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Pyruvate Transport in Isolated Cardiac Mitochondria from Two Species of Amphibian Exhibiting Dissimilar Aerobic Scope: *Bufo marinus* and *Rana catesbeiana*

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ABSTRACT Cardiac mitochondria were isolated from *Bufo marinus* and *Rana catesbeiana*, two species of amphibian whose cardiovascular systems are adapted to either predominantly aerobic or glycolytic modes of locomotion. Mitochondrial oxidative capacity was compared using VO_2 max and respiratory control ratios in the presence of a variety of substrates including pyruvate, lactate, oxaloacetate, β -hydroxybutyrate, and octanoyl-carnitine. *B. marinus* cardiac mitochondria exhibited VO_2 max values twice that of *R. catesbeiana* cardiac mitochondria when oxidizing carbohydrate substrates. Pyruvate transport was measured via a radiolabeled-tracer assay in isolated *B. marinus* and *R. catesbeiana* cardiac mitochondria. Time-course experiments described both α -cyano-4-hydroxycinnamate-sensitive (MCT-like) and phenylsuccinate-sensitive pyruvate uptake mechanisms in both species. Pyruvate uptake by the MCT-like transporter was enhanced in the presence of a pH gradient, whereas the phenylsuccinate-sensitive transporter was inhibited. Notably, anuran cardiac mitochondria exhibited activities of lactate dehydrogenase and pyruvate carboxylase. The presence of both transporters on the inner mitochondrial membrane affords the net uptake of monocarboxylates including pyruvate, β -hydroxybutyrate, and lactate; the latter potentially indicating the presence of a lactate/pyruvate shuttle allowing oxidation of extramitochondrial NADH. Intra-mitochondrial lactate dehydrogenase and pyruvate carboxylase enables lactate to be oxidized to pyruvate or converted to anaplerotic oxaloacetate. Kinetics of the MCT-like transporter differed significantly between the two species, suggesting differences in aerobic scope may be in part attributable to differences in mitochondrial carbohydrate utilization.

Profound differences in the aerobic scope of anuran amphibians are well documented. Species such as *Scaphiopus hammondi* and *Bufo marinus* show large aerobic capacities, species such as *Hyla regilla* and *Rana catesbeiana* rely to a much greater extent on glycolytic production of adenosine triphosphate (ATP; Seymore, '73; Hillman, '91). Cardiovascular bases for these interspecific differences have been proposed by Hillman ('76) and Withers and Hillman ('88): increases in maximal organismal oxygen uptake (VO_2 max) are strongly correlated with increased cardiac output based on increased stroke volumes, and increased corpuscular hemoglobin concentrations and/or increased hematocrit.

Sustained increased cardiac output in species displaying greater aerobic scope requires the maintenance of high adenylate ratios via oxidative ATP production. Variation in mitochondrial oxidative capacities has been shown to correlate with interspecific differences in aerobic capacity. For example, isolated cardiac mitochondria from

tuna (highly aerobic species) and carp (less aerobic species) exhibited a two-fold difference in VO_2 max (Moyes et al., '92). It is conceivable that similar differences may be present in anuran cardiac mitochondria and provide a partial biological basis for organismal aerobic endurance. Differences in mitochondrial VO_2 max during periods of high activity may be a function of increased volume and surface densities of mitochondrial clusters (Johnston et al., '98) or differences in substrate availability and/or utilization.

Pyruvate and other monocarboxylates play a central role in aerobic, glycolytic, and post-exercise oxidative metabolism. The role of mitochondrial substrate transporters is to provide substrate at rates that sustain ATP production at levels commensurate with cellular demand. Vertebrate cardiac mitochondria generally transport and oxidize carbohydrate substrates, specifically pyruvate and lactate (Bremer, '65; Gleeson, '96; Stanley et al., '97). During burst or sustained exercise, amphibians exhibit elevated muscle and blood lactate levels (Gleeson, '96). In both reptiles and amphibians, blood lactate produced during exercise is removed and converted into glycogen by oxidative skeletal muscle fibers (Fournier and Guderley, '92; Donovan and Gleeson, 2001). In mammals, lactate produced by glycolytic tissues is either shuttled to oxidative tissues, including the heart, as a readily available energy source (Laughlin et al., '93; Brooks et al., '99) and/or converted to glycogen in the liver.

Transport of pyruvate and lactate into mitochondria has been studied extensively in mammalian tissues. Both monocarboxylates traverse the mitochondrial inner membrane in four ways: (1) via free diffusion; (2) via a monocarboxylate transporter (MCT) that acts both as a pyruvate/ H^+ or lactate/ H^+ antiporter (Poole and Halestrap, '93); (3) via a lactate/pyruvate antiporter (Valenti et al., 2002); and (4) via a pyruvate/oxaloacetate antiporter (deBari et al., 2004).

Monocarboxylate uptake via MCT proteins has been described in lizard skeletal muscle (Donovan and Gleeson, 2001). However, in *Xenopus* oocytes, pyruvate and other monocarboxylates are transported via a novel broad-specificity Na^+ -dependent mechanism (Tosco et al., 2000). Presently, there are no studies on mitochondrial transport mechanisms in adult amphibians.

Insights into the range of mitochondrial transporters in the energy metabolism of amphibian cardiac mitochondria may be obtained by a comparative study of two species of anurans

adapted to disparate modes of locomotion and exhibiting differing aerobic capacity. Our objective was to characterize the utilization of metabolic fuels in isolated cardiac mitochondria from *B. marinus* (high aerobic, low anaerobic) and *R. catesbeiana* (low aerobic, high anaerobic) by (1) evaluating mitochondrial VO_2 max in the presence of a variety of substrates, (2) describing pyruvate transport mechanisms, and (3) evaluating key intermediate enzyme activities.

METHODS AND MATERIALS

Animals

B. marinus (marine toads) and *R. catesbeiana* (bullfrogs) were purchased from commercial suppliers and maintained in glass aquaria with free access to water. Animals were used within 3 weeks of arrival and maintained on a cricket diet.

Isolation of mitochondria

Heart muscle mitochondria were isolated using a modified skeletal muscle mitochondria isolation technique (Duerr and Hillman, '91). Animals were doubly pithed and hearts quickly excised. Cardiac tissue ranging from 500 to 1000 mg was placed into ice-cold isolation buffer containing 225 mM sucrose, 20 mM TES (N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid), 5 mM EGTA (ethyleneglycol-bis- β -aminoethyl ether), and 0.5% bovine serum albumin (BSA) at pH 7.2. The final osmotic pressure of 270 mOsm was verified using a vapor pressure osmometer (Wescor Inc, Model 5500, Logan, UT). Muscle tissue was manually minced with scissors into small fragments followed by homogenization in a Potter-type glass homogenizer (GlasCol, LLC Terre Haute, IN). Large cellular debris was removed in a low-speed spin for 5 min at 500g. A crude separation of mitochondria was obtained in a 10 min high-speed spin at 10,200g. The crude mitochondrial pellet was resuspended in isolation buffer and subjected to a repeat low-speed spin at 700g to remove remaining large debris, if any, and the supernatant was centrifuged at 10,200g for 10 min to produce a final mitochondrial pellet. High levels of citrate synthase enrichment (12-fold) and minimal alkaline phosphatase enrichment (1.2-fold) indicated a relatively pure mitochondrial isolation. The citrate synthase enzyme assay was adapted from Putnam and Bennett ('83) and the alkaline phosphatase assay from Berner and Kinne ('76).

Measurement of respiration

Oxygen uptake was measured at 20°C with a Diamond General Chemical (Ann Arbor, MI) Microsensor Model 1251 calibration cell. Attached to the 0.9 mL reaction chamber was a Clark-style oxygen microelectrode, which utilized the polarographic principle to measure oxygen tension within the chamber (see Estabrook, '67). The output voltage was recorded on a Macintosh G3 computer using Warthog Systems LabHelper data acquisition software (Mark Chappell, University of California, Riverside, CA). For all assays, the reaction chamber was filled with oxygen uptake buffer consisting of 90 mM KCl, 55 mM mannitol, 20 mM sucrose, 10 mM Tris, 10 mM KH₂PO₄, and 2 mM malic acid (osmotic pressure = 270 mOsm) at variable pH adjusted with either KOH or HCl. Malic acid was used as a "sparker" substrate and because rank order comparisons are made, the endogenous rate of malate oxidation is cancelled. In combination with oxygen uptake buffer, 50 µg of mitochondria and 10 mM pyruvate (saturating) were present. Maximal oxidative activity was initiated with the addition of 4 mM ADP. Oxidative metabolism was measured as the difference in oxygen tension within the reaction chamber per minute. This rate of oxygen consumption is considered state 3 or VO₂ max. Respiratory control ratios (RCRs) were calculated as the rate of oxygen consumption in the presence of ADP (state 3) to the rate of oxygen consumption after exhaustion of ADP (state 4) or state3/state4 (Estabrook, '67).

Effect of substrate on O₂ uptake

Freshly isolated mitochondria were placed into a 0.9 mL respiration chamber with oxygen uptake medium including pyruvate. In some conditions, either 2 mM α -cyano-4-hydroxycinnamate (α -chc) or 2.5 mM lactate, β -hydroxybutyrate (β -HB), octanoyl-carnitine, or oxaloacetate was added. The addition of ADP to the medium initiated state 3 respiration or VO₂ max.

¹⁴C-pyruvate uptake

Isolated mitochondria pyruvate uptake assays were adapted from previously described radiolabeled tracer transport assays in membrane vesicles and mitochondria (Duerr and Ahearn, '96; Klein and Ahearn, '99). Assays were conducted at 20°C using mitochondria in state 4 and started by diluting mitochondria into uptake buffer containing trace amounts of ¹⁴C-pyruvate and unlabeled pyruvate. In addition, furfural, a pyruvate

dehydrogenase (PDH) inhibitor, was added to prevent loss of pyruvate as CO₂. Pyruvate uptake was terminated by placing the mitochondria into 2 mL of ice-cold stop medium (uptake medium + 2 mM α -chc and 20 mM phenylsuccinate) utilizing the Millipore filtration technique developed by Hopfer et al. ('73). Filters were then rapidly dissolved in liquid scintillation cocktail and radioactivity counted in a Beckman LS 6000SC scintillation counter. Pyruvate uptake is expressed as picomoles per milligram protein per minute. Pyruvate uptake into mitochondria was corrected for nonspecific binding by subtracting "blank" values where mitochondria and radiolabeled incubation medium were simultaneously injected into ice-cold stop medium without prior mixing (see Hopfer et al., '73).

Time course of pyruvate uptake into isolated mitochondria

Pyruvate accumulation into mitochondria was measured in the presence and absence of α -chc and phenylsuccinate over a 20 min time course at neutral pH. Uptake was initiated when mitochondria were added to uptake medium with 5 mM K-¹⁴C-pyruvate either with or without α -chc or phenylsuccinate. Mitochondria were sampled at 0.25, 1, 2, 5, 10, and 20 min, then assayed for radioactivity.

Kinetics of the anuran mitochondrial pyruvate transporters

A time course of ¹⁴C-pyruvate uptake for periods of 15, 30, 45, 60, and 120 sec was examined to determine a range over which uptake remained linear. The maximum time point for linear uptake was 60 sec. The kinetics of ¹⁴C-pyruvate uptake at 1 min as a function of external pyruvate was examined for K-pyruvate concentrations ranging from 0 to 20 mM in isolated mitochondria from both species. Mitochondria were suspended in uptake buffer and variable pyruvate concentrations either in the presence or absence of α -chc and phenylsuccinate. Final influx values reflect the difference between uptake in the presence and absence of α -chc and phenylsuccinate, and thereby both α -chc and phenylsuccinate-sensitive accumulation. At higher pyruvate concentrations, osmotic pressure was maintained at 270 mOsm by reducing sucrose concentration. To determine the effect of pH on pyruvate uptake, experiments were conducted in which pyruvate concentration was maintained at 5 mM at pH ranging from 5.3 to

8.2. Samples were taken at 1 min and assayed for radioactivity.

Effect of other carboxylates on ¹⁴C-pyruvate transport by anuran cardiac mitochondria

Isolated anuran cardiac mitochondria were placed into uptake medium including 5 mM ¹⁴C-pyruvate and in some cases either α -chc (1 mM), K-lactate (2 mM), K-oxaloacetate (2 mM), or K- β -HB (2 mM) were also present. Samples were taken at 1 min and assayed for radioactivity.

Enzyme assays

Briefly, frozen mitochondrial pellets were thawed and resuspended in a potassium buffer (oxygen uptake buffer). Measurements of PDH activity were conducted based on a protocol described by Robertson et al. ('86). Aliquots (100 μ g) of solubilized mitochondria were suspended in assay medium producing a final reaction mixture containing (in mM) 50 Hepes (pH 7.8), 2.5 NAD⁺, 0.5 acetyl-CoA, 0.5 thiamine pyrophosphate, 0.5 dithiothreitol, 5 MgCl₂, and 5 pyruvate in a final volume of 800 μ L. Enzyme activity is indirectly inhibited by furfural and was measured by following the furfural-sensitive accumulation of NADH at 340 nm in a Fisher Scientific (Pittsburgh, PA) SpectroMaster Model 715 at 20°C. Lactate dehydrogenase (LDH) and catalase (CAT) activities were determined using methods described in Bergmeyer ('63). Briefly, CAT was measured in an H₂O₂-phosphate buffer at pH 7. Enzymatic degradation of H₂O₂ was monitored at 240 nm. The LDH-dependent decrease in NADH was measured at 340 nm in a pyruvic acid-phosphate buffer at pH 7.4. Cytochrome *c* oxidase (COX) activity was determined based on the method of Hodges and Leonard ('74) using a phosphate buffer with reduced cytochrome *c*. The oxidation of cytochrome *c* in the presence of solubilized mitochondria was measured at 550 nm. Pyruvate carboxylase (PC) activity was determined using the method of Payne and Morris ('69), in which the oxidation of DTNB (5,5'-dithio-bis (2-nitrobenzoic acid)) by liberated acetylCoA was measured at 412 nm.

Statistical comparisons

All respiratory and enzyme rates were normalized relative to total protein (determined using the BioRad Protein Assay, BioRad Laboratories, Hercules, CA) to accommodate differences in preparatory yield. Statistical analyses were

conducted using SPSS v11.0 (Chicago, IL) for Macintosh. Each experiment was repeated two to three times using mitochondria from different animals. Either four or five replicate measures (*n*) for each treatment in each experiment were obtained. Significant differences between species and among treatments were determined using two-way analysis of variance (ANOVA) with repeated measures followed by Tukey's honestly significant differences (HSD) post hoc tests for interspecific comparisons, unless otherwise noted. A value of *P* < 0.05 was considered significant.

Chemicals and radionuclides

Common reagents, organic acids, phenylsuccinate, and α -chc were obtained from Sigma Chemical Co. (St. Louis, MO). All organic acids were neutralized with the addition of KOH and used as potassium salts. ¹⁴C-pyruvate and scintillation cocktail were obtained from Amersham Biosciences (Piscataway, NJ).

RESULTS

Purity and functional integrity of isolated mitochondria

Activities of citrate synthase, a mitochondria-specific enzyme, and CAT, an enzyme specific to peroxisomes (a common co-isolate of mitochondrial fractions) are displayed in Table 1. Citrate

TABLE 1. Initial biochemical and physiological characteristics of isolated anuran cardiac mitochondria

	<i>Bufo marinus</i>	<i>Rana catesbeiana</i>
VO ₂ max ^a	144 ± 10.2	67 ± 3.5
RCR ^b	7.6 ± 1.3	7.8 ± 1.1
Citrate synthase		
Homogenate ^c	0.239 ± 0.04	0.314 ± 0.04
Mitochondrial pellet ^c	2.9 ± 0.3	3.3 ± 0.4
Enrichment factor ^d	12.1	10.5
Catalase		
Homogenate ^c	395 ± 31	286 ± 20
Mitochondrial pellet ^c	293 ± 29	246 ± 17
Enrichment ^d	0.74	0.86

^aVO₂ expressed as nanomoles O₂/mg protein per minute, measured at pH 6.9 with pyruvate as substrate, means significantly different (*P* < 0.05).

^bMeasured as state 3/state 4 as described in Estabrook ('67).

^cEnzyme activities in micromoles product released per milligram protein per minute and are mean ± SEM, *n* = 3.

^dValues indicate difference in activity relative to crude homogenates and are mean ± SEM, *n* = 3–9.

synthase is enriched by 10–12-fold, whereas CAT enrichment is less than one indicating a relatively pure preparation of mitochondria. Mitochondrial integrity was assessed by RCR values, which varied between 6 and 8. Higher RCRs corresponded with the use of carbohydrate substrates.

Isolated cardiac mitochondria utilize a variety of substrates

Figure 1 illustrates oxygen consumption rates of mitochondria from both anuran species in the presence of a variety of substrates. Measurements were subjected to analysis by two-way ANOVA revealing significant differences between the two species and with substrate. However, there was also evidence of a species \times substrate interaction. Though the pattern of response to changes in substrate differed between the two species, interactions make post hoc tests inappropriate. Therefore, each species was examined independently using one-way ANOVA followed by Tukey's HSD.

For *B. marinus*, pyruvate, lactate and β -HB sustained oxygen consumption at equivalent rates relative to each other and at rates that were statistically greater rates relative to octanoyl-carnitine and oxaloacetate. For *R. catesbeiana*, all substrates sustained oxygen consumption at statistically equivalent rates with the exception of one significant difference between β -HB and oxaloacetate. Furthermore, toad heart mitochondria exhibited a significantly greater respiratory rate relative to *R. catesbeiana* heart mitochondria when provided with either pyruvate, lactate, or β -HB, but not with octanoylcarnitine or oxaloacetate.

We elected to closely examine pyruvate uptake as this substrate is oxidized at high rates in many tissues and results could be compared with a variety of other tissues and organisms. Initial uptake experiments (Fig. 2) indicated pyruvate uptake into isolated mitochondria is linear for the first minute, thus establishing a time point for subsequent kinetic assays.

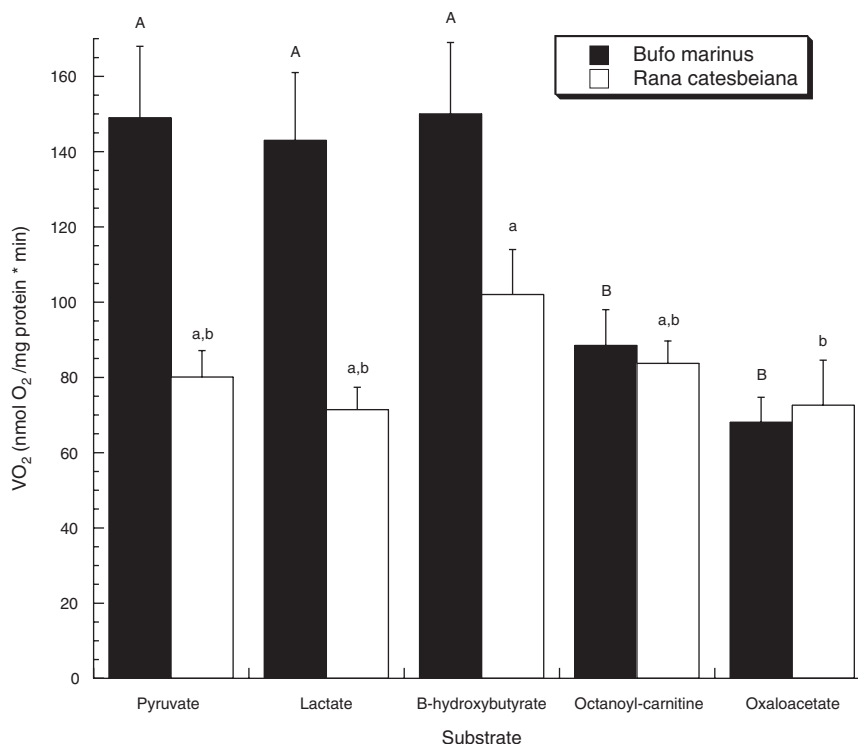


Fig. 1. Maximal VO₂ performance of isolated *Bufo marinus* and *Rana catesbeiana* heart muscle mitochondria as a function of substrate. Isolated mitochondria were placed into a 0.9 mL reaction chamber fitted with a Clark-style oxygen microelectrode and filled with oxygen uptake buffer with one of five possible substrates. Oxygen consumption was monitored following the addition of ADP. O₂ consumption rates were collected and calculated using Warthog Data-Acquisition software on a Power Macintosh. Values are mean \pm SEM ($N = 5$). In a given cell of the figure, columns with different alphabets are significantly different (uppercase = *B. marinus*, lowercase = *R. catesbeiana*).

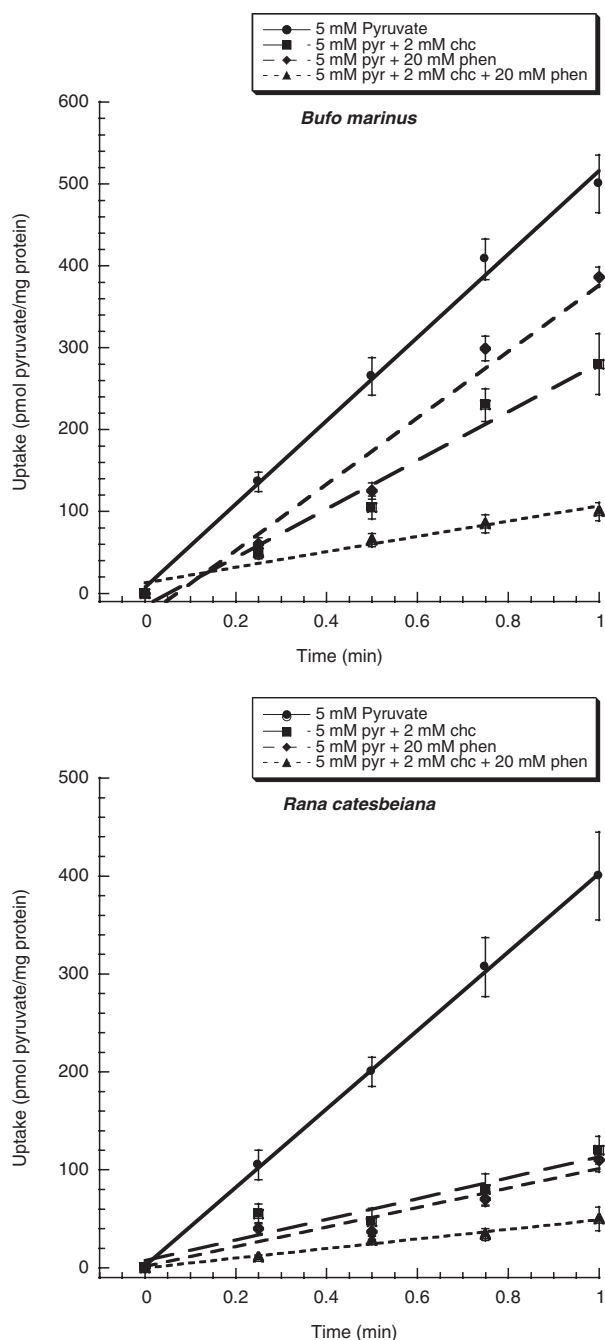


Fig. 2. Linearity of ^{14}C -pyruvate uptake by anuran cardiac muscle mitochondria during a 1-min interval. Mitochondria isolated from *Bufo marinus* (upper panel) and *Rana catesbeiana* (lower panel) were incubated for 0.25, 0.50, 0.75, and 1.0 min time intervals in an uptake medium containing 225 mM sucrose, 20 mM Tris, 5 mM ethyleneglycol-bis- β -aminoethyl ether, and 0.5% bovine serum albumin, pH 7.0, and 5 mM radiolabeled K-pyruvate either with or without 2 mM α -cyano-4-hydroxycinnamate or 20 mM phenylsuccinate. Values are mean \pm SEM, $N = 3$. Linearity of data was determined using linear curve fitting software (Kaleidagraph 4.0.2). R^2 values ranged between 0.94 and 0.99 for data obtained from both species.

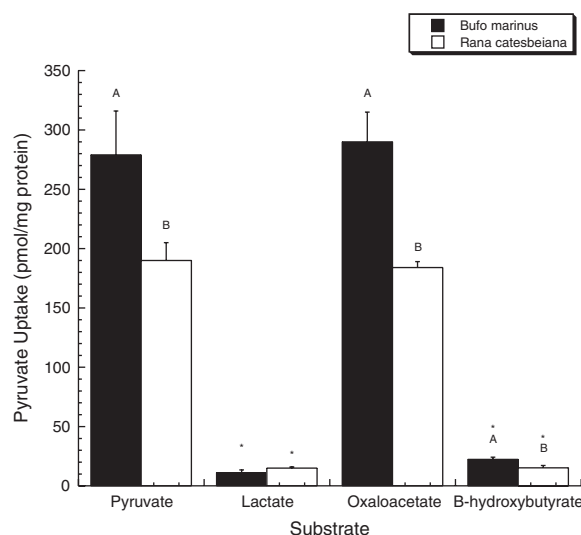


Fig. 3. Cis-inhibition of ^{14}C -pyruvate uptake by anuran cardiac muscle mitochondria. Mitochondria isolated from *Bufo marinus* and *Rana catesbeiana* were incubated in uptake medium for 60 sec containing 5 mM radiolabeled K-pyruvate and in some cases 50 mM of the following K-salts: lactate, oxaloacetate, or β -hydroxybutyrate. Data for both plots represent mean \pm SEM, $N = 5$. In a given cell of the figure, different letters are significantly different. Among cells, asterisks indicate significant difference from pyruvate uptake.

Effect of other carboxylates on pyruvate transport by anuran cardiac mitochondria

Figure 3 illustrates a significant reduction (two-way ANOVA, Tukey HSD, $P < 0.05$) in the mitochondrial accumulation of pyruvate in both species when coincubated with either lactate or β -HB (monocarboxylates) but not oxaloacetate (a dicarboxylate). These data suggest that lactate, β -HB and pyruvate may share one or more transport mechanisms. Because monocarboxylates have previously been shown to be transported via multiple carriers (Passarella et al., '85), we predicted that pyruvate uptake in anuran mitochondria was mediated by at least two different transport mechanisms.

Carrier-mediated transport of pyruvate occurs in anuran cardiac mitochondria

Pyruvate accumulation was measured over a 20-min period in the presence and absence of α -chc and phenylsuccinate, both known inhibitors of pyruvate-specific MCTs in mammalian heart mitochondria (Halestrap and Denton, '74; Valenti et al., 2002). Pyruvate entry into isolated mitochondria exhibited a mild overshoot at 5 min

(Figs. 4a and b). Application of either α -chc or phenylsuccinate abolished overshoots and pyruvate entered at an attenuated rate. Pyruvate uptake in the presence of both inhibitors was essentially linear, indicating pyruvate entry via passive diffusion solely. These data strongly suggest the presence of two pharmacologically distinct facilitative transport mechanisms for pyruvate.

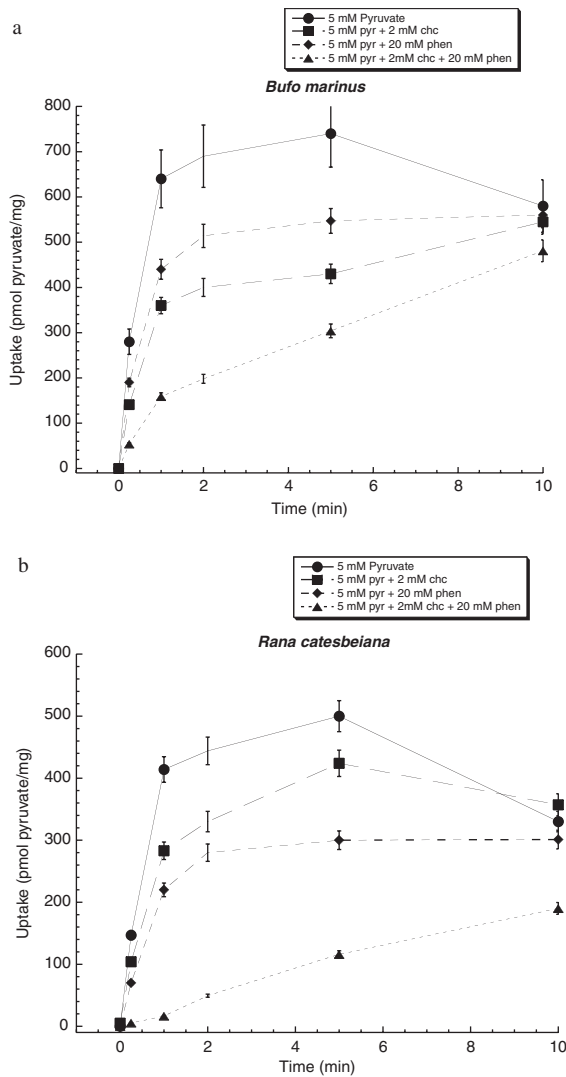


Fig. 4. Time course of ^{14}C -pyruvate uptake by anuran cardiac muscle mitochondria. (a) Mitochondria isolated from *Bufo marinus* were incubated in an uptake medium containing 225 mM sucrose, 20 mM Tris, 5 mM ethyleneglycol-bis- β -aminoethyl ether, and 0.5% bovine serum albumin, pH 7.0, and 5 mM radiolabeled K-pyruvate either with or without 2 mM α -cyano-4-hydroxycinnamate or 20 mM phenylsuccinate. Values are mean \pm SEM, $N = 5$. (b) Mitochondria isolated from *Rana catesbeiana* and treated as described for (a).

Kinetics of the anuran mitochondrial pyruvate transporters

Pyruvate influx occurring via both mechanisms could be described as a function of two distinct saturable carriers exhibiting Michaelis-Menten kinetic characteristics. These systems could be quantitatively described by the following equation:

$$J_{\text{pyr}} = J_{\text{max}} \times [\text{Pyr}] / \{K_t + [\text{Pyr}]\} \quad (1)$$

where J_{pyr} is the inward pyruvate flux in picomoles per milligram protein per minute, J_{max} is the maximal carrier-mediated pyruvate flux, and K_t is the concentration of pyruvate resulting in $0.5 J_{\text{max}}$ and $[\text{pyr}]$ is the pyruvate concentration (in millimolar). The hyperbolic curve in Figures 5a and b represents α -chc-sensitive carrier-mediated pyruvate uptake calculated using an iterative curve-fitting algorithm in Kaleidagraph v4.02 and gives the following kinetic constants: $K_t = 1.8 \pm 0.2 \text{ mM}$ (*B. marinus*) and $0.95 \pm 0.07 \text{ mM}$ (*R. catesbeiana*), $J_{\text{max}} = 298 \pm 11 \text{ pmol mg}^{-1} \text{ protein per minute}$ (*B. marinus*) and $193 \pm 34 \text{ pmol mg}^{-1} \text{ protein per minute}$ (*R. catesbeiana*). Both the K_t and J_{max} values are significantly different between the two species (Student's t -test, $n = 5$). The hyperbolic curve in Figures 6a and b represents the phenylsuccinate-sensitive carrier-mediated pyruvate uptake which exhibits a $K_t = 2.3 \pm 0.7 \text{ mM}$ (*B. marinus*) and $2.3 \pm 0.4 \text{ mM}$ (*R. catesbeiana*), $J_{\text{max}} = 411 \pm 49 \text{ pmol mg}^{-1} \text{ protein per min}$ (*B. marinus*) and $391 \pm 25 \text{ pmol mg}^{-1} \text{ protein per min}$ (*R. catesbeiana*).

Effect of external pH on pyruvate influx

Freshly isolated mitochondria were added to isolation buffer including ^{14}C -pyruvate at variable pH ranging from 6.2 to 8.2 in the presence and absence of α -chc. Plotted in Figures 7a and b are the α -chc-sensitive uptake rates as a function of extramitochondrial pH. A stimulatory relationship between pyruvate uptake and pH is observed in which acidic conditions increase substrate transport. The data were curve-fitted according to the Hill equation:

$$J_{\text{pyr}} = J_{\text{max}} [1 + (10^{\text{pHe}} / 10^{\text{pK}})^n] \quad (2)$$

where J_{pyr} is the inward pyruvate flux and J_{max} is the maximal carrier-mediated pyruvate flux in picomoles per milligram protein, pK is the pH at which pyruvate flux is $0.5 J_{\text{max}}$, and n is the Hill coefficient for inhibition. The kinetic constants calculated using Kaleidagraph v4.02 give the

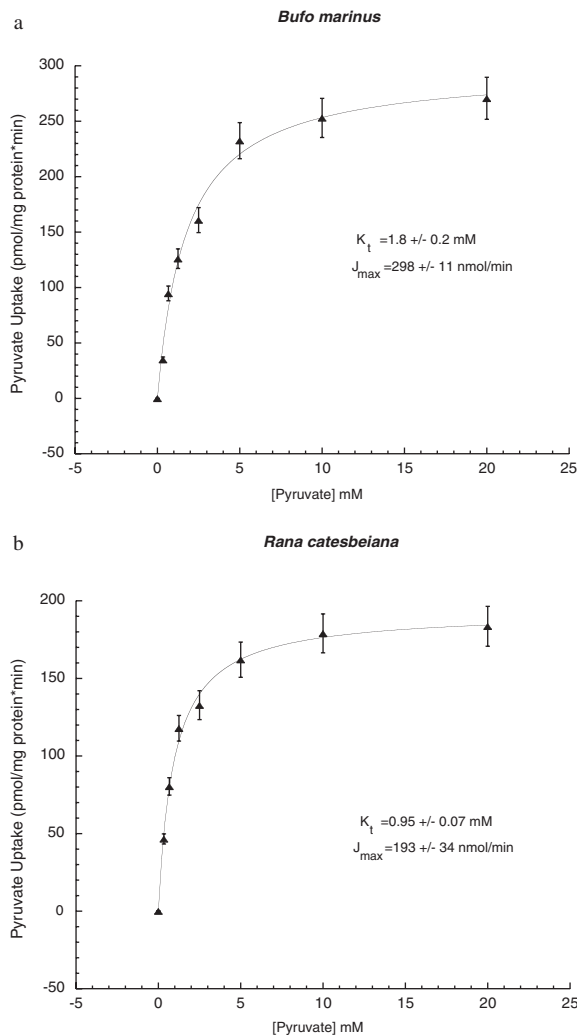


Fig. 5. ^{14}C -pyruvate uptake kinetics for the α -chc-sensitive transporter in anuran cardiac muscle mitochondria. (a) Mitochondria isolated from *Bufo marinus* were incubated for 60 sec in phenylsuccinate uptake medium at pH 7 containing concentrations of radiolabeled K-pyruvate ranging from 0 to 20 mM either with or without α -cyano-4-hydroxycinnamate. (b) Mitochondria isolated from *R. catesbeiana* and treated as described for *B. marinus*. Data for both plots represent mean \pm SEM, $N=4$, and the hyperbolic curve and kinetic constants were calculated using iterative curve-fitting software (KaleidaGraph 4.0.2).

following values: $\text{pK} = 7.2 \pm 0.1$ for *B. marinus* and $\text{pK} = 7.1 \pm 0.1$ for *R. catesbeiana*. Hill coefficients of 4.0 ± 0.5 and 2.3 ± 0.3 were calculated for *B. marinus* and *R. catesbeiana*, respectively. Plotted in Figures 8a and b are the phenylsuccinate-sensitive uptake rates as a function of extramitochondrial pH. An inhibitory relationship between pyruvate uptake and pH is observed in which acidic conditions reduce substrate transport. For *B. marinus* pK is 6.0 ± 0.3 and

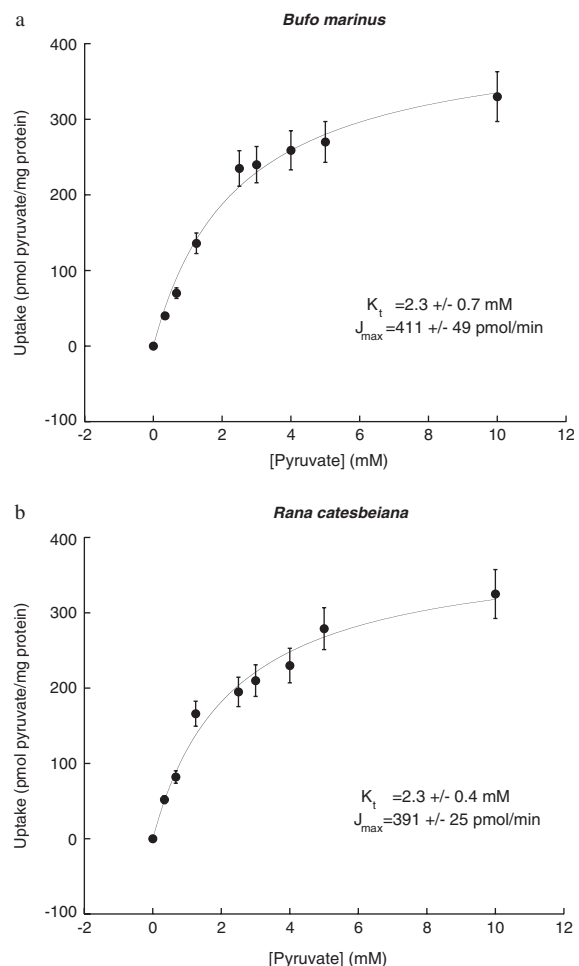


Fig. 6. ^{14}C -pyruvate uptake kinetics for the phenylsuccinate-sensitive pyruvate transporter in anuran cardiac muscle mitochondria. (a) Mitochondria isolated from *Bufo marinus* were incubated for 60 sec in α -cyano-4-hydroxycinnamate uptake medium at pH 7 containing concentrations of radiolabeled K-pyruvate ranging from 0 to 10 mM either with or without phenylsuccinate. (b) Mitochondria isolated from *Rana catesbeiana* and treated as described for *B. marinus*. Data for both plots represent mean \pm SEM, $N=4$, and the hyperbolic curve and kinetic constants were calculated using iterative curve-fitting software (KaleidaGraph 4.0.2).

6.8 ± 0.1 for *R. catesbeiana*. Phenylsuccinate-sensitive pyruvate transport displayed a Hill coefficient of 2.6 ± 0.6 and 2.0 ± 0.4 for *B. marinus* and *R. catesbeiana*, respectively. The pK values for *B. marinus* and *R. catesbeiana* were not significantly different (Student's t test, $n=5$).

Enzyme activities in anuran cardiac mitochondria

The activities of four enzymes were measured to examine their potential role with that of the

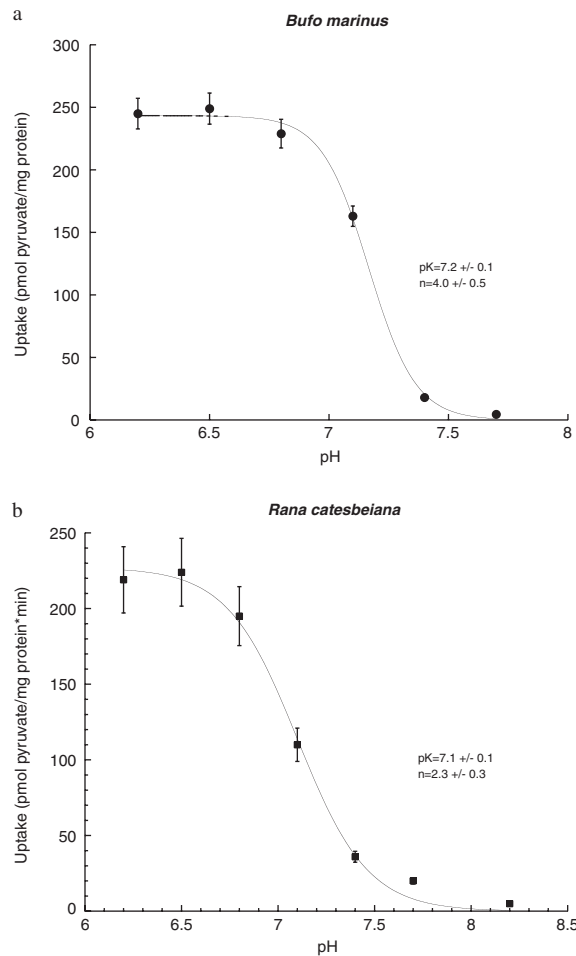


Fig. 7. Effect of extramitochondrial pH on ^{14}C -pyruvate uptake for the α -chc-sensitive transporter in anuran cardiac muscle mitochondria. (a) Mitochondria isolated from *Bufo marinus* were incubated for 60 sec in phenylsuccinate-uptake medium containing 5 mM radiolabeled K-pyruvate at pH ranging from 6.0 to 7.6 either with or without α -cyano-4-hydroxycinnamate. (b) Mitochondria isolated from *Rana catesbeiana* and treated as described for *B. marinus* at pH ranging from 6.2 to 8.2. Data for both plots represent mean \pm SEM, $N=4$, and the hyperbolic curve and kinetic constants were calculated using iterative curve-fitting software (KaleidaGraph 4.0.2).

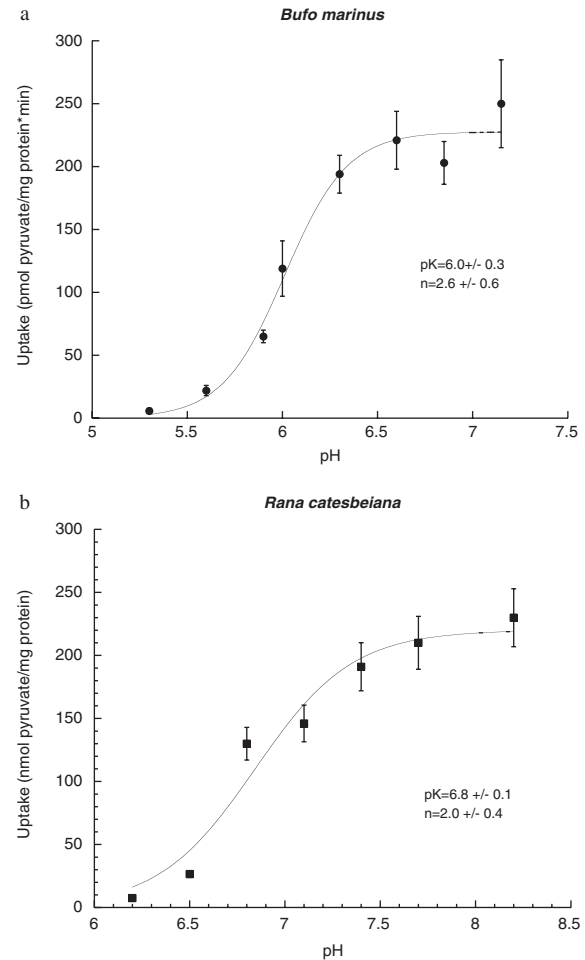


Fig. 8. Effect of extramitochondrial pH on ^{14}C -pyruvate uptake kinetics for the phenylsuccinate-sensitive pyruvate transporter in anuran cardiac muscle mitochondria. (a) Mitochondria isolated from *Bufo marinus* were incubated for 60 sec in α -chc-uptake medium containing 5 mM radiolabeled K-pyruvate at pH ranging from 5.3 to 7.0 either with or without α -cyano-4-hydroxycinnamate. (b) Mitochondria isolated from *Rana catesbeiana* and treated as described for *B. marinus* at pH ranging from 6.2 to 8.4. Data for both plots represent mean \pm SEM, $N=4$, and the hyperbolic curve and kinetic constants were calculated using iterative curve-fitting software (KaleidaGraph 4.0.2).

DISCUSSION

two pyruvate transporters, which may underlie differences in aerobic scope of the two species. The activities of PDH, COX, LDH, and PC are presented in Table 2. All four enzymes were highly expressed in mitochondria isolated from both species and exhibited comparable activities. There were no interspecific differences in enzyme activity, however, the activity of LDH and PC were significantly higher than that of PDH.

Aerobic metabolism in amphibians varies with individual species and is strongly associated with behavioral patterns. Foraging semi-terrestrial toads including *B. marinus* exhibit higher organismal VO_2 max relative to that of *R. catesbeiana*, which demonstrates sudden and brief bursts of activity when evading potential predators (Hillman, '76). This investigation describes two distinct pyruvate transporters in isolated anuran cardiac mitochondria and demonstrates

TABLE 2. Enzyme activities measured in isolated anuran cardiac mitochondria

Animal	Pyruvate dehydrogenase	Cytochrome c oxidase	Lactate dehydrogenase	Pyruvate carboxylase
<i>Bufo marinus</i>	6.3 ± 0.7	8.9 ± 0.9	24 ± 3	13.2 ± 2.6
<i>Rana catesbeiana</i>	5.9 ± 0.4	9.1 ± 1.1	34 ± 10	17.1 ± 1.1

Values are mean ± SEM, $N = 3-5$.

Enzyme activities in micromoles product released per milligram protein per minute.

differences in carbohydrate, ketone body, and fatty acid substrate utilization during aerobic metabolism.

Mitochondrial VO_2 max was measured using a variety of substrates generally utilized by cardiac tissue. Pyruvate, lactate, and β -HB were oxidized at consistently higher rates compared with octanoyl-carnitine and oxaloacetate in *B. marinus* mitochondria (Fig. 1). *R. catesbeiana* mitochondria preferred β -HB only when compared with oxaloacetate, otherwise oxidation rates among carbohydrates and octanoyl-carnitine were similar. Anuran cardiac mitochondria display substrate preference patterns that are consistent with previous findings in fish heart tissue (Sidell et al., '87), which utilized carbohydrate or fatty acid fuels, and elasmobranch heart tissue (Moyes et al., '90), which preferred either pyruvate or β -HB to meet maximal energetic demands. This is in contrast with the aerobic turtle heart which preferentially extracts free fatty acids even in the presence of exogenous glucose (Brachfeld et al., '72). Mitochondrial VO_2 max in *B. marinus* was measured at rates twice that of *R. catesbeiana* mitochondria in the presence of either pyruvate or lactate, and based on citrate synthase activities mitochondrial densities in frog and toad heart are predicted to be equivalent. Taken together, these data suggest interspecific differences in carbohydrate and fatty acid oxidative metabolism in anuran cardiac mitochondria.

Pyruvate transport into actively respiring mitochondria is mediated by more than one transmembrane-spanning carrier protein. The earliest description of a pyruvate transporter was by Halestrap and Denton ('74) in rat liver mitochondria. Pyruvate was shown to be cotransported with a proton into the mitochondrial matrix and specifically inhibited by α -chc. In addition to pyruvate, the carrier was also shown to transport lactate, acetoacetate, β -HB, and other monocarboxylates (Halestrap, '78) and named the mitochondrial MCT (mMCT). Passarella et al. ('85) described a second pathway for pyruvate uptake via a phenylsuccinate-sensitive pyruvate/oxaloac-

tate antiporter in rat kidney mitochondria. Pyruvate influx into mammalian mitochondria has also been demonstrated via a pyruvate/malate antiporter and pyruvate efflux can occur via a pyruvate/lactate antiporter (deBari et al., 2004). Our studies indicate that pyruvate transport into anuran amphibian cardiac mitochondria is accommodated by at least two distinct saturable transporters: one α -chc-sensitive and the other phenylsuccinate-sensitive (Figs. 4a and b). To our knowledge, this is the first description of pyruvate transport into amphibian mitochondria.

The α -chc-sensitive transporter described for *B. marinus* and *R. catesbeiana* exhibited a $K_t = 1.8$ and 0.95 mM, respectively. Both anuran constants are higher than kinetic constants (0.15 mM) reported for the mammalian mitochondrial pyruvate transporter (Halestrap, '75), but do fall within the range ($0.63-8.5$ mM) reported for mammalian plasma membrane pyruvate transporters (see Poole and Halestrap, '93). This transporter is likely an amphibian mMCT, orthologous to the mammalian transporter. The anuran mMCT exhibits kinetic parameters reflecting the aerobic capacities of the mitochondria: the anuran mMCT in *R. catesbeiana* only transports pyruvate at 65% the rate of toad potentially acting as a limiting factor in aerobic metabolism. Alternatively, this may imply that although pyruvate is oxidized at maximal rates in both species, pyruvate may not be the predominant or sole substrate in vivo. The phenylsuccinate-sensitive transporter described for *B. marinus* and *R. catesbeiana* exhibited a $K_t = 2.3 \pm 0.7$ and 2.3 ± 0.4 mM, respectively. The J_{\max} values were greater in both species relative to the mMCT (411 ± 49 pmol mg^{-1} protein per minute for *B. marinus* and 391 ± 25 pmol mg^{-1} protein per minute for *R. catesbeiana*).

Our data suggest that lactate and β -HB may compete directly with pyruvate as substrate for the mMCT and that lactate may also enter the mitochondrion via exchange with pyruvate via a second transporter. First, a significant reduction in pyruvate uptake was recorded in the presence

of lactate especially, and β -HB (Fig. 3). Second, we have detected both an α -chc-sensitive uptake mechanism (Figs. 4a and b) that is stimulated by an inwardly directed pH gradient (Figs. 7a and b) (mMCT) and a phenylsuccinate-sensitive transporter which has been described in rat heart mitochondria as a lactate/pyruvate exchanger. Given these data, we propose that β -HB, pyruvate, and lactate are specifically transported into cardiac mitochondria and are important fuels for the amphibian heart.

β -HB transport into cardiac mitochondria can provide acetyl-CoA when rates of glycolysis are low. The high rate of oxygen consumption exhibited by cardiac mitochondria in the presence of β -HB identifies it as a significant source of energy in amphibians. This result is consistent with rat, pigeon, and turtle heart, which utilize β -HB at high rates (Moyes et al., '90). Pyruvate represents a more common source of acetyl-CoA and is the end product of glycolysis, which is located in the cytosol. Pyruvate must then be transported to the mitochondrial matrix where it is completely oxidized in the tricarboxylic acid (TCA) cycle when P_{O_2} levels are adequate. During periods of activity, P_{O_2} levels may fall below a critical level and some tissues will rely on glycolytic production of ATP, thereby producing lactate as a metabolic end product.

The fate of lactic acid in vertebrates is varied. In mammals, blood lactate is both converted to glycogen in the liver (Con cycle) and oxidized in skeletal muscle (Brooks, '98). Brooks et al. ('99) further delineate a role for mitochondrial LDH and lactate oxidation as comprising an intracellular "lactate shuttle", which balances the production of lactate by glycolytic tissues during exercise and oxidation of lactate by oxidative skeletal and/or heart muscle. In reptiles, the liver does not participate significantly in the clearance of blood lactate, but instead muscle glycogen replenishment is favored (Gleeson and Dalessio, '89). Similarly, the liver plays a negligible role in lactate disposal in amphibians (Fournier and Guderley, '92). Lactic acid produced by glycolytic muscle fibers is exported into the extracellular space and subsequently transported into oxidative skeletal muscle, where it is either oxidized or converted into glycogen during recovery (Fournier and Guderley, '92; Gleeson, '96; Withers et al., '88).

Coincident with increased blood lactate levels during and following exercise, lactate is found to have a stimulatory effect on turtle (Watson et al.,

'94) and toad (Pinz and Portner, 2002) myocyte metabolic rate. Both investigations provide evidence that blood lactate is transported into cardiac cells, an event followed by a transient rise in metabolic rate and postulate that lactate entry into myocytes is accompanied by increased Na^+/H^+ exchange and therefore increase Na^+/K^+ ATPase activity. Lactate may be oxidized following conversion to pyruvate by LDH thereby providing a metabolic fuel. Furthermore, organs in the freeze-tolerant frog *Rana sylvatica* survive hypoxia-anoxia use of glycolytic energy production. Maximal lactate concentrations accumulated during freezing were found in heart and brain, suggesting either a slow clearance or active utilization of lactate flushed out of other tissues (Storey, '87). We detected substantial LDH activity in *B. marinus* and *R. catesbeiana* cardiac mitochondria. Our data indicate anuran cardiac mitochondria can effectively transport and readily interconvert pyruvate and lactate, reflecting their substrate availability and usage in vivo.

The mMCT and the putative pyruvate/lactate antiporter may act as a pyruvate-lactate-NADH shuttle, in which electrons associated with cytosolic NADH are brought into the mitochondria. In the presence of elevated cytosolic NADH, extramitochondrial pyruvate is converted to lactate via LDH, which is subsequently exchanged for intramitochondrial pyruvate. Lactate is then reconverted to pyruvate yielding intramitochondrial NADH. A similar model of such a shuttle has been proposed for rat heart mitochondria by Valenti et al. (2002).

It is interesting to note that in *R. catesbeiana*, the K_t of the phenylsuccinate-sensitive pyruvate transporter (2.3 mM) is considerably higher than the K_t of the mMCT (0.95 mM). This suggests that the mMCT is a high affinity low rate transporter that may be the primary pyruvate transporter in operation when cardiac workloads are lighter. The low affinity high rate phenylsuccinate-sensitive pathway would exhibit increased activity when extramitochondrial lactate concentrations increase during or immediately following exercise. In both species, high mitochondrial LDH activity is consistent with a high volume of NADH transfer via the "lactate shuttle" and with a lower mitochondrial NAD/NADH ratio during normoxic periods of high ATP turnover. The combined action of both transporters and intramitochondrial LDH provides a potentially effective mechanism by which lactate may be oxidized by anuran heart tissue.

The oxidation of acetyl-CoA to CO₂ is the main function of the TCA cycle. However, intermediates in the TCA cycle also participate in biosynthetic reactions and as a consequence, TCA intermediates (TCAI) are in a constant state of flux with 2-oxoglutarate, malate, and oxaloacetate possessing the lowest concentrations. Anaplerosis is defined as the replenishment of TCAI. PC, which synthesizes oxaloacetate from pyruvate, is the major anaplerotic enzyme in rat heart mitochondria. According to Compton et al. ('97), a significant source of oxaloacetate in rat heart mitochondria is pyruvate. In the working rat heart, changes in TCAI precede functional declines, which are rapidly reversed upon addition of anaplerosis substrate (Gibala et al., 2000; Panchal et al., 2000). The activity of PC in isolated anuran cardiac mitochondria suggests that anaplerosis is potentially an important aspect of mitochondrial oxidative physiology. Though toads have been shown exhibit greater cardiovascular endurance (Hillman, '91) and resistance to dehydration (Hillman, '87), differences in PC levels between *B. marinus* and *R. catesbeiana* are not apparent. The concomitant transport of lactate and pyruvate via the anuran mMCT provides acetate via LDH and PDH, as well as oxaloacetate via PC, to the TCA. During periods of fat breakdown, β -HB could serve as a major source of acetate in heart when endogenous levels of glycolysis are low and exogenously produced lactate could serve to replenish oxaloacetate. Thus, the anuran heart manifests a metabolic profile enabling continuous operation in a variety whole-animal physiological states.

The effect of an imposed extramitochondrial pH gradient (inside alkaline) accelerated pyruvate uptake by the mMCT suggesting pyruvate-proton symport, consistent with the secondary active transport mechanism described for the mMCT in mammals (Halestrap, '75). Although no difference exists between K_t values, the Hill coefficient for the mMCT in *B. marinus* (4.0) is nearly twice that relative to *R. catesbeiana* (2.3). One consequence of a higher Hill coefficient for *B. marinus* is a greater rate of pyruvate transport at neutral pH, consistent with the greater J_{\max} exhibited by the mMCT in *B. marinus*. In contrast, as the pH is lowered from 7.6 to 5.3, transport by the putative pyruvate/lactate antiporter is inhibited. Valenti et al. (2002) originally reported a similar decline in lactate/pyruvate transport between pH 6 and 8. The sensitivity of pyruvate/lactate antiport to acidic conditions is greater in *R. catesbeiana*

relative to *B. marinus*. In *R. catesbeiana*, transport activity is half maximal at pH 6.8, whereas in *B. marinus* half-maximal activity is achieved at pH 6.0. During exercise, both blood CO₂ and lactate levels increase and cardiac contractility has been shown to decline in *Bufo arenarum* (Mattiuzzi and Cingolani, '77) at intracellular pH <7.1. During exhaustive exercise, *Rana pipiens* has been shown to exhibit both a blood and intracellular cardiac myocyte lactate concentration equal to 10.6 mM (Warren and Jackson, 2005) suggesting profuse entry of lactic acid into cardiac myocytes. In *Rana temporaria* skeletal muscle, fatiguing stimulation produced an intracellular lactate concentration of 18 mM and an intracellular pH of between 6.3 and 6.7 (Usher-Smith et al., 2006). These data are interesting in light of the observed pH dependence of the pyruvate/lactate (NADH shuttle) transporter. In *B. marinus*, pyruvate/lactate antiporter rates do not begin to diminish sharply until pH <6.5 and for *R. catesbeiana*, the pyruvate/lactate antiporter is functioning only at 15% of maximal pH 6.5, within the pH range reported for fatigued skeletal muscle *R. temporaria*. Taken together, these data suggest a potential correlation, though not necessarily causal, between pyruvate/lactate antiporter activity and cardiac performance in *B. marinus* and *R. catesbeiana*. It should be noted that the negative inotropic effect of hypercapnic acidosis on the mammalian heart has been shown to be the result of decreased myofilament responsiveness to Ca²⁺ (Orchard and Kentish, '90). However, if the pyruvate/lactate antiporter is also acting as an NADH shuttle, it may be playing an important role in the maintenance of the intramitochondrial redox potential.

In summary, our investigation into the role of mitochondrial transporters in the energy metabolism of amphibian cardiac mitochondria has led to the identification of two pyruvate transporters located on the inner mitochondrial membrane. One transporter closely resembles the mitochondrial α -chc-sensitive MCT described previously in rat heart mitochondria, the other transporter is a phenylsuccinate-sensitive transporter that is likely functioning as a pyruvate/lactate antiporter. In addition to PDH and COX, anuran cardiac mitochondria also exhibit LDH and PC activity. The presence of these two enzymes provides for the reduction of lactate to pyruvate and the carboxylation of pyruvate to oxaloacetate, respectively. Oxidation of pyruvate, lactate, and β -HB are comparably high relative to oxaloacetate and octanoyl-carnitine, except in *R. catesbeiana* where

octanoyl-carnitine was also oxidized at comparably high rates. Our data also suggest that the anuran heart could utilize lactate from glycolytic tissues via a lactate shuttle mechanism. Anuran mitochondria also have the capability of replenishing oxaloacetate via anaplerosis, thereby preventing potential declines in cardiac contractility during times of greater flux in the TCA intermediate pool. The kinetics of the MCT-like transporter differed significantly between the two species with *B. marinus* exhibiting a higher pyruvate transport maximum, suggesting that if substrate availability is non-limiting, differences in aerobic scope may be partially attributable to differences in substrate delivery mechanisms of mitochondria.

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