Erratum

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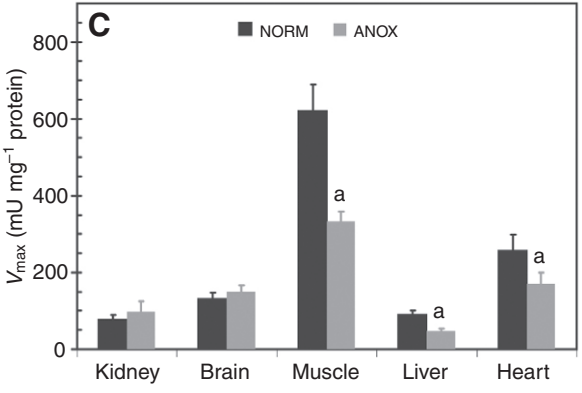
In Fig. 1C, the bars depicting V_{max} values for kidney and brain should not have had significance indicators on them. These were accidentally introduced during the production process. As stated in the Results and Discussion of the paper, the only tissues in which V_{max} values differed between normoxia and anoxia were muscle, liver and heart.

The correct version of the figure is shown below.



This error does not affect the results or conclusions of the paper.

We apologise to authors and readers for this error.

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Regulation of sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) in turtle muscle and liver during acute exposure to anoxia

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SUMMARY

The freshwater turtle *Trachemys scripta elegans* naturally tolerates extended periods of anoxia during winter hibernation at the bottom of ice-locked ponds. Survival in this anoxic state is facilitated by a profound depression of metabolic rate. As calcium levels are known to be elevated in anoxic turtles, and ion pumping is an ATP-expensive process, we proposed that activity of the sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) would be reduced in muscle and liver of *T. s. elegans* during acute (up to 20 h) exposure to anoxia. SERCA activity decreased ~30% in liver and ~40% in muscle after 1 h anoxia exposure and was ~50% lower after 20 h of anoxia exposure in both tissues, even though SERCA protein levels did not change. SERCA kinetic parameters (increased substrate K_m values, increased Arrhenius activation energy) were indicative of a less active enzyme form under anoxic conditions. Interestingly, the less active SERCA in anoxic turtles featured greater stability than the enzyme from normoxic animals as determined by both kinetic analysis (effect of low pH and low temperatures on K_m MgATP) and conformational resistance to urea denaturation. The quick time course of deactivation and the stable changes in kinetic parameters that resulted suggested that SERCA was regulated by a post-translational mechanism. *In vitro* experiments indicated that SERCA activity could be blunted by protein phosphorylation and enhanced by dephosphorylation in a tissue-specific manner.

Key words: sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA), anoxia, phosphorylation, metabolic rate depression, *Trachemys scripta elegans*.

INTRODUCTION

Several species of turtles have a superb ability for prolonged survival without oxygen. The freshwater red-eared slider, *Trachemys scripta elegans*, can live for as long as 3 months submerged in cold deoxygenated water, a feature that supports winter hibernation under water in ice-locked lakes (Jackson, 2004; Ultsch, 2006). Multiple biochemical adaptations contribute to anoxia tolerance, the most important of these being a profound anoxia-induced depression of resting metabolic rate (RMR) (Storey and Storey, 2004; Storey and Storey, 2007). In turtles, metabolic depression lowers RMR to just 10–20% of the corresponding aerobic rate at the same temperature (Herbert and Jackson, 1985). Depression of RMR includes both coordinated global suppression of all ATP-producing and ATP-consuming cell functions as well as a reprioritization of ATP use to sustain vital activities (e.g. ion pumping to sustain membrane potential difference) while shutting down optional activities such as protein synthesis and the cell cycle (Storey and Storey, 2007). For example, studies with turtle hepatocytes showed a net 94% decrease in ATP turnover under anoxia but a strong proportional increase in the fractional use of ATP by Na^+/K^+-ATPase (62% of total ATP turnover under anoxia *versus* 28% in normoxia) (Hochachka et al., 1996). Calcium pumping *via* the sarcoendoplasmic reticulum (SER) Ca^{2+}-ATPase (SERCA) is a major ATP expenditure of cells, and we propose that, like Na^+/K^+-ATPase, SERCA must be differentially regulated under anoxic conditions in turtle tissues.

Calcium is a dynamic signaling molecule that triggers a variety of responses across all cell types (Shimoda et al., 2006). Cytoplasmic calcium concentration is tightly controlled, and usually maintained at low levels between 40 and 100 nmol l^{-1} (Collins and Thomas, 2001). Influx of Ca^{2+} into the cytoplasm is gated by calcium channels, whereas calcium is actively transported out of the cytoplasm *via* plasma membrane Ca^{2+}-ATPase (PMCA) and SERCA. In myocytes, depolarization triggers calcium release from SER stores and cytoplasmic Ca^{2+} functions in cross-bridge formation and contraction (Endoh, 2006). Cytoplasmic Ca^{2+} is then pumped, at the cost of one ATP per two Ca^{2+} ions moved, into the SER lumen by SERCA to re-establish resting conditions (MacLennan et al., 1997). SERCA activity is most prominent in muscle, accounting for 10–25% of basal ATP turnover (Clausen et al., 1991; Simonides et al., 2001), but SERCA is ubiquitous and consumes varying amounts of ATP in cells depending on cell type and physiological state.

In anoxia-intolerant species such as mammals, anoxia triggers an immediate and drastic increase in intracellular calcium due to ATP depletion. For example, cytoplasmic [Ca^{2+}] rises by more than 10-fold in mammalian neurons within minutes when oxygen is depleted (Bickler et al., 2001) and multiple events triggered by Ca^{2+} are responsible for many of the injuries to mammalian cells caused by anoxia or ischemia (Hochachka, 1986). By contrast, turtle neurons show a much lower rise in cytoplasmic Ca^{2+} of ~2-fold within the first few hours of anoxia exposure, before stabilizing at this level over subsequent weeks of anaerobiosis (Bickler, 1998). Extracellular calcium levels also rise in anoxic turtles, due to calcium released from shell and bone matrices to provide buffering of the byproducts of anaerobic fermentation (Jackson, 2002; Jackson, 2004). This

mobilization of calcium results in increased [Ca^{2+}] in plasma and cerebrospinal fluid over the duration of anoxia exposure (Cserr et al., 1998; Reese et al., 2001).

The present study examined the regulation of SERCA in response to anoxic submergence in *T. s. elegans* tissues. A stable decrease in activity occurred in several tissues and significant differences in SERCA kinetic and stability properties were identified when aerobic *versus* anoxic forms were analyzed in skeletal muscle and liver. The molecular basis of changes in SERCA properties between the two states was traced to reversible phosphorylation of the enzyme.

MATERIALS AND METHODS

Animals and chemicals

This research was approved by the Carleton University Animal Care Committee. Adult male red-eared slider turtles (*T. s. elegans* Schoepff) of ~20–25 cm shell length were acclimated in large tanks filled with running dechlorinated water (5–7°C) and fed *ad libitum* on a diet of trout pellets, lettuce and egg shells for ~6 weeks prior to experimentation. Control (aerobic) turtles were sampled from this condition. For anoxia exposure, some turtles were transferred to 40 l tanks (three animals per tank) filled with deoxygenated water (previously bubbled with nitrogen gas for several hours) at 5–7°C. A wire mesh placed 20 cm below the water surface prevented the animals from surfacing. Nitrogen gas was continuously bubbled for the duration of the anoxic treatment (1, 5 or 20 h). Animals were killed by decapitation; tissues were immediately removed and frozen in liquid nitrogen and then stored at –80°C until use.

The mouse anti-chicken IgG serum SERCA antibody (CaF2-5D2) was purchased from the Developmental Studies Hybridoma Bank (DSHB, University of Iowa, Iowa City, IA, USA), and HRP-conjugated goat anti-mouse secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Okadaic acid was purchased from Calbiochem (La Jolla, CA, USA). All other chemicals were obtained from Sigma Corporation (St Louis, MO, USA).

Sample preparation

Frozen tissue samples were homogenized 1:5 (w:v) using a Polytron PT1000 homogenizer (Brinkmann Instruments, Rexdale, ON, Canada) in pre-chilled (4°C) extract buffer containing 25 mmol l^{-1} imidazole pH 7.2, 10% (v:v) glycerol, 0.2% (w:v) sodium deoxycholate, 250 mmol l^{-1} sucrose and 10 mmol l^{-1} 2-mercaptoethanol, with the following additions included to inhibit endogenous kinases and phosphatases: 2 mmol l^{-1} EDTA, 5 mmol l^{-1} spermine and 25 mmol l^{-1} β-glycerol phosphate (βGP). A few crystals of phenylmethylsulfonyl fluoride (PMSF), 1:1000 (v:v) of Sigma Protease Inhibitor Cocktail (P8340) and 1:1000 (v:v) of Sigma Phosphatase Inhibitor Cocktail 1 (P2850) were added just prior to homogenization. Homogenates were centrifuged at 10,000 ***g*** for 20 min at 4°C. Both the pellet [insoluble protein, resuspended in an equal volume of extract buffer containing 1% (w:v) sodium deoxycholate] and supernatant (containing soluble protein) fractions were desalted by low speed centrifugation through small columns of Sephadex G25 (300 µl of supernatant per 3 ml of G25) equilibrated in extract buffer to remove endogenous ions, small molecules and free phosphate, and assayed immediately.

Optimization of extraction conditions

General serine/threonine and tyrosine phosphatase inhibitors (NaF and Na_3VO_4) were omitted from the homogenizing buffer because they reduced recoverable SERCA activity, and alternative reagents were used (1 mmol l^{-1} EDTA, 5 mmol l^{-1} spermine, 25 mmol l^{-1} βGP, and Sigma Phosphatase Inhibitor Cocktail 1 to inhibit endogenous kinases and phosphatases). Different detergents (deoxycholate, Brij-35, CHAPS) were tried during homogenization to solubilize turtle SERCA and 0.2% sodium deoxycholate gave the highest recoverable activity in the soluble fraction with negligible activity remaining in the pellet. Thus, the data reported are for SERCA in soluble extracts homogenized in the presence of 0.2% sodium deoxycholate.

SERCA assay

SERCA activity was determined from assays in the absence *versus* presence of 50 nmol l^{-1} thapsigargin (TG), a specific inhibitor of SERCA (Lytton et al., 1992). The difference in activity was attributed to SERCA. Initial experiments determined that 50 nmol l^{-1} TG was sufficient to abolish 100% SERCA activity in crude extracts. Assays were initiated by adding 25 µl of desalted extract to paired reaction tubes (±50 nmol l^{-1} TG) in 250 µl final reaction volumes containing 25 mmol l^{-1} imidazole pH 7.0, 2 mmol l^{-1} MgATP, 400 µmol l^{-1} $CaCl_2$, 50 mmol l^{-1} KCl, 5 mmol l^{-1} $MgCl_2$ and 2 µmol l^{-1} calcium ionophore. The reaction proceeded for 20 min at 22°C. Blank reaction tubes contained no calcium and 5 mmol l^{-1} EGTA. Thus, *T. s. elegans* SERCA was defined as calcium-dependent, TG-sensitive, ATPase activity. Initial experiments validated that enzyme activity was linear with respect to time and extract volume, and optimized assay conditions with respect to pH, ATP and Ca^{2+}concentration. SERCA activity was assayed over a range of MgATP (0–250 µmol l^{-1}) and Ca^{2+} (0–5 µmol l^{-1}) concentrations to determine kinetic constants. SERCA activity was assayed spectrophotometrically by measuring the amount of inorganic phosphate produced using a malachite green/ammonium molybdate dye reagent; this red dye reagent turns green when it forms a complex with inorganic phosphate, and intensity is quantified (OD_{595}). The reagent was prepared as previously described (MacDonald and Storey, 1999). SERCA assays were stopped by removing 50 µl aliquots of the assay mix and mixing these into tubes containing 750 µl of ddH_2O and 200 µl of the dye reagent. After thorough mixing, color was allowed to develop for ~10 min followed by measurement of OD_{595} using an MR5000 microplate reader (Dynatech Laboratories, Chantilly, VA, USA) and Biolinx 2.0 software. Phosphate quantity was determined from a standard curve prepared with known amounts of KH_2PO_4.

Protein quantification

Soluble protein content was quantified using the Coomassie Blue dye binding method and the BioRad prepared reagent (Hercules, CA, USA) with a standard curve of bovine serum albumin. SERCA activity was reported as mU mg^{-1} soluble protein (1 mU=1 nmol phosphate released per min); soluble protein content of extracts did not change between aerobic and anoxic conditions.

Temperature studies

For studies characterizing the Michaelis constant (K_m) for MgATP and maximal velocity (V_{max}) as a function of temperature, assay reaction tubes were incubated in water baths at the appropriate temperature (4–68°C) for ~10 min before initiating the reaction. Assays were stopped and phosphate production determined as previously described. For V_{max} *versus* temperature studies, activation energy (E_a) values were determined (in kJ mol^{-1}) from the linear portion of Arrhenius plots.

Incubation to study effects of phosphorylation state

To assess the effects of phosphorylation state on enzyme activity, soluble tissue extracts were prepared in extract buffer and desalted

on G25 Sephadex equilibrated in extract buffer (without phosphatase and kinase inhibitors) as previously described. Then aliquots were incubated *in vitro* at 22°C for 8 h under conditions that promoted either protein phosphorylation or protein dephosphorylation (Ramnanan and Storey, 2008). Desalted tissue extracts were incubated 1:3 (v:v) with 25 mmol l^{-1} imidazole pH 7.2, 10% (v:v) glycerol, 250 mmol l^{-1} sucrose and 10 mmol l^{-1} 2-mercaptoethanol with additions as follows.

Control incubations: 5 mmol l^{-1} spermine, 25 mmol l^{-1} βGP, 2 mmol l^{-1} EDTA, and 1:1000 (v:v) Sigma Phosphatase Inhibitor Cocktail 1 (P2850) to inhibit all kinase and phosphatase activities.

Stimulation of endogenous kinase activities: 5 mmol l^{-1} MgATP, 5 mmol l^{-1} spermine, 25 mmol l^{-1} βGP, 1:1000 P2850, and one of the following: (i) 1 mmol l^{-1} cAMP to stimulate protein kinase A (PKA); (ii) 1 mmol l^{-1} cGMP to stimulate protein kinase G (PKG); (iii) 1.3 mmol l^{-1} $CaCl_2$ + 7 μg ml^{-1} phorbol myristate acetate to stimulate protein kinase C (PKC); (iv) 1 U calmodulin activity per incubation tube + 1.3 mmol l^{-1} $CaCl_2$ to stimulate calcium/calmodulin-dependent protein kinase (CAMK); or (v) 20 mmol l^{-1} AMP to stimulate AMP-activated protein kinase (AMPK).

Stimulation of endogenous protein phosphatase (PP) activities: (i) for total PP activity: 5 mmol l^{-1} $CaCl_2$ and 5 mmol l^{-1} $MgCl_2$; (ii) for total PP minus PP2A: 5 mmol l^{-1} $CaCl_2$ and 5 mmol l^{-1} $MgCl_2$ + 10 nmol l^{-1} okadaic acid (abolishes 100% of PP2A activity); (iii) for PP1/PP2A activity: 2 mmol l^{-1} EGTA + 10 nmol l^{-1} cypermethrin + 2 mmol l^{-1} EDTA; (iv) for total PP minus PP1/PP2A activity: 5 mmol l^{-1} $CaCl_2$ and 5 mmol l^{-1} $MgCl_2$ + 1 μmol l^{-1} okadaic acid (abolishes 100% of PP1 and PP2A activity); (v) for PP2B activity: 5 mmol l^{-1} $CaCl_2$ + 1 U of calmodulin activity per tube + 2 mmol l^{-1} EDTA + 1 μmol l^{-1} okadaic acid; (vi) for PP2C activity: 5 mmol l^{-1} $MgCl_2$ + 1 μmol l^{-1} okadaic acid + 10 nmol l^{-1} cypermethrin; or (vii) to promote full dephosphorylation: 1 U commercial calf intestinal alkaline phosphatase (AP) per incubation tube + 5 mmol l^{-1} $CaCl_2$ and 5 mmol l^{-1} $MgCl_2$.

Following incubation, extracts were desalted using low speed centrifugation through small columns of G25 Sephadex equilibrated in extract buffer and SERCA activity was assayed immediately.

Determination of kinetic data for substrate curves

Kinetic analysis to determine K_m, V_{max}, E_a and I_{50} (inhibition constant for 50% inhibition) values used a Kinetics computer program (Brooks, 1992). Kinetic constants were calculated using the concentration of free ions during assay conditions as determined by the Bound and Determined (BAD) computer program (Brooks and Storey, 1992).

SDS-PAGE and western blotting

Isolation, electrophoresis, blotting and immunodetection of SERCA protein were performed essentially as described previously (Ramnanan and Storey, 2008) with the following minor modifications. Aliquots containing 35 μg of soluble protein were subjected to electrophoresis and proteins were subsequently wet-transferred to PVDF membranes. Membranes were blocked with 5% non-fat dried milk in Tris-buffered saline containing Tween-20 (TBST: 10 mmol l^{-1} Tris-base pH 7.0, 150 mmol l^{-1} NaCl, 0.1% v:v Tween 20) for 1 h at room temperature. Membranes were incubated overnight at 4°C with mouse serum anti-SERCA (CaF2-5D2) diluted 1:1000 v:v in TBST. After 3×5 min washes with TBST, membranes were then incubated with HRP-conjugated goat anti-mouse secondary antibody (diluted 1:2000 v:v in TBST) for 1 h at room temperature. Following 3×5 min washes in TBST, immunodetection was performed using Western Lightning Chemiluminescence Plus reagents (Perkin Elmer, Boston, MA, USA) following the manufacturer's protocols, and imaged and quantified using the Syngene ChemiGenius bio-imaging system (Frederick, MD, USA) and associated GeneTools software (v3.00.02). Samples were normalized for slight variations in protein loading as described previously (Ramnanan and Storey, 2006; Ramnanan and Storey, 2008).

Pulse proteolysis to determine protein stability

We assessed the structural stability of SERCA in muscle and liver by treating extracts with increasing concentrations of denaturant (urea) followed by pulse proteolysis incubation with thermolysin, as described previously (Ramnanan and Storey, 2006). Tissue extracts were prepared as described above with the exception that protease inhibitors were omitted during homogenization. Aliquots of soluble crude extract were incubated for 24 h in urea solutions (0–3.4 mol l^{-1} final concentration) prior to treatment with 10 mg ml^{-1} thermolysin (stock prepared with 2.5 mol l^{-1} NaCl and 10 mmol l^{-1} $CaCl_2$). Thermolysin activity was stopped after 10 min by the

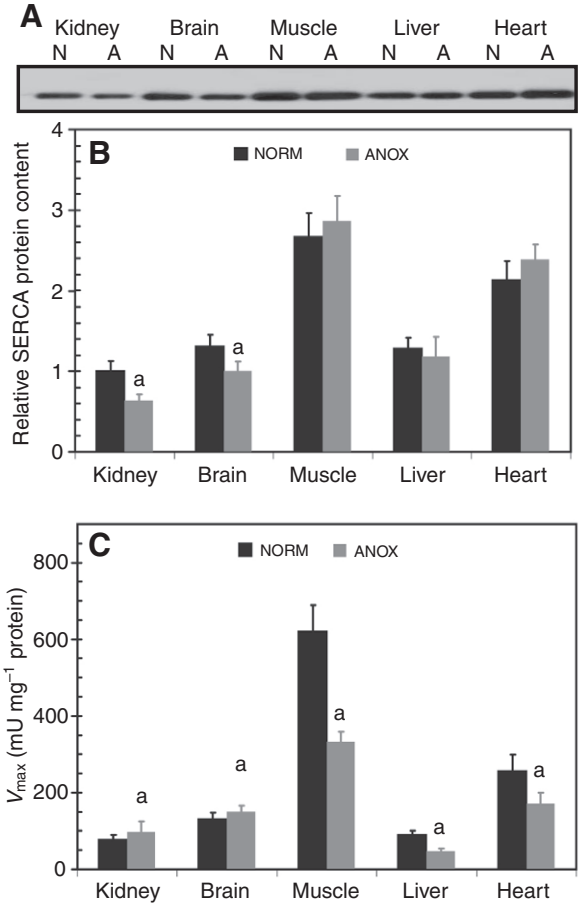

Fig. 1. SERCA protein and activity profile of tissues of *Trachemys scripta elegans*. (A) Representative (of *N*=4–6) western blot of SERCA (immunoreactive band at 120 kDa) from normoxic (N) and 20 h anoxic (A) turtle tissues. (B) SERCA protein content and (C) SERCA maximal velocities (V_{max}) for normoxic (NORM) and 20 h anoxic (ANOX) tissues. Enzyme assays were conducted under optimal conditions at 22°C as described in Materials and methods. Data are means ± s.e.m., *N*=6 independent enzyme preparations for liver and muscle, *N*=4 for other tissues. [a]Significantly different from normoxic tissue value, $P<0.05$.

addition of 20 µl of 50 mmol l^{-1} EDTA (pH 8.0). SERCA protein content was assessed by western blotting and parameters of protein unfolding (C_m) were calculated.

Data statistics

Statistical analysis was performed using Graph-Pad Prism 5.0. All data are expressed as means ± s.e.m. For comparision of two groups, Student's *t*-test was used. For comparison of more than two groups, ANOVA was performed followed by *post hoc* Tukey's Multiple Comparison to determine significance between groups.

RESULTS

SERCA protein level and maximal activity

Total SERCA protein content and maximal activity (V_{max}) were assessed in several tissues of normoxic and anoxic turtles (Fig. 1). Immunoblotting detected a single band corresponding to the typical size of vertebrate SERCA (110 kDa). After 20 h of anoxic submergence, SERCA protein levels remained unchanged in skeletal muscle, liver and heart, but decreased by 36% and 24% in kidney and brain, respectively (Fig. 1A,B). SERCA V_{max} decreased by 46% in muscle, 47% in liver and 35% in heart of anoxic turtles, but did not change significantly in other tissues (Fig. 1C). Subsequent experiments focused on characterizing SERCA in skeletal muscle and liver.

Temperature effects on SERCA enzyme kinetics

SERCA V_{max} and K_m for MgATP and Ca^{2+} substrates were characterized as a function of temperature using enzyme extracts from both tissues, and similar trends were observed in liver (Fig. 2) and muscle (data not shown). When assayed at intermediate temperatures (15–25°C), the K_m for MgATP was significantly higher for SERCA from 20 h anoxic turtles as compared with the enzyme from liver of normoxic control turtles. Conversely, when assayed at low temperatures (4–10°C), K_m MgATP values for SERCA from 20 h anoxic turtles were significantly lower than those for the normoxic control enzyme (Fig. 2A). K_m for MgATP was generally stable (between 31 and 37 µmol l^{-1} MgATP) in anoxic liver at all assay temperatures, but K_m MgATP values for SERCA from normoxic liver were generally elevated at low temperatures. No temperature-dependent or anoxia-induced effects were observed for calcium kinetics of the liver (Fig. 2B) or muscle enzyme. The effect of temperature on SERCA V_{max} was then analyzed and is shown as an Arrhenius plot (log V_{max} *versus* inverse temperature in Kelvin) in Fig. 2C. This revealed a linear relationship from 4 to 25°C, with a break point apparent at 25°C (Fig. 2C). The calculated E_a from the linear portions of these plots was increased by 60% and 41% for muscle and liver SERCA, respectively, of turtles subjected to 20 h anoxic submergence (Fig. 2D).

Effect of pH on SERCA activity

Cellular pH decreases in tissues when turtles are anoxic because of metabolic acidosis associated with glycolytic ATP production and lactic acid accumulation. We examined SERCA activity as a function of pH and similar results were generally observed in muscle (Fig. 3) and liver (data not shown). Normoxic SERCA was more sensitive to assay pH than anoxic SERCA in muscle. Anoxic muscle SERCA was relatively stable (~280–360 mU mg^{-1} protein) between pH 6.6 and pH 8.0 (Fig. 3A). Conversely, normoxic SERCA showed

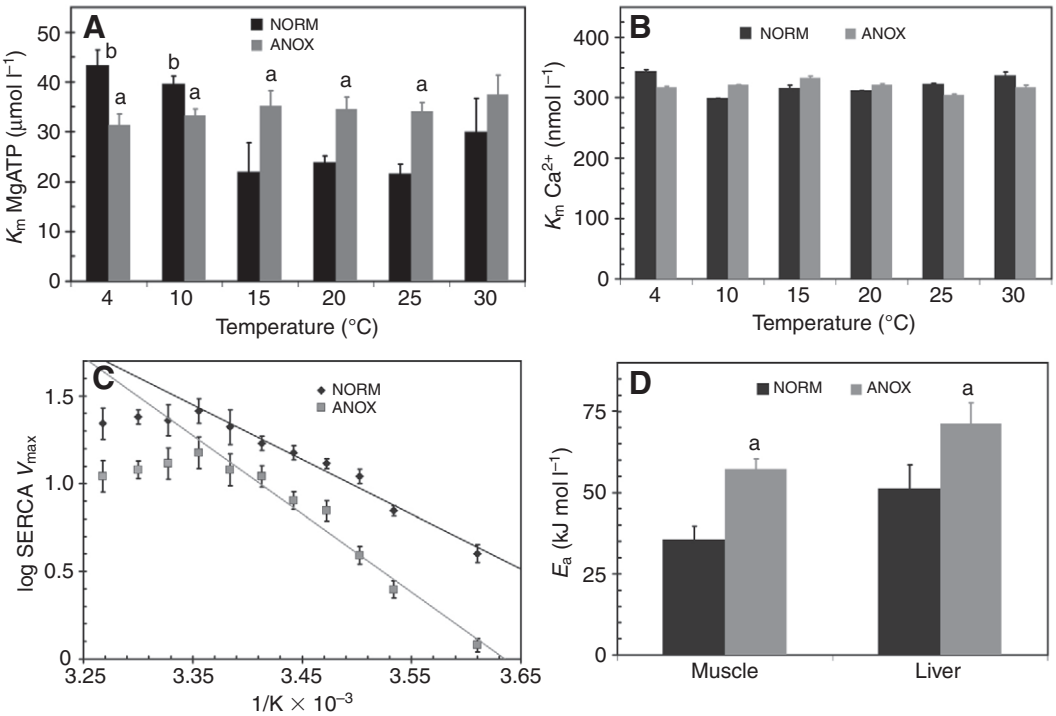

Fig. 2. SERCA enzyme kinetics as a function of temperature in normoxic (NORM) and 20 h anoxic (ANOX) *T. s. elegans* tissues. (A) K_m for MgATP *versus* temperature for SERCA activity from liver extracts. (B) Michaelis constant (K_m) for Ca^{2+} *versus* temperature for SERCA from liver extracts. (C) Arrhenius plots of SERCA maximal activity as a function of temperature (Kelvin) for liver extracts. (D) Arrhenius activation energy (E_a) for SERCA from liver and muscle extracts, calculated from the linear part of the relationship in C. Assays were conducted under V_{max} conditions and data are means ± s.e.m., N=5–7 independent SERCA preparations for liver data, and N=4–5 for muscle data. [a]Significantly different from corresponding normoxic tissue value; [b]significantly different from values at other temperatures for the same enzyme preparation, P<0.05.

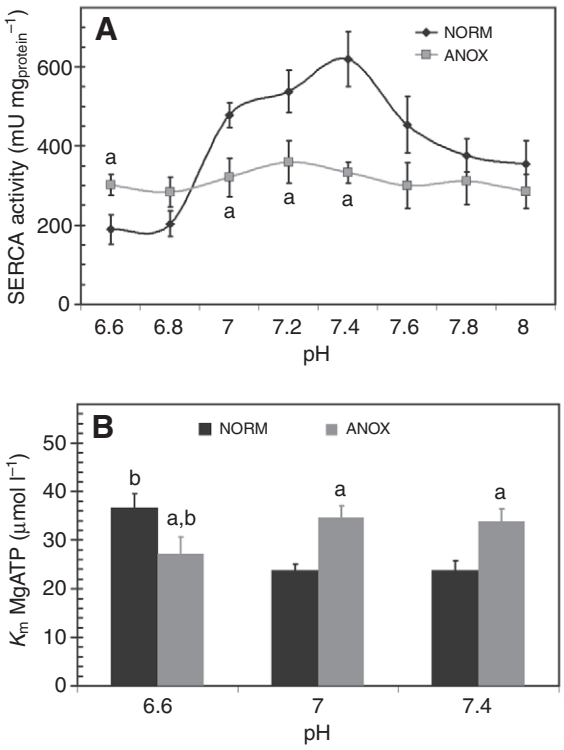

Fig. 3. Effect of pH on normoxic (NORM) and 20 h anoxic (ANOX) turtle muscle SERCA (A) maximal activity and (B) K_m MgATP. Data are means ± s.e.m. of N=4–5 independent preparations. [a]Significantly different from corresponding normoxic tissue value at same pH; [b]significant difference from values assayed at pH 7.0 and 7.4 for the same enzyme preparation, P<0.05.

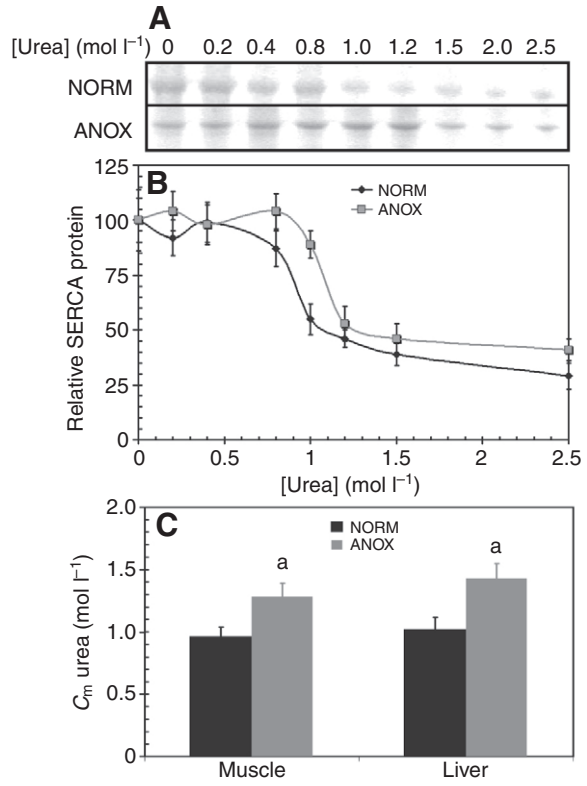

Fig. 4. Structural unfolding of SERCA from liver extracts from normoxic and 20 h anoxic *T. s. elegans* liver. Extracts were denatured overnight with different concentrations of urea and subsequently treated with thermolysin. (A) Representative western blots detecting the amount of the 120 kDa SERCA band remaining after proteolysis as a function of urea concentration (0–2.5 mol l^{-1}). (B) Plot of mean (±s.e.m., N=4) SERCA protein content remaining after overnight urea denaturation and thermolysin proteolysis and (C) corresponding C_m values (concentration of urea required to denature 50% of SERCA protein) in turtle liver and muscle (means ± s.e.m., N=4). [a]Significantly different from normoxic C_m, P<0.05.

a broad peak of activity that was greater than anoxic muscle SERCA activity between pH 7.0 and 7.4, but normoxic SERCA activity sharply declined on either side of that range, and was less than that of anoxic muscle SERCA at low pH (6.6–6.8). K_m MgATP of muscle SERCA was also assayed over a range of pH values (Fig. 3B). K_m MgATP of anoxic SERCA was significantly higher (i.e. lower affinity) than that of the normoxic enzyme at higher pH values (7.0, 7.4) but the situation was reversed at pH 6.6 when K_m of the anoxic enzyme decreased (i.e. affinity decreased).

SERCA protein stability

To determine whether there was a difference in the structural stability of SERCA between normoxic and anoxic states, the liver and muscle enzymes from both conditions were incubated with varying concentrations of the denaturant urea, followed by pulse proteolysis with thermolysin to selectively degrade denatured (unfolded) protein. The remaining amount of native protein was then assessed by immunoblotting with quantification of the intensity of the 120 kDa band. As shown in representative western blots (Fig. 4A) and a plot of relative protein *versus* urea concentration (Fig. 4B), the population of SERCA in liver from anoxic animals was more stable after urea treatment/thermolysin-mediated proteolysis. The kinetic constant for unfolding (C_m; the urea concentration required to denature 50% of test protein) was 33% and 41% higher, respectively, for SERCA from muscle and liver of anoxic *versus* normoxic turtles (Fig. 4C).

Time course of anoxia effects on SERCA activity

Fig. 1 showed that SERCA activity was strongly suppressed after 20 h of anoxia exposure. To determine how quickly enzyme activity was shut down, turtles were sampled over a time course of anoxic submergence. In both tissues examined, SERCA activity decreased rapidly, by 39% and 32% in muscle and liver, respectively, within 1 h of anoxia exposure (Fig. 5). Subsequently, SERCA activity decreased further over time to reach levels that were 46% and 47% of the corresponding normoxic values in muscle and liver, respectively, after 20 h of anoxia treatment.

Effect of phosphorylation state on SERCA activity

To determine whether the differences in SERCA enzyme kinetics during oxygen deprivation were due to anoxia-induced phosphorylation of the enzyme, tissue extracts were incubated under conditions that promoted the activity of specific endogenous protein kinases or protein phosphatases. Data are presented relative to SERCA activity recovered from control incubations that contained inhibitors of all kinases and phosphatases. In muscle, incubations promoting PKA or PKG activity reduced SERCA activity in extracts from normoxic turtles by 65% and 83%, respectively, to levels more typical of anoxia-treated animals (Fig. 6A). Incubation under conditions that stimulated PKG also decreased SERCA activity in extracts of anoxic muscle by ~50%. Stimulation of PKC activity had no effect on SERCA activity. Incubations stimulating individual endogenous protein phosphatases failed to affect recoverable muscle SERCA activity (data not shown). However,

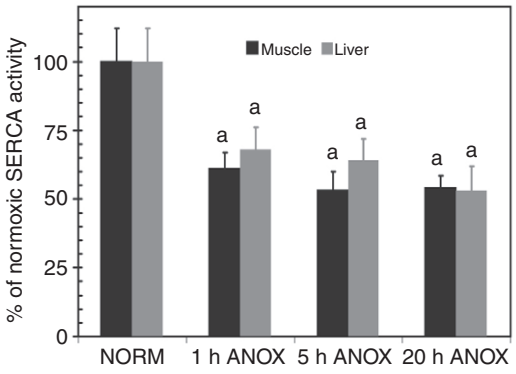

Fig. 5. Time course of anoxia-induced effects on SERCA activity in liver and muscle. Animals were subjected to anoxia treatment for 1, 5 and 20 h (as detailed in Materials and methods) and recoverable SERCA activity from tissue extracts was assayed at V_{max} conditions compared with that of normoxic control animals. Data are means ± s.e.m., N=4. [a]Significantly different from normoxic SERCA activity, P<0.05.

incubation with a commercial heat-stable AP led to 56% and 246% increases in SERCA activity in extracts from normoxic and anoxic muscle, respectively (Fig. 6A). The changes in muscle SERCA activity were related to changes in substrate kinetics (Fig. 6B), where PKG stimulation increased K_m MgATP (reduced affinity) for both normoxic and anoxic SERCA, and incubation with AP decreased K_m MgATP (increased affinity) of the anoxic enzyme.

With liver extracts, incubations promoting PKA and PKC led to 41% and 59% reductions in SERCA activity from normoxic animals, but did not affect recoverable SERCA activity in extracts from anoxic turtles (Fig. 6C). Incubations to stimulate PKG were ineffective in modifying liver SERCA activity (data not shown). Stimulation of endogenous ion-independent phosphatases (PP1/PP2A) led to an increase in recoverable SERCA activity of 40% and 100% for the normoxic and anoxic enzyme, respectively (Fig. 6C), but incubations that promoted ion-dependent phosphatases were ineffective (data not shown). This stimulatory effect was blocked in an incubation containing 10 nmol l^{-1} okadaic acid, which inhibits endogenous PP2A but not PP1 (i.e. Total PP2A, Fig. 6C) and was also blocked in all other incubations that contained higher okadaic acid (1 µmol l^{-1}) which inhibited both PP2A and PP1. Hence, the stimulatory effect of endogenous phosphatases was localized to the action of PP2A. Again, changes in SERCA activity in liver were consistent with changes in K_m MgATP (Fig. 6D); incubations promoting PKA increased the K_m MgATP of normoxic SERCA to levels associated with anoxic SERCA, whereas promoting PP2A activity decreased K_m MgATP in anoxic extracts to levels similar to those observed for normoxic SERCA.

DISCUSSION

Pronounced depression of RMR is crucial for survival without oxygen by anoxia-tolerant species, and must be carefully regulated to achieve the overall suppression of metabolism while still fueling vital cellular processes. It stands to reason that ion-motive ATPases, due to their energetically demanding functions in maintaining the transmembrane ion gradients that are necessary for life, are critical loci for regulation during transitions to or from states of hypometabolism. For example, the overall metabolic rate of turtle hepatocytes was reduced by 95% in response to anoxia, but the fraction of ATP consumed by the ATP-dependent sodium–potassium pump (Na^+/K^+-ATPase) rose from 28% to 75%

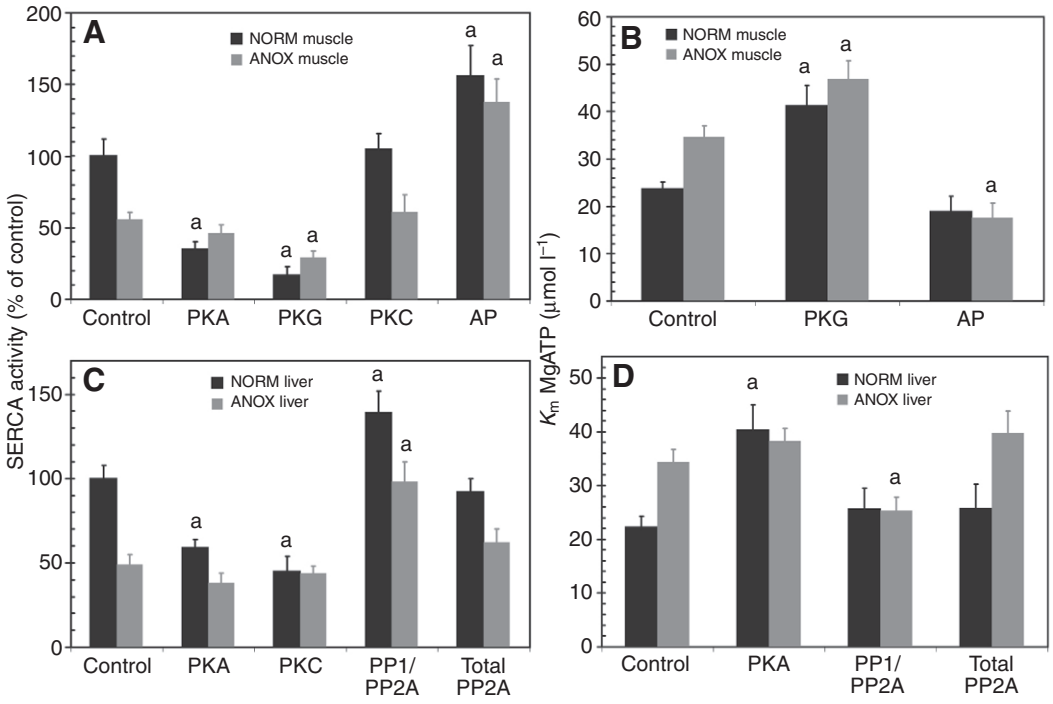

Fig. 6. Effect of *in vitro* incubations to stimulate endogenous protein kinases (PK) and protein phosphatases (PP) on *T. s. elegans* SERCA (A) maximal activity, (B) MgATP K_m in muscle extracts, (C) maximal activity and (D) MgATP K_m in liver extracts from normoxic and 20 h anoxic conditions. Incubation conditions are detailed in Materials and methods. Data are means ± s.e.m., N=4, and expressed as percentage of control incubation values for activity in A and C. [a]Significantly different from SERCA activity for corresponding control incubation, P<0.05.

of overall ATP hydrolyzed (Hochachka et al., 1996). Indeed, it has been shown that Na^+/K^+-ATPase and SERCA are closely regulated and are strongly suppressed in hypometabolic states including hibernation, estivation and diapause (MacDonald and Storey, 1999; Hemmings and Storey, 2001; Malysheva et al., 2001; Ramnanan and Storey, 2006; Ramnanan and Storey, 2008; McMullen and Storey, 2008). Anoxia has been shown to suppress SERCA activity in neurons (Henrich and Buckler, 2008) and hypoxia decreased SERCA expression in hearts (Pei et al., 2003) of rats. If SERCA is sensitive to low oxygen in mammals, then it is likely to be a major target for energy savings in anoxia-tolerant species. We proposed that SERCA would be differentially regulated in response to anoxia exposure in tissues of *T. s. elegans*, a well-established model of vertebrate facultative anaerobiosis (Lutz, 1992; Hochachka and Lutz, 2001; Jackson, 2002; Storey and Storey, 2007), as part of the coordinated transition to the hypometabolic state.

A degree of ATP conservation would be expected in skeletal muscle during winter hibernation of turtles because of the overall infrequency of muscle movements. However, ATP consumption by ion-motive ATPases is a major part of RMR for all animals. In particular, it is well established that thyroid hormone increases RMR by stimulating SERCA expression and activity. The RMR of hypothyroid and hyperthyroid mammals is ~70% and ~150% of euthyroid values, respectively, and is associated with proportional changes in SERCA activity (Clausen et al., 1991; Simonides et al., 2001). Therefore, it is logical to propose that the anoxia-induced transition to a hypometabolic state must include strong suppression of SERCA activity as a key part of long-term anaerobiosis and the present study shows that this is indeed true for the best-studied vertebrate facultative anaerobe, the freshwater turtle *T. s. elegans*.

We characterized SERCA activity in crude extracts of *T. s. elegans* by using the SERCA-specific inhibitor TG to differentiate between SERCA and PMCA activities; TG binds SERCA (but not PMCA) and is widely used to study SERCA activity without the need for SERCA purification (Treiman et al., 1998). After 20 h of anoxia exposure, SERCA maximal activity decreased in muscle, liver and heart, without a change in SERCA total protein (as measured by immunoblotting). Conversely, SERCA protein levels decreased in brain and kidney of anoxic turtles without causing a change in SERCA activity (Fig. 1). Hence, SERCA activity in turtle organs was not strictly determined by the amount of SERCA protein.

Substrate affinity of liver SERCA for MgATP differed between normoxic and anoxic states, and at higher assay temperatures (15–25°C) the higher K_m MgATP of the anoxic enzyme would suggest a less active enzyme form under anoxic conditions (Fig. 2A). At low temperatures (4–10°C), however, the opposite was true which could be an advantage during winter hibernation in cold water. Notably, the K_m value of the anoxic enzyme form was actually stable across the temperature range whereas the K_m of normoxic SERCA changed 2-fold over the same temperature range; this feature demonstrates a substantial difference between the two enzyme forms.

The activity of anoxic SERCA showed good stability with respect to pH change, whereas normoxic SERCA activity declined sharply below pH 7.0. However, at a typical physiological pH (pH 7.0–7.4), SERCA from anoxia-exposed animals had a lower V_{max} (Fig. 3A) and decreased substrate affinity for MgATP (Fig. 3B) relative to normoxic SERCA, consistent with a less active form of the enzyme. At pH values <7.0, however, the situation was reversed and anoxic SERCA activity was higher and its K_m MgATP was lower than the normoxic form when assayed at pH 6.6. While this pH is subphysiological, physiological pH in turtles does gradually decrease over long (weeks to months) periods of anoxia. Thus, anoxic SERCA activity is conferred a degree of stability by an early (within 20 h of initial anoxia submergence) modification that may be beneficial for maintaining activity during longer bouts of anoxia exposure that include a substantial decline in the pH of body fluids.

The enhanced kinetic stability of SERCA with respect to temperature and pH in anoxic tissues also corresponded to increased structural and conformational stability. Thus, SERCA from liver of 20 h anoxic turtles was more resistant to urea denaturation and unfolding than was the normoxic form (Fig. 4). Thus, whereas the SERCA population in muscle and liver was dominated by the lower activity form of the enzyme after 20 h of anoxia exposure, this low activity conformation had increased stability in terms of substrate kinetics, susceptibility to urea denaturation, and ability to function efficiently (greater affinity for ATP) at the low temperatures and low pH values that *T. s. elegans* tissues would encounter during extended bouts of winter hibernation under water.

The Arrhenius relationship (Fig. 2C) was generally linear from the lowest temperature assayed (4°C) up to 22°C, which correlates well with the temperature range naturally encountered by turtles in their native habitat. A break in an Arrhenius plot is indicative of a temperature-dependent shift in a rate-limiting aspect of the enzymatic reaction, and can be a marker for a deleterious shift in the local, membrane lipid environment of an ATPase. Lipid membranes are well established as regulators of SERCA activity (Lee, 1998). Previous studies have established that Arrhenius breaks in SERCA plots are tightly correlated with environmental temperatures faced by the organism and this pattern holds true across the animal kingdom, including (i) rabbits where the break occurred at 20°C (Rubstov et al., 1986); (ii) cold water invertebrates like the deep sea scallop *Placopecten magellanicus* and lobster *Homarus gammarus* that show Arrhenius breaks at 10°C (Madeira et al., 1974; Kalobokis and Hardwicke, 1998); (iii) several species of fish where the break points were related to ambient living temperatures (Godiksen and Jessen, 2001; Godiksen and Jessen, 2002); and (iv) the desert snail *Otala lactea* where the break occurred above 30°C (Ramnanan and Storey, 2008). Calculated from the linear part of the relationship, the activation energy for turtle SERCA was increased in both tissues after 20 h of anoxia exposure, consistent with a less active form of the enzyme (Fig. 2D).

The transition to anoxia-induced hypometabolism in *T. s. elegans* has been shown to be associated with substantial changes in the phosphorylation state of enzymes and the activities of protein kinases/phosphatases (Greenway and Storey, 2000; Rider et al., 2009). Therefore, in light of previous studies of the regulation of ATP-dependent ion pumps in hypometabolic states (MacDonald and Storey, 1999; Ramnanan and Storey, 2006; Ramnanan and Storey, 2008), we proposed that the mechanism responsible for SERCA regulation in turtle tissues during anoxia exposure would be reversible protein phosphorylation.

Strong suppression (39% and 32% in muscle and liver) of SERCA activity was evident within 1 h of anoxia submergence (Fig. 5) and activities remained low after 20 h of anoxia exposure. Changes in SERCA activity occurred without a decrease in SERCA protein (Fig. 1) indicating that proteolysis was not a factor. This suggested that the stable suppression of SERCA activity in response to acute anoxia exposure was due to a post-translational modification of the protein, presumably by reversible protein phosphorylation. This is consistent with the observation that substantial changes in the phosphorylation state of proteins

involved in signal transduction (Greenway and Storey, 2000) and protein synthesis (Rider et al., 2009) are also evident within hours of anoxia exposure in this model.

Strong evidence for this mechanism in the regulation of turtle SERCA was obtained from *in vitro* incubations that stimulated the activities of specific endogenous kinases and phosphatases and measured the consequent effects on SERCA activity. Stimulation of PKA and PKG in muscle (Fig. 6A) and PKA and PKC in liver (Fig. 6C) significantly reduced SERCA activity compared with that in normoxic extracts, similar to the effects of *in vivo* anoxia exposure on the enzyme. PKG stimulation also further reduced SERCA in anoxic muscle extracts, whereas no kinases affected SERCA activity in anoxic liver, suggesting that the enzyme was already in a maximally phosphorylated and inhibited state. Thus, regulatory phosphorylation of SERCA seems to occur in a tissue-specific manner that includes differential responses to the three kinases evaluated. Furthermore, these kinases generally increased the K_m MgATP for SERCA in normoxic extracts to levels that were approximately equal to the K_m values of anoxic SERCA in muscle (Fig. 6B) and liver (Fig. 6D).

Incubation with AP strongly increased SERCA activity in both normoxic and anoxic muscle extracts (Fig. 6A), and decreased K_m MgATP in anoxic extracts (Fig. 6B), indicating that SERCA activity can be enhanced by dephosphorylation. In anoxic liver extracts, SERCA activity also increased in response to phosphatase action (Fig. 6C) and K_m MgATP decreased (Fig. 6D). Both of these responses in liver appeared to be specifically stimulated by PP2A, and these effects were blocked under any incubation condition that involved inhibition of PP2A with okadaic acid. These data suggest that regulatory phosphorylation mediates the anoxia-dependent changes in SERCA function in both turtle liver and muscle, under tissue-specific kinase and phosphatase action. The change in SERCA activity seems to be related to a change in substrate affinity for MgATP, and the *in vivo* dephosphorylation of liver SERCA appears to be mediated by PP2A.

Transiently phosphorylated catalytic intermediates have been established for SERCA (Ryan et al., 2004). While liver SERCA is not typically known to have sites that are targets for stable, covalently bound phosphate with regulatory properties, our findings do have a precedent in muscle (Hawkins et al., 1994). Additionally, the presence of covalently bound phosphate on bay scallop SERCA was shown to increase stability of both structure and enzymatic activity (Ryan et al., 2004), comparable to the results seen in anoxic turtle tissues at low pH and low temperature.

Thus, it is apparent that SERCA activity is suppressed in liver and muscle in *T. s. elegans* early (within 1 h) into anoxia exposure *via* stable alterations to enzyme activity and kinetic properties. Our data are consistent with the mechanism being protein phosphorylation with dephosphorylation of the anoxic enzyme reinstating properties that are like the normoxic enzyme. This post-translational mechanism of regulating SERCA also stabilizes enzyme structure and could help to protect/stabilize the enzyme during long-term anoxic submergence while also being quickly reversible when animals transition back to breathing air again. Thus, there is a conserved drive in vertebrates to protect SERCA protein content and/or activity. Regulatory phosphorylation allows for quick attenuation of enzyme function, as evident in anoxic turtles where SERCA was suppressed after 1 h of anoxia exposure. This fits in with the overall move to suppress RMR under anoxic conditions, as SERCA consumes a considerable percentage of cellular ATP even in tissues at rest.

LIST OF ABBREVIATIONS

AMPK	AMP-activated protein kinase
AP	alkaline phosphatase
C_m	constant for protein unfolding
CAMK	calcium/calmodulin-dependent protein kinase
E_a	Arrhenius activation energy
I_{50}	inhibition constant
K_m	Michaelis constant
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PMCA	plasma membrane Ca^{2+}-ATPase
PP	protein phosphatase
PP1	type 1 protein phosphatase
PP2	type 2 protein phosphatase (A,B,C)
RMR	resting metabolic rate
SER	sarcoendoplasmic reticulum
SERCA	sarcoendoplasmic reticulum Ca^{2+}-ATPase
TG	thapsigargin
V_{max}	maximal enzyme velocity

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