CALCIUM UPTAKE BY MITOCHONDRIA ISOLATED FROM
MUSKRAT AND GUINEA PIG HEARTS

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Summary

Subsarcolemmal and interfibrillar mitochondria were isolated from the hearts of
the diving muskrat and non-diving guinea pig and direct and indirect measure-
ments of calcium uptake were examined in vitro. The calcium-stimulated
respiration rate and 45Ca uptake were measured and found to be greater in
muskrat than in guinea pig mitochondria. Muskrat mitochondria were able to
endure a greater external calcium concentration than guinea pig mitochondria
before exhibiting indications of inner membrane damage. Calcium uptake by
muskrat heart mitochondria was inhibited more by 1 mmol l−1 MgCl2 than was
uptake by guinea pig mitochondria. No differences were detected between the
interfibrillar and subsarcolemmal populations of mitochondria within species. An
increased ability to sequester calcium by mitochondria without causing them
damage may aid an animal during recovery from hypoxia, ischemia or acidosis.

Introduction

Muskrats (Ondatra zibethicus) are among the most common North American
freshwater diving mammals. Although muskrats are small (1000 g) and thus have a
large mass-specific oxygen consumption (Fish, 1983), their submersion time in a
laboratory setting is about 4 min, as measured by the time taken to reach a flat
electroencephalogram (Jones et al. 1982). In a more natural setting their maximum
dive time is estimated to be 9–12 min (Andersen, 1966). Muskrats have large
oxygen stores in the lower range of those reported for marine diving mammals
(Snyder and Binkley, 1985). They have a well-developed diving response to either
forced or voluntary submergence (Jones et al. 1982; MacArthur and Karpan,
1989). This response consists of a bradycardia and a redistribution of blood flow
primarily to the heart, brain and adrenal gland (Jones et al. 1982). In spite of
oxygen conserving mechanisms, diving mammals may experience extreme hypox-
emia during long dives. An arterial \( P_{O_2} \) of 2 kPa (15 mmHg) has been measured
in animals forced to dive, with the animal showing complete recovery following the
dive (McKean, 1982). By comparison, an arterial \( P_{O_2} \) of 3.3 kPa (25 mmHg)

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may be fatal to terrestrial animals (Hattingh et al. 1986). Muskrat hearts are adapted to these hypoxic conditions (McKean and Landon, 1982; McKean, 1984) and one of the adaptations is a high potential for glycolysis (McKean et al. 1986). Sordahl et al. (1983) examined oxidative function in seal hearts by measuring mitochondrial respiration rates and cytochrome concentrations and compared these values to those obtained from dog heart mitochondria. The study showed that seal hearts have fewer mitochondria per gram of tissue, lower active respiratory rates and lower cytochrome contents than dog hearts. This study, however, failed to take into account the effect of scaling according to body mass: the smaller animal (dog) would be expected to have a greater oxidative capacity (Driedzic et al. 1987). McKean (1990) showed that mitochondrial marker enzyme activities were similar in muskrat and guinea pig hearts and that respiration rates and respiratory control ratios of muskrat heart mitochondria did not differ from rates and ratios in guinea pig mitochondria.

Hypoxia followed by reoxygenation and ischemia followed by reperfusion may damage the myocardium. In both of these conditions intracellular calcium concentration is known to rise (Poole-Wilson et al. 1984). These is some controversy over whether the rise in calcium exacerbates the damage (Marban et al. 1989; Poole-Wilson et al. 1984) or simply accompanies it (Cheung et al. 1986). Mitochondria sequester calcium by means of a uniporter driven by the electrical gradient established by the extrusion of protons from electron transfer in the respiratory chain (Denton and McCormack, 1985). Mitochondria extrude Ca\(^{2+}\) by means of an electrically neutral Ca\(^{2+}/2\)Na\(^+\) antiport. At physiological concentrations of calcium there is cycling of Ca\(^{2+}\) between the cytosol and the mitochondrial matrix. When calcium enters the matrix, it stimulates respiration by reducing the electrical potential difference across the mitochondria. Calcium also strongly activates several important mitochondrial enzymes (Denton and McCormack, 1985; Wan et al. 1989). At high concentrations of cytosolic Ca\(^{2+}\), the capacity of the uptake system exceeds that of the extrusion system and there is a net uptake of calcium by the mitochondria (Nichols and Akerman, 1982). If mitochondria accumulate calcium, yet remain coupled, the myocardium may be protected against calcium overload. With large calcium loads, however, the mitochondria themselves become damaged (Palmer et al. 1986).

Cardiac muscle of the rat is reported to have two functionally different populations of mitochondria that can be isolated by a combination of mechanical and proteolytic disruption of cardiac tissue (Palmer et al. 1977). Interfibrillar mitochondria are found among the myofibrils and have a high respiration rate and calcium-sequestering capability compared with the subsarcolemmal mitochondria found adjacent to the cardiac sarcolemma (Palmer et al. 1977, 1986). Several studies, however, have shown that there are no functional differences between these populations of mitochondria when respiration studies are conducted in a medium of high ionic strength (Matlib et al. 1981; McKean, 1990).

The purpose of this study was to determine (1) whether muskrat heart mitochondria sequester calcium at a greater rate than heart mitochondria from a
Muskrat heart mitochondria

Muskrats were obtained by live-trapping in Washington and Idaho. The animals were fed carrots and were used within 2 days of capture. Muskrat body mass was 947±44 g. Guinea pigs were obtained from licensed animal dealers or were bred in the laboratory. Body mass was 690±50 g. Both sexes of animals were used. Animals were anesthetized with ethyl ether and their hearts removed and the atria and right ventricle dissected free.

Interfibrillar and subsarcolemmal mitochondria were prepared from the left ventricle according to the method of Palmer et al. (1977). The method involves dislodging subsarcolemmal mitochondria by relatively gentle disruption with a Polytron homogenizer. Interfibrillar mitochondria are released by enzymatic digestion with the bacterial protease Nagarse (Enzyme Development Corp., New York, NY), and then centrifuged. The final two washes and subsequent suspension of mitochondria were carried out in solutions previously described (McKean, 1990) but prepared without EGTA. Protein content was determined using the Bio-Rad (Richmond, CA) protein assay with bovine serum albumin as a standard. All samples and standards contained 0.1% Triton X-100.

To determine if there was soluble protein contamination in the mitochondrial suspension, it was centrifuged and the protein content of the supernatant was determined. To determine if there was insoluble protein contamination in the mitochondrial suspension, mitochondria were fixed in half-strength Karnovsky's phosphate-buffered fixative and then centrifuged. They were then rinsed in a 0.2 mol l⁻¹ cacodylate buffer and postfixed in 1% buffered OsO₄. The samples were subsequently dehydrated in ascending concentrations of ethanol. The pellets were embedded in Medcast, stained with uranyl acetate and lead citrate and examined in a Zeiss EM10A electron microscope.

Respiration rates were determined in some preparations to confirm that the mitochondria were healthy by placing 50 μl of mitochondrial suspension into an Instech Laboratories (Horsham, PA) oxygen uptake system. Chamber volume was 600 μl; the suspension was stirred and maintained at 27°C. Rates were determined using a reaction medium consisting of 250 mmol l⁻¹ sucrose, 10 mmol l⁻¹ KH₂PO₄, 5 mmol l⁻¹ Hepes, 1.7 mmol l⁻¹ malate and 10 mmol l⁻¹ glutamate, pH 7.2. Three minutes after the introduction of mitochondria, 500 nmol of ADP was added and state 3 respiration rate measured. Only mitochondria having a respiration rate exceeding 200 nanoatoms O min⁻¹ mg⁻¹ protein were included in the study. The studies in which respiration rate was stimulated by the addition of calcium were performed with a reaction medium of 220 mmol l⁻¹ mannitol, 70 mmol l⁻¹ sucrose, 5 mmol l⁻¹ Mops, 1.7 mmol l⁻¹ KH₂PO₄, 1.7 mmol l⁻¹ succinate and 2.5 μg ml⁻¹ rotenone at a pH of 7.2. Mitochondria were
added to the chamber containing the reaction medium and after 2 min calcium was added over a range of 0.05–2.5 mmol l\(^{-1}\) and the respiration rate determined.

Calcium uptake was determined in intact coupled mitochondria by adding 0.18 mg of mitochondrial protein to 0.5 ml of the Mops reaction medium for 2 min at 28°C. Calcium uptake was then initiated by the addition of a solution of 6 mmol l\(^{-1}\) \(^{45}\)CaCl\(_2\) (specific activity 0.83 Ci mol\(^{-1}\)). The initial concentration of calcium was 134 mmol l\(^{-1}\), which decreased with time as the mitochondria accumulated calcium. In some experiments MgCl\(_2\) was added to the reaction medium at a concentration of 1 mmol l\(^{-1}\) or 1 \(\mu\)mol l\(^{-1}\) to determine the effect of magnesium on calcium uptake. The uptake was stopped by the addition of 3 ml of ice-cold MgCl\(_2\) (100 mmol l\(^{-1}\)). The mitochondria were then filtered onto Millipore 0.45 HA filters and rinsed three times with 3 ml of a solution of 0.18 mol l\(^{-1}\) KCl and 0.5 % bovine serum albumin, pH 7.0. Calcium uptake was negligible in the presence of the stop solution and accumulated calcium did not efflux with the addition of either the stop or the wash solutions. The filters were placed in Ecolite liquid scintillation cocktail (Interchem Enterprises, Inc., San Diego, CA) and counted in a Packard liquid scintillation spectrometer. Citrate synthase, a mitochondrial marker enzyme, was determined in the supernatants from mitochondria that had been charged in the reaction medium for 2 min at 28°C and then incubated in various concentrations of added calcium. The method of Shepard and Garland (1969) was used. Data are presented as the mean±one standard error unless otherwise indicated. Analysis of variance or the Student’s t-test was used to determine statistical significance. Significance was established if the \(P\) value was less than 0.05.

**Results**

The morphological appearance of mitochondria examined under the electron microscope was similar for the two species. The mitochondria did not appear swollen and both the inner and outer membranes were intact. There was a very small amount of debris or broken mitochondria in the samples examined. The soluble protein in the mitochondrial suspension (after centrifugation and removal of the pellet) was less than 1 % of the total mitochondrial protein for both muskrat and guinea pig mitochondria. This was corrected for the bovine serum albumin that had been added to the suspension medium.

Fig. 1 shows the calcium-stimulated respiration rates for interfibrillar and subsarcolemmal mitochondria. The calcium-stimulated respiration rate was greater in muskrat mitochondria than in guinea pig mitochondria. No difference was found in calcium-stimulated respiration rate between the two populations of mitochondria within a species. Following the addition of small amounts of calcium, respiration rate was stimulated and then returned to the control rate within 10 s. If larger amounts of calcium were added, the respiration rate remained elevated. Muskrat mitochondria could be exposed to a greater calcium concentration before developing a sustained elevated respiration rate than could guinea
Fig. 1. Calcium-stimulated respiration rate of muskrat (triangles) and guinea pig (circles) mitochondria, N=6, for guinea pig and N=5 for muskrat. 0.21–0.29 mg of mitochondrial protein was incubated in the Mops-succinate buffer for 2 min and then the indicated concentration of calcium was added. Respiration rate was determined from the slope of the O₂ electrode vs time tracing. Data are presented as mean±standard error. There is no statistical difference between populations of mitochondria but there is a difference between species (two-way analysis of variance).

pig mitochondria. For the muskrat mitochondria a sustained increase occurred at a calcium concentration of 0.7±0.11 mmol⁻¹ (N=12) and for the guinea pig mitochondria it occurred at a calcium concentration of 0.4±0.03 mmol⁻¹ (N=10). The calcium concentrations are the initial concentrations in the 600 μl chamber; they decrease with time as the mitochondria accumulate calcium. Citrate synthase appeared in the supernatant of these mitochondria at a concentration ranging between 2 and 13% of the total citrate synthase present in the Triton-X 100 solubilized mitochondria. There was a very weak correlation between citrate synthase released into the supernatant and the amount of calcium added over a range of calcium concentration of 0–1.13 mmol⁻¹. The slope of the regression lines that relate calcium concentration to citrate synthase release did not differ significantly from zero (P=0.106, muskrat; P=0.206, guinea pig). The regression lines extrapolated to 6% for both muskrat and guinea pig mitochondria at zero added calcium. This is the percentage of the total citrate synthase that leaked from the mitochondria during the course of the experiments.

Fig. 2 shows that there was no difference between subsarcolemmal and interfibrillar mitochondrial calcium uptake but that uptake was significantly greater in muskrat mitochondria than in guinea pig mitochondria. The difference in uptake is most evident after 60 s of incubation. The results shown in Fig. 2 were obtained from mitochondria that were nominally free of magnesium, although
Fig. 2. Uptake of $^{45}$Ca by muskrat and guinea pig heart mitochondria; $N=5$ for both species. Data are presented as mean±standard error. There is no statistical difference between populations but there is a statistical difference between species (two-way analysis of variance).

Magnesium is normally present in the intracellular fluid. Fig. 3 shows the effect of 1 mmol l$^{-1}$ and 1 $\mu$mol l$^{-1}$ magnesium on calcium uptake by muskrat and guinea pig heart mitochondria. Magnesium has little effect on calcium uptake in guinea pig mitochondria and a significant effect at 1 mmol l$^{-1}$ in the muskrat heart mitochondria.

**Discussion**

Calcium-stimulated respiration rate and calcium uptake by mitochondria are expressed per milligram of mitochondrial protein. It is paramount to the interpretation of the results of this study that the purity of mitochondrial preparations should be comparable between mitochondrial preparations. The results of a previous study (McKean, 1990) indicated that the yield in isolating mitochondria from guinea pig and muskrat hearts was similar (41 vs 43 %), but the distribution of marker enzyme (citrate synthase) in the different fractions obtained during the isolation procedure varied markedly. Thus, it appeared that Polytron homogenization and Nagarse digestion did not affect the hearts of the two species in a quantitatively similar fashion. This was probably due to differences in the friability of the tissue from the two species. The respiration rate of these mitochondria per milligram protein did not differ between species or between populations of mitochondria, and the rates indicated that the mitochondria were healthy. A similar isolation procedure was used in this study. Mitochondria were examined under the electron microscope and were found to be almost entirely free of contaminating debris, as was also reported for other studies using this isolation
Fig. 3. The effect of magnesium on calcium uptake in muskrat and guinea pig mitochondria. The magnesium was added to the incubation solution so that mitochondria were exposed for 2 min prior to the addition of calcium. Calcium was added, and the uptake was stopped after 15 s by the addition of stop solution. Similar results (not shown) were obtained after 30 s of uptake. Two populations of mitochondria each were isolated from three guinea pigs and two muskrats and run in triplicate, giving \( N = 9 \) (data were not obtained from all populations) for guinea pigs and \( N = 12 \) for muskrats. Since no differences in calcium uptake were observed between populations, the results from different mitochondrial populations were pooled for graphical display. Controls (100%) had no added magnesium.

The mitochondria were also free of contaminating soluble proteins. This was determined by centrifuging the final mitochondrial suspension and measuring the protein content of the supernatant. When the supernatant was assayed for citrate synthase at the end of the experiments, enzyme content was 6.6±1.36% of the total citrate synthase content in the suspension for guinea pig mitochondria and 6.0±0.38% for muskrat mitochondria. Therefore, the mitochondria used in this study are relatively pure: because (1) respiration studies (respiration rate, respiratory control ratio and phosphorous to oxygen ratio) show the mitochondria to be healthy, (2) a minimal amount of contaminating insoluble protein in the suspension is visible under the electron microscope, (3) only a small amount of soluble protein is present in the suspension at the time of the initial protein determination, and (4) only 6% leakage of marker enzyme occurs over the course of the experiments.

Palmer et al. (1977) demonstrated that two populations of mitochondria can be isolated from rat left ventricle by Polytron treatment followed by Nagarse digestion. Interfibrillar mitochondria had respiration rates and citrate synthase activities about 1.5 times higher than rates and activities found in the subsarcolemmal mitochondria. About 15% of the total citrate synthase activity in the heart was found in the supernatant fraction and the authors argue that this attests to the
suitability of their isolation procedure. Matlib et al. (1981) showed that, when subsarcolemmal mitochondria are assayed in high ionic strength medium, their state 3 respiration rate is low compared with the rate measure in low ionic strength medium. A previous study on muskrat, guinea pig and rat heart mitochondria (McKean, 1990) also failed to show a difference in respiration rates between interfibrillar and subsarcolemmal mitochondria or among mitochondria from different species when low ionic strength media were used during the respiration assays.

Palmer et al. (1986) also examined the calcium uptake capacity of rat heart subsarcolemmal and interfibrillar mitochondria. They found that calcium-stimulated respiration rates were about 50% greater in the interfibrillar mitochondria. The respiration rate in subsarcolemmal mitochondria was mildly elevated following the addition of low concentrations of calcium (0.05–0.4 mmol l⁻¹). At higher concentrations of calcium (0.6–0.8 mmol l⁻¹), the respiration rate in both populations of mitochondria remained elevated. In this study, no difference was found between subsarcolemmal and interfibrillar mitochondria in their calcium-stimulated respiration rates. However, there were differences in the calcium-stimulated respiration rates between muskrat and guinea pig mitochondria, with those of the muskrat having an approximately 20% larger $V_{\text{max}}$. The $V_{\text{max}}$ for muskrat mitochondria is also about 20% greater than the $V_{\text{max}}$ reported for rat heart mitochondria (Palmer et al. 1986). Guinea pig $V_{\text{max}}$ values are close to those reported for the rat. This study also showed that the concentration of calcium necessary for a sustained increase in calcium-stimulated respiration rate is about twice as great in muskrat mitochondria as in guinea pig mitochondria. Palmer et al. (1986) showed that there was an increase in citrate synthase release by mitochondria after exposure to calcium. The increase was greatest in subsarcolemmal mitochondria, with an increase from the control level of 5% of total activity to 10–30% of total activity in mitochondria that had been exposed to 0.9 mmol l⁻¹ CaCl₂. No such increase was observed in interfibrillar mitochondria, even though the respiration rate was sustained following exposure to calcium and visible structural damage was evident in electron micrographs (Palmer et al. 1986). In this study there was no increased release of citrate synthase in muskrat heart mitochondria following exposure to 1.2 mmol l⁻¹ calcium. Only at the highest concentration of calcium was there increased release of citrate synthase in guinea pig mitochondria. However, the study by Palmer et al. (1986) demonstrates that it is possible to have both physiological and structural damage to mitochondria without mitochondrial marker enzyme release. The difference observed in this study in the concentration necessary for a sustained increase in calcium-stimulated respiration suggests that the guinea pig mitochondria were more easily damaged by calcium than were the muskrat mitochondria.

The calcium uptake experiments show that there is no difference in uptake between populations of mitochondria but that calcium uptake in muskrat heart mitochondria is about 30% greater than uptake in guinea pig mitochondria. This increased uptake was most evident 40–120 s after the addition of calcium.
uptake studies using different magnesium concentrations show that 1 mmol l\(^{-1}\) Mg\(^{2+}\) has a greater inhibitory effect on calcium uptake in muskrat heart mitochondria than in guinea pig mitochondria. Magnesium is a normal constituent of intracellular fluid and has been reported to inhibit the uptake of calcium by mitochondria (Crompton et al. 1976). The exact concentration of magnesium in intracellular fluid is not known because convenient and reliable methods for its determination are lacking (Rotevan et al. 1989). Nuclear magnetic resonance studies indicate that chick heart cells have a free magnesium concentration of about 0.5 mmol l\(^{-1}\), but this concentration is also affected by changes in the intracellular concentration of calcium (Rotevan et al. 1989). The concentration of free magnesium to which the mitochondria were exposed in this study is unknown because (1) there may be contamination of the mitochondrial matrix by magnesium, even though the mitochondria were isolated in a nominally magnesium-free medium, and (2) the 1 mmol l\(^{-1}\) and 1 \(\mu\)mol l\(^{-1}\) magnesium concentrations are based on the amount of added magnesium and do not take into account magnesium binding by various negatively charged groups. It is not known if the greater calcium uptake seen in muskrat heart mitochondria in a Mg\(^{2+}\)-free medium would be offset by the greater inhibition that these mitochondria would experience in the presence of magnesium. The results of this study indicate that, under low-magnesium conditions, calcium uptake of muskrat heart mitochondria is greater than that of guinea pig mitochondria. Based on the threshold for a sustained Ca\(^{2+}\)-stimulated respiration rate, muskrat mitochondria may be able to sequester more calcium without mitochondrial damage than guinea pig mitochondria. Any differences in calcium uptake by individual mitochondria are not offset by greater numbers of mitochondria in guinea pig hearts for it appears that there are approximately equal numbers of mitochondria in muskrat hearts and in guinea pig hearts (McKean, 1990). If there were a potential for a greater in vivo uptake of calcium by muskrat heart mitochondria without damage to the mitochondria, this would be of benefit to the animal during the conditions of hypoxia and acidosis that would occur during a prolonged dive.

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References


