration at a high rate and diverting respiratory energy from ADP phosphorylation. The proton pore is closed in the presence of guanine nucleotides, providing a mechanism for the cellular control of heat production (Nicholls and Locke, 1984). The mitochondria in heater tissue, unlike their counterparts in brown fat, lack uncoupling protein (Block, 1986) and must maintain high oxidative activity in another way. The presence of an extensive sarcoplasmic reticulum (SR) and T-tubule network in heater tissue indicates a Ca^{2+}-based mechanism for stimulation (Block, 1986, 1987, 1991). Structural and

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biochemical evidence suggests that thermogenesis in heater cells is initiated by 'excitation–thermogenic coupling,' a Ca^{2+}-activated thermogenic cycle under nervous control (Block, 1994). This cycle is based on the release of Ca^{2+} through Ca^{2+}-release channels in the extensive SR network with a consequent increase in metabolic activity in the heater cell.

The interaction of Ca^{2+} and mitochondria during the thermogenic cycle is the focus of this study. Vertebrate mitochondria are known to possess energy-dependent Ca^{2+} uptake and efflux pathways. Ca^{2+} is taken up by an electrophoretic Ca^{2+} uniporter that responds to the mitochondrial membrane potential (Crompton, 1985). Ca^{2+} enters through the uniporter with two positive charges at the expense of the membrane electrical gradient. By collapsing the electrical gradient, this process stimulates respiration and competes with ATP synthesis. Ca^{2+} taken up through the uniporter is lost from mitochondria through a number of routes. The most active efflux pathway in mitochondria from excitable cells is an electrogenic $n Na^+/Ca^{2+}$ exchanger (Jung *et al.* 1995) which is, in turn, driven by an energy-consuming Na^+/H^+ antiporter (Carafoli, 1982; McCormack *et al.* 1990). The activities of the Ca^{2+} uptake and efflux systems normally maintain matrix Ca^{2+} levels below 100 nmol l^{-1}, but this can rise substantially under conditions involving increases in average cytosolic Ca^{2+} concentration such as hormonal activation, muscular activity and ischaemia (McCormack *et al.* 1990). Such changes in matrix free Ca^{2+} concentration have been identified as an important mediator in the regulation of mitochondrial oxidative phosphorylation in vertebrate tissues (McCormack *et al.* 1990; McCormack and Denton, 1993).

The very close apposition of heater organ mitochondria with the extensive SR, along with the small cytoplasmic space (Block and Franzini-Armstrong, 1988), raises the possibility that released Ca^{2+} may be taken up directly into the mitochondria during the Ca^{2+} release cycle. If the activity of the uptake uniporter and an efflux pathway were sufficiently high, respiration could then be uncoupled during transient high Ca^{2+} levels. This would result in enhanced respiration and heat production. Rapid uptake of extramitochondrial Ca^{2+} added at high concentration has been observed in isolated mitochondria from the heater organ of swordfish (Ballantyne *et al.* 1992), indicating that heater organ mitochondria have an active Ca^{2+} uptake mechanism. Thus, Ca^{2+} uncoupling could be a mechanism for activating thermogenesis in heater cells, but would result in a reduced output of ATP. On the other hand, if the mitochondria were relatively insensitive to physiological levels of Ca^{2+}, stimulation of energy consumption and heat production would depend on other processes such as cellular consumption of ATP and the modulation of the supply of substrate and NADH.

In this study, we have examined energetic aspects of Ca^{2+} cycling in marlin heater organ mitochondria. Red locomotory muscle mitochondria were studied for comparison. We found that mitochondria from both tissues actively take up Ca^{2+} from the medium, but that significant uncoupling occurs in heater mitochondria only at free Ca^{2+} concentrations higher than those predicted to be found in the heater cell. These results support a model in which heat production is stimulated by ATP consumption in the heater cell. A futile Ca^{2+} cycle involving the SR Ca^{2+}-release channel and the SR Ca^{2+}-ATPase is discussed.

Materials and methods

Isolation of mitochondria

Heater organs from blue marlin (*Makaira nigricans* Lacépède) were dissected from recently caught specimens and chilled in ice-cold 0.9 % NaCl. Samples (3 g) of heater tissue were dissected from the center of the organ and trimmed free of attached rete and extraocular muscle. The tissue piece was minced in 10 volumes of ice-cold homogenizing buffer containing 0.25 mol l^{-1} sucrose, 5 mmol l^{-1} K-Hepes, pH 7.2, 2 mmol l^{-1} EGTA, 1 % (w/v) serum albumin. The minced tissue was homogenized with three passes of a loose-fitting Potter-Elvhjem homogenizer and centrifuged for 5 min at 120 ***g*** in a Sorvall centrifuge. The supernatant was filtered through two layers of cheesecloth and centrifuged for 5 min at 9750 ***g***. The resulting pellet was resuspended in 0.2 mol l^{-1} sucrose, 50 mmol l^{-1} KCl, 5 mmol l^{-1} K-Hepes, pH 7.2, 2 mmol l^{-1} EGTA and 1 % serum albumin (wash buffer) and centrifuged for 5 min at 9750 ***g***. The wash step was repeated once and the final pellet resuspended in 0.5 ml of wash buffer per gram starting tissue. Mitochondrial protein content was assayed using the BCA (bicinchoninic acid) method (Pierce) with serum albumin as the protein standard. Mitochondrial preparations were kept on ice and maintained respiratory control for up to 8 h. However, most experiments were performed within 3 h of isolation.

Mitochondria from red swimming muscle were prepared in the same way. Approximately 5 g of red muscle was dissected from the base of the tail and trimmed free of white muscle and connective tissue. The homogenization procedure was sufficient to release a large number of mitochondria, however, it is likely that the majority of interfibrillar mitochondria remained with the myofibrils and were discarded. We chose to accept this bias in favor of subsarcolemmal mitochondria as the possible damage to the mitochondria caused by more vigorous homogenization methods would be detrimental to the study.

The time between capture of the fish by sportfishing boats and dissection of tissues varied and influenced the characteristics of the mitochondria. Experimental fish were selected using a variety of criteria including time of capture, evidence of muscle rigor and skin discoloration. Preparations were made from the freshest fish possible and were not used if the respiratory control ratio was below 2.5.

Respiration measurements

Respiration rates were measured using a Strathkelvin 1302 oxygen electrode and Strathkelvin 781 oxygen meter. The electrode was mounted in a glass 2 ml side-port water-jacketed chamber maintained at 25 °C using a circulating water bath.

Additions were made through a groove in the Teflon chamber plug and the volume was adjusted to compensate for added liquid. Mitochondria were suspended at approximately 0.5 mg ml^{-1} in an assay buffer containing 0.15 mol l^{-1} KCl, 5 mmol l^{-1} K-Hepes, 5 mmol l^{-1} potassium phosphate, pH 7.2 at 25 °C. The assay mixture contained, as carryover from the wash medium, 10 mmol l^{-1} sucrose, 100 µmol l^{-1} EGTA and 0.05 % serum albumin. Substrates and inhibitors were added as indicated in the Figure legends. P/O ratios were calculated using the method of Estabrook (1967) from total oxygen consumed. Levels of oxygen and adenylates were assayed as described previously (O'Brien and Vetter, 1990).

Ca^{2+} flux measurements

For Ca^{2+} flux measurements, the chamber plug of the respiration chamber described above was modified to fit a Radiometer F2112Ca calcium electrode. The calomel reference electrode (Radiometer K401) was kept in a separate water-jacketed chamber filled with 0.15 mol l^{-1} KCl and was connected to the assay chamber by means of an agar bridge made with 0.15 mol l^{-1} KCl. The 95 % response time of the Ca^{2+} electrode to step addition of 10 µmol l^{-1} Ca^{2+} in respiration buffer was 2.1 min, but increased to 5.5±0.8 min in the presence of mitochondria and EGTA. Somewhat faster responses were obtained at higher Ca^{2+} concentrations. Ca^{2+} fluxes and oxygen consumption were measured simultaneously for all experiments. The Ca^{2+} solutions used were calibrated using the Ca^{2+} electrode by comparison to standard curves made in 0.15 mol l^{-1} KCl with a Radiometer Ca^{2+} standard. The solutions were checked daily by comparison of known additions of the Ca^{2+} solution to mitochondria assay buffer with comparable additions of the Ca^{2+} standard. Each individual assay performed was followed by an addition of 50 nmoles of the Ca^{2+} standard after addition of 2 µmol l^{-1} Ruthenium Red to the assay mixture to inhibit mitochondrial Ca^{2+} uptake. This served as an internal control for endogenous buffering of added Ca^{2+}.

Statistical comparisons

Unless otherwise noted, the data are means ± S.E.M. of measurements from 4–8 preparations from different fish. Comparisons between treatments were made using a Kruskal-Wallis one-way analysis of variance (ANOVA). Differences at the $P \leq 0.05$ level were considered significant.

Results

Characteristics of isolated mitochondria

Mitochondria isolated from both heater organs and red locomotory muscle were well coupled and respired on a variety of substrates. Isolated heater organ mitochondria typically displayed respiratory control ratios (RCRs) above 3.0 and red muscle mitochondria frequently showed higher values (Table 1). With heater organ mitochondria, it was necessary to omit Mg^{2+} from the assay medium as Mg^{2+}-stimulated ATPase activity was present in the preparations and interfered with P/O ratio measurements. This activity was very low in red muscle mitochondrial preparations, but Mg^{2+} was omitted from all experiments in order to maintain similar conditions throughout. Preliminary experiments with red muscle mitochondria (results not shown) indicated that P/O ratios and RCRs were not different in media containing Mg^{2+}.

Table 1. *Respiratory characteristics of marlin muscle mitochondria*

	Heater organ (*N*=9)	Red muscle (*N*=7)
Respiratory control ratio	3.44±0.69	6.52±3.54
P/O ratio	2.41±0.42*	2.78±0.24
Respiratory rate (nanoAtoms oxygen min^{-1} mg^{-1} protein)		
State 4	26.7±6.6	24.5±3.8
State 3	104.4±25.0	141.6±72.7

*Indicates a statistically significant difference (P<0.05).

The P/O ratios measured using 10 mmol l^{-1} glutamate plus 5 mmol l^{-1} malate as the substrate were within the range reported for mammalian mitochondria (Table 1). The P/O ratio measured for red muscle mitochondria was significantly higher than that measured for heater organ mitochondria, but state 4 and state 3 respiration rates and respiratory control ratios were not significantly different.

Ca^{2+} uptake and efflux pathways exist in heater organ mitochondria

Free Ca^{2+}, when added to heater organ mitochondria at micromolar concentrations, was taken up in an energy-dependent fashion. In Fig. 1, the respiration-dependent Ca^{2+}

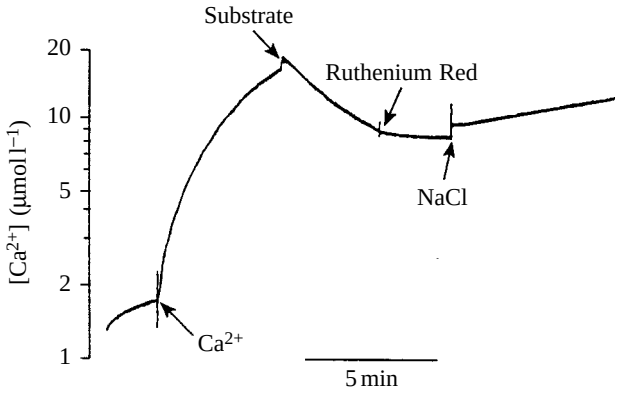

Fig. 1. Ca^{2+} electrode recording of Ca^{2+} fluxes in heater organ mitochondria. Mitochondria were suspended in assay medium at 0.72 mg ml^{-1} and $CaCl_2$ was added to give a free Ca^{2+} concentration of 1.2 µmol l^{-1} (beginning of trace). Additions indicated by the arrows were: Ca^{2+}, 55 nmoles $CaCl_2$ calculated to give a free Ca^{2+} concentration of 19 µmol l^{-1}; substrate, 10 mmol l^{-1} glutamate plus 5 mmol l^{-1} malate (potassium salts); Ruthenium Red, 1 µmol l^{-1} Ruthenium Red; NaCl, 5 mmol l^{-1} NaCl. Preparation 91BM20HO.

uptake pathway and Na^+-induced efflux pathway in heater organ mitochondria are followed using a Ca^{2+} electrode. Ca^{2+} added to non-energized mitochondria was not taken up until a respiratory substrate was provided. Upon addition of $5\,mmol\,l^{-1}$ malate plus $10\,mmol\,l^{-1}$ glutamate, oxygen consumption (results not shown) and Ca^{2+} uptake were stimulated. The respiration-dependent Ca^{2+} uptake was completely abolished by $1\,\mu mol\,l^{-1}$ Ruthenium Red. Ca^{2+} efflux from the Ruthenium-Red-inhibited respiring mitochondria could not be measured until $5\,mmol\,l^{-1}$ NaCl was added to the medium. After Na^+ addition, Ca^{2+} was released at a steady rate.

Effects of Ca^{2+} on respiratory coupling in heater organ and red muscle mitochondria

To study the effects of Ca^{2+} on respiratory coupling, we measured the P/O ratios and respiratory rates of mitochondria over a range of concentrations of added Ca^{2+}. Fig. 2 shows the effects of $11.1\,\mu mol\,l^{-1}$ added free Ca^{2+} on respiration and oxidative phosphorylation by red muscle mitochondria. Ca^{2+} was added in these experiments to mitochondria respiring in state 4 in the presence of excess substrate ($10\,mmol\,l^{-1}$ glutamate plus $5\,mmol\,l^{-1}$ malate). Following stabilization of state 4 respiration, ADP was added to stimulate state 3 respiration. In Fig. 2A, most of the added Ca^{2+} was rapidly taken up (Ca^{2+} electrode trace) with a concomitant increase in state 4 respiration. The mitochondria were still able to phosphorylate ADP in the presence of free Ca^{2+}, but the P/O ratio measured (1.99) was well below the control P/O ratio of 2.88 measured in the absence of Ca^{2+}. The addition of $2\,\mu mol\,l^{-1}$ Ruthenium Red (Fig. 2B) prevented Ca^{2+} uptake and stimulation of state 4 respiration. The P/O ratio measured under these conditions was 2.82, showing that the reduction in the P/O ratio caused by added Ca^{2+} is due to uptake through the Ruthenium-Red-sensitive Ca^{2+} uniporter.

Heater organ mitochondria showed similar effects of added Ca^{2+} on the P/O ratio to those found in red muscle mitochondria. Fig. 3 shows the concentration-dependence of these effects. As levels of free Ca^{2+} and Ca^{2+} loaded into the mitochondria increased, the P/O ratio was progressively reduced (significant reductions are indicated by an asterisk at each Ca^{2+} concentration) and the mitochondrial preparations tended to lose respiratory control. The addition of $2\,\mu mol\,l^{-1}$ Ruthenium Red before Ca^{2+} addition prevented this effect. The effects of Ca^{2+} levels on eight heater organ (Fig. 3A) and six red muscle (Fig. 3B) mitochondrial preparations are shown. However, a number of red muscle preparations lost respiratory control at higher Ca^{2+} concentrations and could not be included in the analysis. The P/O ratios plotted represent only those from preparations that had measurable respiratory control at the designated Ca^{2+} concentration. The reductions in P/O ratio compared to the Ruthenium-Red-treated controls were statistically significant above $10\,\mu mol\,l^{-1}$ added Ca^{2+} for both types of mitochondria, while at $1.1\,\mu mol\,l^{-1}$ free Ca^{2+}, only red muscle mitochondria showed a significantly reduced P/O ratio.

The Ca^{2+}-dependent reductions in the P/O ratio described above were associated with a loss of respiratory control caused, in part, by a decrease in the state 3 respiratory rate. Fig. 4 shows the reduction in state 3 respiratory rates compared to Ruthenium-Red-treated controls over the range of Ca^{2+} concentrations tested. Due to the variation between preparations in absolute respiratory rate, the state 3 respiratory rate of each preparation was normalized to the state 3 rate of the same preparation in the absence of added Ca^{2+} for statistical comparisons. Both heater organ (Fig. 4A) and red muscle (Fig. 4B) mitochondria showed declines in state 3 respiration rate relative to Ruthenium-Red-treated controls. In red muscle, a significant reduction in state 3 respiratory rate occurred at $1\,\mu mol\,l^{-1}$ free Ca^{2+} as well as at each higher Ca^{2+}

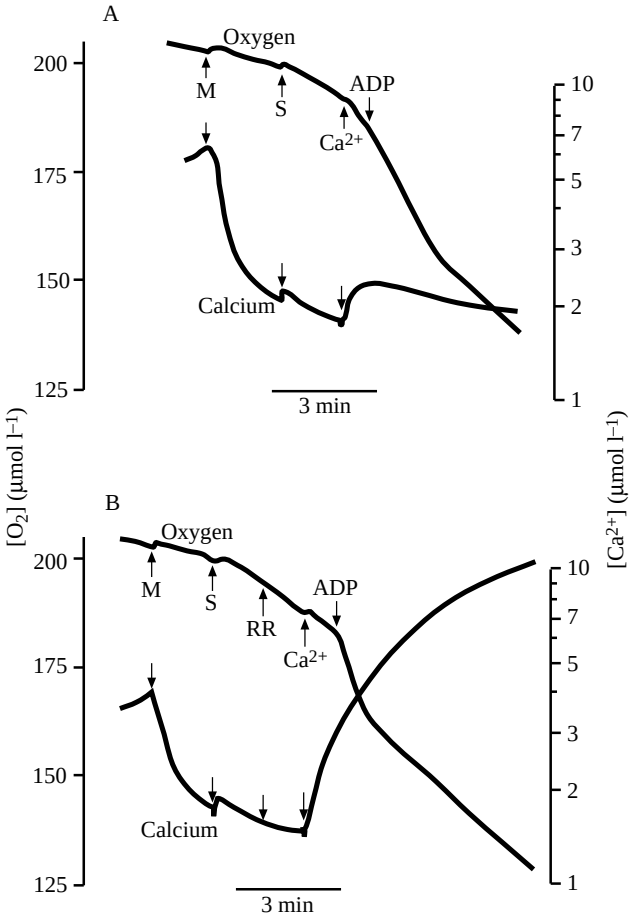

Fig. 2. Effects of Ca^{2+} and Ruthenium Red on the respiratory properties of red muscle mitochondria. Red muscle mitochondria were suspended at $0.46\,mg\,ml^{-1}$ in assay medium and oxygen consumption and Ca^{2+} fluxes were recorded simultaneously. (A) Measurement of the P/O ratio in the presence of $11.1\,\mu mol\,l^{-1}$ Ca^{2+}. Additions indicated by the arrows are: M, 0.92 mg mitochondria; S, $10\,mmol\,l^{-1}$ glutamate plus $5\,mmol\,l^{-1}$ malate; Ca^{2+}, 198 nmoles $CaCl_2$; ADP, 235 nmoles ADP. The P/O ratio measured in this experiment was 1.99. (B) Measurement of the P/O ratio in the presence of $2\,\mu mol\,l^{-1}$ Ruthenium Red and $11.1\,\mu mol\,l^{-1}$ $CaCl_2$. Additions indicated by arrows are as in (A) but include: RR, $2\,\mu mol\,l^{-1}$ Ruthenium Red. The P/O ratio measured in this experiment was 2.82. Preparation 92BM17RM for both examples.

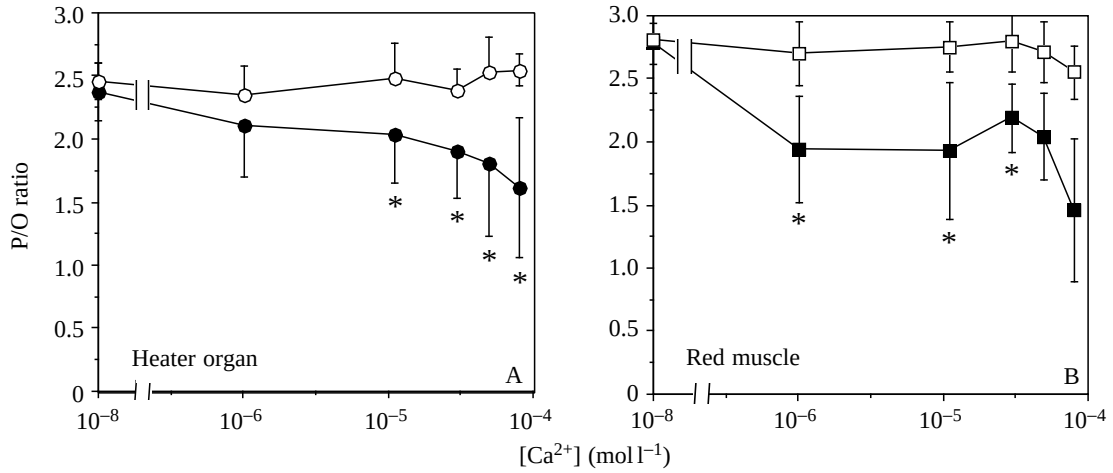

Fig. 3. Effects of free Ca^{2+} concentration on the P/O ratio. Mitochondria preparations were treated as in Fig. 2, and the P/O ratio was measured with (open symbols) and without (filled symbols) addition of 2 μmol l^{-1} Ruthenium Red. An asterisk below a pair of points indicates that the P/O ratio was significantly different (P<0.05) between Ruthenium-Red-treated and untreated mitochondria. Only preparations with measurable respiratory control values were included in the analysis. (A) Heater organ mitochondria. Data are means ± S.E.M. for eight preparations. (B) Red muscle mitochondria. Data are means ± S.E.M. for six preparations. Some error bars are omitted for clarity.

concentration. Heater organ mitochondria showed a significant reduction in state 3 respiration rate compared to the Ruthenium-Red-treated control at free Ca^{2+} levels above 10 μmol l^{-1}.

In heater organ mitochondria, the respiration rates in Ruthenium-Red-treated controls were significantly higher in the presence of added Ca^{2+} than in its absence (Fig. 4A). This was not the case for red muscle mitochondria (Fig. 4B). This effect counteracts the inhibition of state 3 respiration by external Ca^{2+} in heater organ mitochondria. In fact, the state 3 respiration rate was significantly different from that of control preparations without Ca^{2+} only at 80 μmol l^{-1} free Ca^{2+}, which is well above the expected physiological range. These observations indicate that two processes occurred together in the heater organ mitochondria experiments: (1) a Ruthenium-Red-sensitive inhibition of respiration caused by added Ca^{2+}, as is the case with red muscle mitochondria, and (2) a Ruthenium-Red-resistant effect that stimulates respiration in the presence of externally added Ca^{2+} in the micromolar range.

Effects of Ca^{2+} on 2-oxoglutarate dehydrogenase

We examined the influence of low concentrations of Ca^{2+} on the activity of 2-oxoglutarate dehydrogenase, an important tricarboxylic acid (TCA) cycle enzyme that is directly modulated by Ca^{2+} (McCormack and Denton, 1990). In this experiment, state 3 respiration of heater organ mitochondria on 2-oxoglutarate was measured under conditions in which the supply of substrate was limiting. A concentration of 200 μmol l^{-1} 2-oxoglutarate was found empirically to give somewhat less than half-maximal state 3 respiration rates in the presence of 75 μmol l^{-1} ADP. Added Ca^{2+} had complex effects on the respiration rate under these conditions. Fig. 5 shows the effect of Ca^{2+} on respiration rate normalized to the

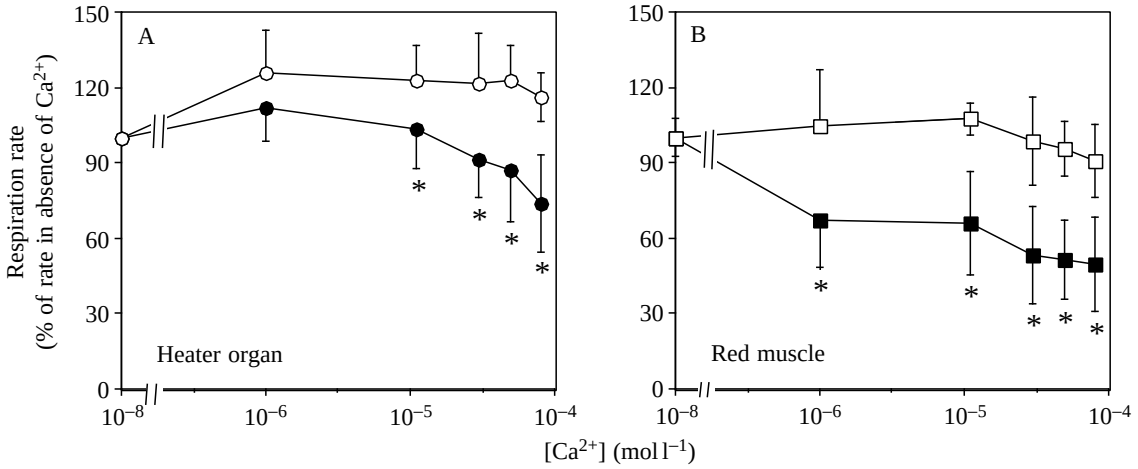

Fig. 4. Effect of free Ca^{2+} concentration on state 3 respiration rates. The maximum state 3 respiratory rates measured from the experiments shown in Fig. 3 in the presence (open symbols) or absence (filled symbols) of 2 μmol l^{-1} Ruthenium Red are plotted *versus* free Ca^{2+} concentration. Values are normalized to the state 3 respiration rate of the same preparation in the absence of added Ca^{2+}. All preparations were included at each point. (A) Heater organ mitochondria. Data are means ± S.E.M. for eight preparations. (B) Red muscle mitochondria. Data are means ± S.E.M. for six preparations. Some error bars are omitted for clarity. An asterisk below a pair of points indicates that the respiratory rates are significantly different (P<0.05).

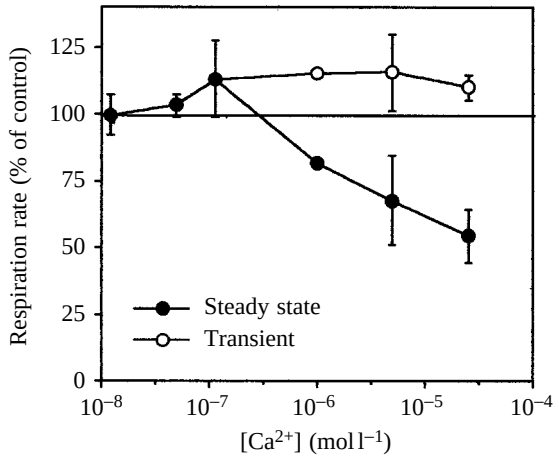

Fig. 5. Effects of free Ca^{2+} concentration on 2-oxoglutarate-supported respiration. Ca^{2+} was added to heater organ mitochondria respiring on 200 μmol l^{-1} 2-oxoglutarate with 75 μmol l^{-1} ADP. The steady-state respiration rate shown (filled symbols) is the Ca^{2+}-influenced rate normalized to the state 3 respiration rate prior to Ca^{2+} addition. At Ca^{2+} concentrations above 10^{-7} mol l^{-1}, respiration showed two stages. The transiently increased respiration rate is shown by the open symbols. Data are means ± S.E.M. for four preparations.

control (no Ca^{2+} addition) state 3 respiration rate for each preparation. The mean ± S.E.M. state 3 respiration rate was 33.0±7.8 nanoatoms oxygen min^{-1} mg $protein^{-1}$. Free Ca^{2+} levels below 1 μmol l^{-1} increased the state 3 respiration rate by up to 15 % above the control value. However, at 1 μmol l^{-1} and above, steady-state respiration was inhibited, as was seen in experiments involving saturating concentrations of glutamate. In the experiments involving Ca^{2+} concentrations of 1 μmol l^{-1} and above, the addition of Ca^{2+} induced a transient increase in the respiration rate that preceded the inhibited steady state. The transient respiration rates are shown in Fig. 5 (open symbols). The transient increases in respiration were not linearly related to Ca^{2+} concentration, but were consistently of the same size as the increased state 3 rate observed at 0.2 μmol l^{-1} Ca^{2+}. The kinetics of respiration rate changes suggest the presence of two processes: (1) a relatively rapid activation by submicromolar Ca^{2+} levels and (2) a slower inhibition by micromolar Ca^{2+} levels similar to that observed with excess glutamate as the substrate.

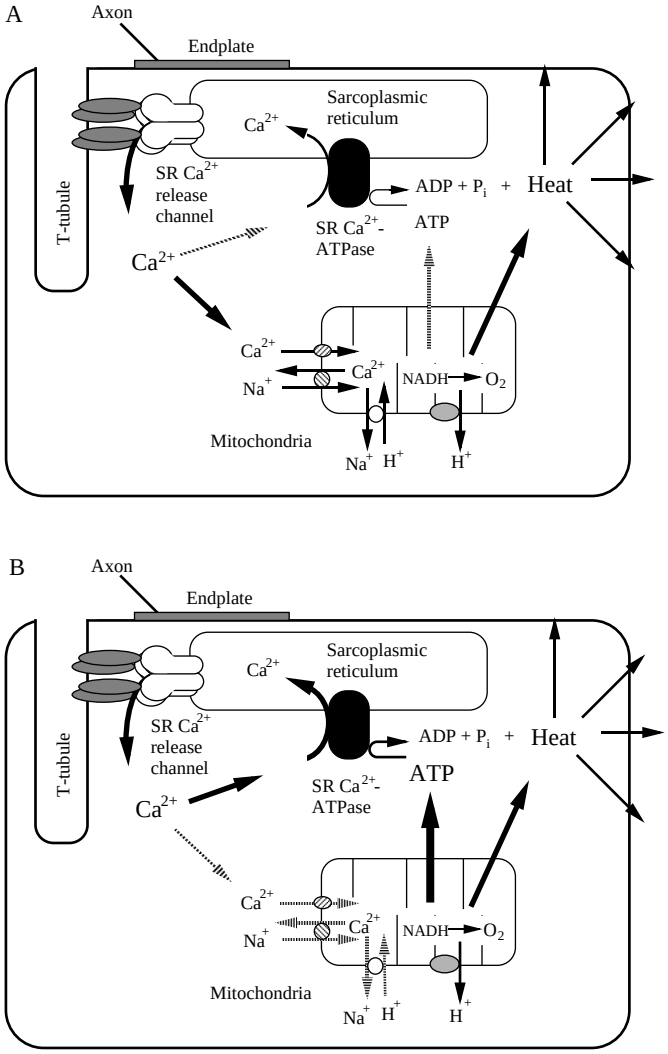

Fig. 6. Two theoretical models describing Ca^{2+} cycling and the mechanism of thermogenesis in heater cells. In A, the activity of the mitochondrial Ca^{2+} uptake and efflux pathways are sufficiently high that Ca^{2+} release can cause significant uncoupling of the mitochondria. The resulting respiratory stimulation contributes to thermogenesis. Relatively large fluxes of ions and heat are indicated by the bold arrows, while relatively smaller fluxes are shown with broken arrows. In B, the mitochondria are insensitive to uncoupling by Ca^{2+}. Release of Ca^{2+} from the sarcoplasmic reticulum (SR) results primarily in stimulation of the SR Ca^{2+}-ATPase and futile cycling of Ca^{2+} through the SR. The high rate of ATP turnover caused by this cycling stimulates mitochondrial respiration and, hence, thermogenesis.

Discussion

Regulation of metabolism by Ca^{2+} is important in many cell types, and in excitable cells the roles of Ca^{2+} are complex. Both the basal cytoplasmic level of free Ca^{2+} and transient Ca^{2+} concentration peaks are of physiological importance, and may serve quite different roles. In muscle cells, the contractile apparatus is triggered by large-amplitude Ca^{2+} release events while the basal, or perhaps time-averaged, myoplasmic Ca^{2+} concentration regulates factors such as the activity of mitochondrial enzymes that affect energy supply (McCormack and Denton, 1990). The heater organ of billfishes is an example of a tissue in which the normal muscle Ca^{2+} release cycle has been modified and harnessed to generate heat (Block, 1987). Heater cells lack the contractile elements of muscle, showing almost no detectable myosin ATPase activity (Block, 1986; Tullis, 1994). However, they retain an extensive network of sarcoplasmic reticulum and T-tubules with a configuration unique among muscle tissues. An average of 63 % of the heater cell volume is occupied by mitochondria, and a large fraction of the remaining cell volume is taken up by SR and T-tubule membrane systems with stacks of sarcoplasmic reticulum

forming a network surrounding each mitochondrion (Block, 1987; Block and Franzini-Armstrong, 1988). In addition, the heater cell plasma membrane is endowed with a rich supply of acetylcholine receptors (Block, 1994). These histological characteristics suggest that heater cells function by a depolarization-induced Ca^{2+} release process, as in normal muscle.

A number of biochemical studies indicate that the heater organ may have Ca^{2+} release and uptake properties similar to fast-twitch oxidative muscle. The heater organ expresses only the α (RyR1) isoform of the SR Ca^{2+}-release channel (Block *et al.* 1994), unlike the majority of other skeletal muscles in non-mammalian vertebrates, which express two different isoforms (α, and β or RyR3) together (Airey *et al.* 1990; Olivares *et al.* 1991; O'Brien *et al.* 1993). We have shown previously that expression of the α isoform of the Ca^{2+}-release channel alone is associated with muscles specialized for high-frequency contraction (O'Brien *et al.* 1993). In addition, the heater organ expresses the SERCA1 or fast isoform of the Ca^{2+}-ATPase (Tullis, 1994). The presence of the fast Ca^{2+} release and uptake proteins suggests that heater organs are capable of performing a rapid cycle of Ca^{2+} release and reuptake.

Knowledge of the interaction between Ca^{2+} and mitochondria in heater organs is also important for understanding the thermogenic cycle. The high density of mitochondria in heater organs and the close proximity of the mitochondria to the SR suggest that Ca^{2+} released from the SR may be taken up directly by the mitochondria. This process could make an important contribution to the mechanism of thermogenesis. In Fig. 6, two models depicting different potential Ca^{2+} cycles in heater cells are shown. Fig. 6A shows a model in which direct Ca^{2+} uptake by mitochondria through the Ca^{2+} uniporter makes a major contribution to the thermogenic cycle. In this model, Ca^{2+} uptake and subsequent Na^{+}-dependent efflux stimulates respiration by consuming the energy in the electrical and proton gradients. A portion of the respiratory energy may be diverted away from oxidative phosphorylation to support this process and this would lead to a reduction in ATP output. Following a transient increase in Ca^{2+} level, oxidative phosphorylation would return to normal as the myoplasmic Ca^{2+} concentration is reduced by the action of Ca^{2+}-ATPase. An elegant demonstration of this sort of effect has been made in cultured neuroblastoma cells (Loew *et al.* 1994). Optical measurements of mitochondrial membrane potential and cytoplasmic Ca^{2+} concentration revealed mitochondrial depolarizations in response to transient increases in cytoplasmic Ca^{2+} levels induced by high extracellular K^{+} levels or hormones. These results suggest that the mitochondrial protonmotive force is reduced during a transient increase in Ca^{2+} level and thus oxidative phosphorylation may be reduced.

An alternative model, shown in Fig. 6B, is one in which the activities of the mitochondrial Ca^{2+} uptake and efflux pathways are relatively low and ATP output is maintained during a transient increase in Ca^{2+} level in the cell. In order to stimulate respiration and thermogenesis, one or several ATP-consuming processes in the cell must operate at a high level. This model would not exclude uptake of a small amount of Ca^{2+} into the mitochondrial matrix, which could activate several matrix dehydrogenases and thus enhance respiration (McCormack *et al.* 1990; McCormack and Denton, 1993). The model in Fig. 6B depicts Ca^{2+} pumping by the SR Ca^{2+}-ATPase as the major ATP-consuming process, although some contribution by the several other ATPases present would be expected. Sustained or frequent Ca^{2+} release events would be needed to maintain the activity of the ATPase and support thermogenesis. The main differences between these models lie in the properties of the mitochondrial Ca^{2+} uptake and release pathways, and characterization of the mitochondrial response to Ca^{2+} will help to choose between the models.

Mitochondria from both the heater organ and red muscle were able to take up Ca^{2+} from the medium, and Ca^{2+} uptake had a negative effect on respiratory coupling. At concentrations above $10\,\mu mol\,l^{-1}$ free Ca^{2+}, the mitochondria showed significantly reduced P/O ratios and hence a reduced efficiency of coupling. At $1\,\mu mol\,l^{-1}$ free Ca^{2+}, near the probable upper limit of physiological Ca^{2+} transients in muscle cells, the P/O ratio of heater organ mitochondria was not significantly reduced. Interestingly, a significant reduction in the P/O ratio was observed in red muscle mitochondria at $1\,\mu mol\,l^{-1}$ free Ca^{2+}. Two factors make it impossible to evaluate this difference directly. Heater organ mitochondria differed significantly from red muscle mitochondria in having both a reduced respiratory control ratio and a lower P/O ratio in control experiments. Thus, a significant reduction in coupling (and, by inference, in protonmotive force) caused by Ca^{2+} uptake may put red muscle mitochondria in a condition similar to that of heater organ mitochondria assayed under control conditions. Nonetheless, it is evident that the heater organ mitochondrial preparations used were capable of maintaining control levels of respiratory coupling and P/O ratio at physiological levels of Ca^{2+}.

One of the primary factors affecting respiratory control was the change in state 3 respiration rate in the presence of added Ca^{2+}. Compared to control preparations treated with $2\,\mu mol\,l^{-1}$ Ruthenium Red, both heater organ and red muscle mitochondria showed reductions in the state 3 respiration rate in the presence of added Ca^{2+}. These reductions were significant at free Ca^{2+} concentrations of $10\,\mu mol\,l^{-1}$ and above, and also at $1\,\mu mol\,l^{-1}$ for red muscle mitochondria. The heater organ mitochondria showed a second effect that was not shown by red muscle mitochondria. In heater organ mitochondria treated with Ruthenium Red, added Ca^{2+} significantly stimulated state 3 respiration. This effect may also occur in the absence of Ruthenium Red. This suggests that a stimulatory effect of Ca^{2+} on state 3 respiration in heater organ mitochondria may counteract the inhibitory effect of Ca^{2+} also observed. The basis for this stimulatory effect is not known. It is possible that the Ruthenium-Red-sensitive Ca^{2+} uniporter was not entirely blocked in the heater organ mitochondrial experiments and that some Ca^{2+} was taken up into the

mitochondrial matrix. This could have the effect of activating matrix dehydrogenases (McCormack *et al.* 1990) and stimulating respiration, as will be discussed further below. However, no uptake of added Ca^{2+} was detectable with the Ca^{2+} electrode under these conditions, so any leak past the Ruthenium Red block must have been small relative to the normal rate of uptake.

The regulatory effects of Ca^{2+} on mitochondrial matrix dehydrogenases have been widely studied (see McCormack *et al.* 1990 for a review). Such effects may be important in the maintenance of elevated metabolism in the heater organ during calcium release events. In the present study, we examined the effects of Ca^{2+} on 2-oxoglutarate dehydrogenase activity in heater organ mitochondria. This experiment showed that Ca^{2+} uptake caused a small but consistent increase in 2-oxoglutarate dehydrogenase activity at concentrations above $0.2\,\mu mol\,l^{-1}$ free Ca^{2+}. This activation was only transiently evident at concentrations above $1\,\mu mol\,l^{-1}$ free Ca^{2+}, as the inhibition of respiration noted with saturating amounts of glutamate as the substrate was also observed under the conditions of this experiment. However, $0.2–1\,\mu mol\,l^{-1}$ free Ca^{2+} produced sustained activation of 2-oxoglutarate-supported respiration, indicating that probable physiological concentrations of Ca^{2+} can stimulate respiration. The degree of 2-oxoglutarate dehydrogenase activation observed, approximately 15 %, is smaller than many values reported for mammalian mitochondria, which can be as high as 200 % (McCormack *et al.* 1990). However, it is possible that the mitochondria used in this study contained elevated matrix concentrations of Ca^{2+} that were not depleted during the isolation procedure. In this case, true basal 2-oxoglutarate dehydrogenase activity may not have been measured and the degree of activation underestimated. In any case, it is evident that sub-micromolar Ca^{2+} levels can stimulate heater organ 2-oxoglutarate dehydrogenase and, by inference, respiratory activity. Thus, it is possible that during thermogenesis, the uptake of a small amount of Ca^{2+} may have an activating effect on mitochondrial oxidative phosphorylation and enhance ATP output. However, the present experiments suggest that this effect is marginal.

In summary, this study indicates that heater organ mitochondria are not significantly uncoupled under conditions likely to occur in the heater cell during a cycle of Ca^{2+} release. This property of heater organ mitochondria suggests that the model shown in Fig. 6B is a more accurate description of the mechanism of metabolic activation in the heater organ: heater organ mitochondria maintain ATP output during high Ca^{2+} transients. Respiration and ATP output may, in fact, be enhanced by the action of Ca^{2+} on mitochondrial matrix dehydrogenases. The biochemical properties of heater tissue indicate a relationship with fast-twitch oxidative muscle and suggest that the heater cell is designed for Ca^{2+} release events that are relatively brief and rapidly repeated. To date, no measurements of cytoplasmic Ca^{2+} transients have been made in heater cells, so the frequency of Ca^{2+} release, peak free Ca^{2+} concentration and average free Ca^{2+} concentration are not known. Nonetheless, the fast-twitch characteristics of the heater cell SR support the contention that released Ca^{2+} is probably quickly returned to the SR and that uptake of Ca^{2+} by the mitochondria is limited. These findings support the hypothesis that mitochondrial ATP output is maintained and that activity of an ATP-consuming process (e.g. Ca^{2+} uptake *via* the SR Ca^{2+}-ATPase) is required to stimulate respiration and generate heat.

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