Introduction

The land snail *Otala lactea* is a native of the seasonally arid lands of the Mediterranean region. When food or water is limiting, this pulmonate gastropod enters estivation. A variety of behavioral, physiological and biochemical adaptations underlie estivation and help to confer prolonged water and energy conservation. The phenomenon is common among both vertebrates and invertebrates and has been well-studied in several terrestrial snails, including *O. lactea* (Brooks and Storey, 1997; Bishop and Brand, 2000; Storey, 2002; Ramos-Vasconcelos and Hermes-Lima, 2003). Because of the limitations of their environments, snails that estivate are typically active for only a few weeks of the year and, during that time, they must accumulate sufficient body fuel reserves to endure many months of dormancy. Indeed, snails can sometimes remain dormant for years (Herreid, 1977).

Strong metabolic rate reduction is a key feature of estivation and serves to greatly extend the time that a fixed reserve of endogenous fuels can support survival. Estivating snails typically reduce their metabolic rate to <30% of the resting rate in nonestivating animals (Rees and Hand, 1990; Brooks and Storey, 1997; Bishop and Brand, 2000). The transition into a hypometabolic state requires coordinated suppression of the rates of both ATP-consuming and ATP-producing reactions. As one of the largest consumers of cellular ATP, the plasma membrane Na⁺/K⁺-ATPase is a potentially key target for regulation during estivation. Na⁺/K⁺-ATPase was investigated in foot muscle and hepatopancreas of the land snail *Otala lactea*, comparing active and estivating states. In both tissues enzyme properties changed significantly during estivation: maximal activity was reduced by about one-third, affinity for Mg,ATP was reduced (*Kₘ* was 40% higher), and activation energy (derived from Arrhenius plots) was increased by ~45%. Foot muscle Na⁺/K⁺-ATPase from estivated snails also showed an 80% increase in *Kₘ* Na⁺ and a 60% increase in *Kₐ* Mg²⁺ as compared with active snails, whereas hepatopancreas Na⁺/K⁺-ATPase showed a 70% increase in *I₅₀* K⁺ during estivation. Western blotting with antibodies recognizing the alpha subunit of Na⁺/K⁺-ATPase showed no change in the amount of enzyme during estivation. Instead, the estivation-responsive change in Na⁺/K⁺-ATPase activity was linked to posttranslational modification. *In vitro* incubations manipulating endogenous kinase and phosphatase activities indicated that Na⁺/K⁺-ATPase from estivating snails was a high phosphate, low activity form, whereas dephosphorylation returned the enzyme to a high activity state characteristic of active snails. Treatment with protein kinases A, C or G could all mediate changes in enzyme properties *in vitro* that mimicked the effect of estivation, whereas treatments with protein phosphatase 1 or 2A had the opposite effect. Reversible phosphorylation control of Na⁺/K⁺-ATPase can provide the means of coordinating ATP use by this ion pump with the rates of ATP generation by catabolic pathways in estivating snails.

**Summary**

Entry into the hypometabolic state of estivation requires a coordinated suppression of the rate of cellular ATP turnover, including both ATP-generating and ATP-consuming reactions. As one of the largest consumers of cellular ATP, the plasma membrane Na⁺/K⁺-ATPase is a potentially key target for regulation during estivation. Na⁺/K⁺-ATPase was investigated in foot muscle and hepatopancreas of the land snail *Otala lactea*, comparing active and estivating states. In both tissues enzyme properties changed significantly during estivation: maximal activity was reduced by about one-third, affinity for Mg,ATP was reduced (*Kₘ* was 40% higher), and activation energy (derived from Arrhenius plots) was increased by ~45%. Foot muscle Na⁺/K⁺-ATPase from estivated snails also showed an 80% increase in *Kₘ* Na⁺ and a 60% increase in *Kₐ* Mg²⁺ as compared with active snails, whereas hepatopancreas Na⁺/K⁺-ATPase showed a 70% increase in *I₅₀* K⁺ during estivation. Western blotting with antibodies recognizing the alpha subunit of Na⁺/K⁺-ATPase showed no change in the amount of enzyme during estivation. Instead, the estivation-responsive change in Na⁺/K⁺-ATPase activity was linked to posttranslational modification. *In vitro* incubations manipulating endogenous kinase and phosphatase activities indicated that Na⁺/K⁺-ATPase from estivating snails was a high phosphate, low activity form, whereas dephosphorylation returned the enzyme to a high activity state characteristic of active snails. Treatment with protein kinases A, C or G could all mediate changes in enzyme properties *in vitro* that mimicked the effect of estivation, whereas treatments with protein phosphatase 1 or 2A had the opposite effect. Reversible phosphorylation control of Na⁺/K⁺-ATPase can provide the means of coordinating ATP use by this ion pump with the rates of ATP generation by catabolic pathways in estivating snails.

Key words: metabolic rate depression, reversible phosphorylation, hepatopancreas, foot muscle, regulation of ion pumps, land snail *Otala lactea*.
states of proteins, often providing essentially on–off control. The importance of reversible protein phosphorylation to the control of estivation in *O. lactea* was first shown in studies of several regulatory enzymes in the ATP-producing pathways of carbohydrate catabolism. Pyruvate kinase (PK), phosphofructokinase (PFK-1), pyruvate dehydrogenase (PDH), and glycogen phosphorylase (GP) all showed reduced activities and altered kinetic properties during estivation that could be traced to changes in their phosphorylation states (Whitwam and Storey, 1990; Whitwam and Storey, 1991; Brooks and Storey, 1992, 1997).

Na7/K+-ATPase has a critical vital function in the maintenance of plasma membrane potential difference in all animal cells, pumping Na+ and K+ against their concentration gradients to maintain high sodium levels outside cells and high potassium inside. The pump consumes a great deal of energy; for example, in resting endotherms it is responsible for 5–40% of total ATP consumption, depending on cell type (Clausen, 1986). The enzyme consists of a large multipass transmembrane catalytic polypeptide (the ~100 kDa α-subunit) and a smaller, associated glycoprotein (the ~40–55 kDa β-subunit). In mammals, four isoforms of the α-subunit are known along with three isoforms of the β-subunit (Clausen, 2003); these combine to create multiple tissue-specific isozymes with different kinetic properties (Lopina, 2001). Homology of the catalytic α-subunit is very high (~85%) indicating a need for strong conservation of functional residues. Na7/K+-ATPase is subject to short-term regulation via reversible phosphorylation of the catalytic subunit (Ewart and Klip, 1995; Lopina, 2001). For example, the α1-isoform of Na7/K+-ATPase, which is the dominant form in mammalian kidney, can be phosphorylated by cAMP-dependent (PKA), cGMP-dependent (PKG) protein kinases, Ca2+ and phospholipid-dependent (PKC) protein kinase and by tyrosine kinase (Lopina, 2001). Phosphorylation can either inhibit or stimulate Na7/K+-ATPase activity, depending on factors such as type of α-isoform present and the calcium concentration (Cheng et al., 1999; Lopina, 2001). The effect of phosphorylation on invertebrate Na7/K+-ATPase activity has yet to be clarified.

In multiple situations of hypometabolism it is clear that transmembrane sodium and potassium gradients are maintained despite much reduced rates of ATP turnover; for example, this has been documented in anoxic turtles (Buck and Hochacha, 1993) and estivating frogs (Flanagan et al., 1993). This requires coordinated suppression of the rates of Na+ and K+ movements through ion channels (termed channel arrest) and oppositely directed ATP-driven ion pumps to match the rates of ATP availability from catabolic pathways (Hochacha, 1986). Controlled suppression of Na7/K+-ATPase activity is one critical element in this process and has been documented in other hypometabolic systems including turtle anaerobiosis and mammalian hibernation (Hochacha et al., 1996; MacDonald and Storey, 1999). In the latter case the mechanism of Na7/K+-ATPase suppression was shown to be protein phosphorylation (MacDonald and Storey, 1999). The same mechanism may also regulate Na7/K+-ATPase suppression during estivation and the present study investigates this in *O. lactea* foot muscle and hepatopancreas. The kinetic properties of Na7/K+-ATPase from active and estivating snails were characterized and *in vitro* incubations with protein kinases and phosphatases indicated that probable changes to the phosphorylation state of the enzyme could account for changes in Na7/K+-ATPase activity between active and estivating states.

**Methods and materials**

**Animals**

*Otala lactea* (Muller) snails from Morocco were purchased from a local retailer. In the laboratory, snails were held at room temperature (~22°C) in plastic containers lined with damp paper towels and fed shredded carrots and cabbage (sprinkled with crushed chalk) every 2–3 days. After 1 month, estivation was induced in one group of snails by placing them in a container with dry paper towels and no food, whereas active snails were maintained under the same conditions as previously. After 10 days, active and estivating snails were sacrificed by decapitation and foot muscle and hepatopancreas were rapidly dissected out, immediately frozen in liquid nitrogen and stored at ~70°C until use.

**Sample preparation**

Frozen samples were homogenized (1:10, w:v) using a Polytron PT1000 homogenizer (Brinkmann Instruments, Rexdale, ON, Canada) in ice-cold buffer A (25 mmol l–1 imidazole pH 8.0, 10% v:v glycerol, 100 mmol l–1 sucrose, 10 mmol l–1 2-mercaptoethanol) that also contained 0.2% (w:v) sodium deoxycholate, 2 mmol l–1 EDTA, 2 mmol l–1 EGTA and 25 mmol l–1 NaF; a sample of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added just before homogenizing. The concentrations of EDTA, EGTA and NaF used were optimized in preliminary studies. After centrifugation at 10 000 g, supernatant samples were removed and desalted by low speed centrifugation through small columns of Sephadex G25 to remove endogenous ions and free phosphate. The columns were typically equilibrated in buffer A containing EDTA, EGTA and NaF but these were omitted in extracts that were to be used in incubations to promote phosphorylation and dephosphorylation of Na7/K+-ATPase. Extracts were stored on ice (4°C) until assay.

**The ammonium molybdate/Malachite Green system of inorganic phosphate detection**

Na7/K+-ATPase activity was measured as the difference between total ATPase activity and ouabain-insensitive (1 mmol l–1) activity, ouabain being a known specific inhibitor of Na7/K+-ATPase. Activity was determined as the amount of free phosphate produced as detected using the ammonium molybdate/Malachite Green phosphate complexing dye. Reagent was prepared and colorimetric assays were performed described by Ekman and Jager (1993). All glassware was
were prepared as 1:1 molar mixtures with MgCl₂. Initial assays were initiated by adding 25·
reaction time, enzyme amount and color development. Experiments were done to optimize and determine linearity of
triplicate and averaged. Activity is reported as nmol
4·h followed by assessing changes in
(Protocol modified from Storey, 1994). Incubation time was
phosphorylation or protein dephosphorylation
then incubated under conditions that promoted either protein
enzyme activity, tissue extracts were prepared in buffer A and
containing 5·mmol·l⁻¹ MgCl₂, 5·mmol·l⁻¹ Mg.ATP and
25·mmol·l⁻¹ imidazole-HCl, pH 7.4. All stock ATP solutions
were prepared as 1:1 molar mixtures with MgCl₂. Initial experiments were done to optimize and determine linearity of reaction time, enzyme amount and color development. Assays were initiated by adding 25·µl of desalted enzyme extract to a final assay volume of 250·µl in an Eppendorf tube. After 10 min at 22°C, a 25·µl sample of reaction mixture was removed and added to a detection tube containing 200·µl dye reagent and 775·µl of ddH₂O. After color development for 10·min, 100·µl samples were transferred to microplates and absorbance at 595 nm was measured using a MR5000 microplate reader (Dynatech Laboratories, Chantilly, VA, USA) and Biolinx 2.0 software. Phosphate production was determined by comparison with a KH₂PO₄ standard curve. All samples were measured in triplicate and averaged. Activity is reported as nmol phosphate released min⁻¹ mg⁻¹ soluble protein (mU mg⁻¹). Protein concentration was quantified using the Coomassie Blue dye binding method and the BioRad prepared reagent with a standard curve of bovine serum albumin. Determination of enzyme kinetic parameters (S₀.₅, Kₘ, Kₐ, I₅₀, Cₐ) used the Microplate Analysis and Kinetics 3.51 software programs (Brooks, 1992; Brooks, 1994).

Phosphorylation and dephosphorylation of Na⁺/K⁺-ATPase

To assess the effects of protein phosphorylation state on enzyme activity, tissue extracts were prepared in buffer A and then incubated under conditions that promoted either protein phosphorylation or protein dephosphorylation in vitro (protocol modified from Storey, 1994). Incubation time was 4·h followed by assessing changes in Vₘₐₓ. Enzyme extracts were mixed 1:2 (v:v) with buffer A with additions as follows,

1. Control incubations: 25·mmol·l⁻¹ NaF, 2·mmol·l⁻¹ EDTA, 2·mmol·l⁻¹ EGTA to inhibit all phosphatase and kinase activities.

2. Stimulation of endogenous protein kinase activities: 5·mmol·l⁻¹ Mg.ATP, 2·mmol·l⁻¹ NaF and either (1) 1·mmol·l⁻¹ cAMP to stimulate protein kinase A (PKA); (2) 1·mmol·l⁻¹ cGMP to stimulate protein kinase G (PKG); (3) 1·3·mmol·l⁻¹ CaCl₂ + 7·µg·ml⁻¹ phorbol myristate acetate to stimulate protein kinase C (PKC); (4) 1·mmol·l⁻¹ AMP to stimulate AMP-activated protein kinase (AMPK); or (5) 1 IU of calmodulin activity/incubation tube + 1·3·mmol·l⁻¹ CaCl₂ to stimulate calcium/calmodulin-dependent protein kinase (CaMK).

3. Stimulation of endogenous protein phosphatases (PPase): (1) for total PPase activity: 5·mmol·l⁻¹ CaCl₂ + 5·mmol·l⁻¹ MgCl₂; (2) for total tyrosine PPase: 25·mmol·l⁻¹ NaF (inhibits Ser/Thr PPases); (3) for PP1: 2·5·mmol·l⁻¹ okadaic acid (inhibits PP2A) + 2·mmol·l⁻¹ EDTA + 2·mmol·l⁻¹ EGTA; (4) for PP1 + PP2A: 2·mmol·l⁻¹ EDTA and 2·mmol·l⁻¹ EGTA; (5) for total PPase minus PP1/PP2A: 1·µmol·l⁻¹ okadaic acid + 5·mmol·l⁻¹ CaCl₂ + 5·mmol·l⁻¹ MgCl₂; (6) for PP2B: 1·µmol·l⁻¹ okadaic acid + 5·mmol·l⁻¹ CaCl₂ + 2·mmol·l⁻¹ EDTA; (7) for PP2C: 1·µmol·l⁻¹ okadaic acid + 5·mmol·l⁻¹ MgCl₂ + 2·mmol·l⁻¹ EDTA (7) for full dephosphorylation: incubation with 1 IU calf intestinal alkaline phosphatase (AP) + 5·mmol·l⁻¹ MgCl₂ + 5·mmol·l⁻¹ CaCl₂.

4. Stimulation of endogenous PKA, PKC, and PKG as a function of CaCl₂ concentration: same conditions as in B, but at concentrations of CaCl₂ ranging from 0–6.25·mmol·l⁻¹.

After incubation, all samples were desalted by low speed centrifugation through small columns of Sephadex G25 equilibrated in buffer A followed by assay under optimum conditions.

Arrhenius analysis

Enzyme assays were performed under Vₘₐₓ conditions over a temperature range from 4–68°C. Activation energy (Eₐ) was determined in kJ mol⁻¹ for linear portions of the relationship.

SDS gel electrophoresis and western blotting

Samples of frozen snail tissue were homogenized 1:10 (w:v) in cold (4°C) buffer that was designed to inhibit endogenous protein phosphatase, protein kinase and protease activities: 25·mmol·l⁻¹ Hepes, pH 7.0, 100·mmol·l⁻¹ sucrose, 10% (v:v) glycerol, 0.2% (w:v) sodium deoxycholate, 2·mmol·l⁻¹ EDTA, 2·mmol·l⁻¹ EGTA, 2·mmol·l⁻¹ NaF, 1·mmol·l⁻¹ Na₃VO₄ and protease inhibitors added at the time of homogenization (1·µmol·l⁻¹ each of PMSF, leupeptin and aprotinin). Homogenates were centrifuged at 10,000·g for 10·min and supernatants were removed. Soluble protein concentration was determined using the Coomassie Blue dye-binding method with the BioRad prepared reagent. A 250·µl sample of supernatant was mixed 1:1 (v:v) with freshly prepared 2× SDS-PAGE loading buffer [100·mmol·l⁻¹ Tris-HCl, pH 6.8, 4% (w:v) SDS, 20% (v:v) glycerol, 0.2% (w:v) Bromophenol Blue, 10% (v:v) 2-mercaptoethanol] and boiled for 5·min. Samples were immediately cold-snaped and stored until use at –20°C. Samples containing 15·µg of soluble protein were loaded onto SDS-polyacrylamide gels (10% resolving gel, 5% stacking gel). Samples were electrophoresed at 200·V for ~1·h until the dye front reached the bottom of the gel in 1× running buffer.
Proteins were wet-transferred to PVDF membrane (300 mA for 3 h at 4°C) using the Bio-Rad Mini Trans-Blot Cell apparatus (Hercules, CA, USA). The transfer buffer contained 25 mmol l⁻¹ Tris-base (pH 8.8), 192 mmol l⁻¹ glycine and 20% (v:v) methanol, chilled to 4°C. Membranes were then blocked with 1% 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% Tween 20) for 1 h at 4°C. Membranes were then incubated overnight at 4°C with α5-Na⁺/K⁺-ATPase primary antibody (mouse anti-chicken IgG serum antibody; DSHB, University of Iowa, USA) diluted 1:1000 (v:v) in TBST. After three 5-min washes with TBST, the membrane was incubated with HRP-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 1:2000 in TBST) for 2–3 h at room temperature, followed by three 5-min washes in TBST. Proteins were visualized using Western Lightning Chemiluminescence Plus reagents (Perkin Elmer, Boston, MA, USA) following the manufacturer’s protocols, and the ECL signal was detected using a ChemiGenius (Syngene, Frederick, MO, USA) after exposures of 5 min (foot muscle) or 15 min (hepatopancreas) and quantified using the associated GeneTools software (v3.00.02). Subsequently, the blot was stained with Coomassie Blue [0.25% (w:v) Coomassie Blue, 50% (v:v) methanol, 7.5% (v:v) acetic acid] for 1 h and destained overnight with 25% (v:v) methanol, 10% (v:v) acetic acid. Three Coomassie-stained bands that did not differ in intensity when scanned between active and estivating conditions were used to normalize the corresponding intensity of the immuno-reacting band in each lane to correct for any unequal protein loading. BioRad Kaleidoscope pre-stained markers were run in one lane of each gel to assess the subunit molecular mass of the test protein.

**Pulse proteolysis of Na⁺/K⁺-ATPase**

To assess the differences in the structural stability of Na⁺/K⁺-ATPase between active and estivating snails, the enzyme was subjected to urea-dependent denaturation and subsequent thermolysin-mediated proteolysis, using methods adapted from Park and Marqusee (2005) and Ramnanan and Storey (2006). Tissue extracts were homogenized in buffer B (buffer A that also contained 0.2% (w:v) sodium deoxycholate, 2 mmol l⁻¹ EDTA, 2 mmol l⁻¹ EGTA and 25 mmol l⁻¹ NaF but without the addition of PMSF), centrifuged, and 50 μl aliquots of supernatant were incubated with 100 μl of a urea solution made up in buffer B. After 12 h incubation at room temperature, extracts were treated with 10 μl of 10 g l⁻¹ thermolysin (Sigma; stock prepared with 2.5 mol l⁻¹ NaCl and 10 mmol l⁻¹ CaCl₂). Thermolysin activity was halted after 10 min by the addition of 20 μl of 50 mmol l⁻¹ EDTA (pH 8.0). Enzyme protein content remaining after protease treatment was measured by western blotting. Parallel experiments were performed to determine enzyme activity as a function of urea concentration. Parameters of protein unfolding (Cₘ) and activity inhibition (I₅₀) by urea were calculated as described above for Na⁺/K⁺-ATPase assay.

**Results**

**Optimization of experimental conditions**

Initial trials were used to optimize the amount of Na⁺/K⁺-ATPase activity that could be recovered from O. lactea hepatopancreas and foot muscle. Inclusion of 2 mmol l⁻¹ EDTA, 2 mmol l⁻¹ EGTA (to inhibit protein kinases) and 25 mmol l⁻¹ NaF (to inhibit protein phosphatases) in the homogenization buffer did not significantly alter recoverable Na⁺/K⁺-ATPase activity but inclusion of β-glycerophosphate or sodium vanadate strongly reduced recoverable activity. Therefore, the standard preparation of tissue extracts included EDTA/EGTA/NaF. Addition of detergents (deoxycholate, Brij-35, CHAPS) was also tested; 0.2% (w:v) deoxycholate increased recoverable activity and was also included in standard preparations. Fig. 1 shows the effect of pH on Na⁺/K⁺-ATPase activity in hepatopancreas. The pH optimum for the enzyme from active snails was 7.4 but the optimum decreased substantially to between 6.8–7.0 for the enzyme isolated from 10-day estivating snails. Foot muscle enzyme behaved similarly (data not shown) with pH optima of 7.2 and 7.0 for the enzyme from active and estivated snails, respectively. Other trials optimized substrate and ion concentrations (20 mmol l⁻¹ KCl, 100 mmol l⁻¹ NaCl, 5 mmol l⁻¹ MgCl₂, and 5 mmol l⁻¹ Mg²⁺-ATP), volume of enzyme extract used (25 μl added to 225 μl assay mixture for 250 μl final volume), reaction time (10 min), and time for color development (10 min).

**Determination of kinetic constants**

The kinetic properties of Na⁺/K⁺-ATPase from both foot muscle and hepatopancreas were characterized in extracts from active and estivating snails. Table 1 shows that the
Na$^+$/K$^+$-ATPase in estivating snails

The activity of Na$^+$/K$^+$-ATPase in estivating snails was reduced significantly compared to active snails. The maximal velocity of the enzyme was reduced to 67% and 65% of the activity in foot muscle and hepatopancreas, respectively. The enzyme displayed sigmoidal kinetics with respect to ATP in both tissues, with Hill coefficients of 1.6–2.7 for the foot enzyme and 1.7–2.2 for hepatopancreas. Generally, kinetic data were consistent with the presence of a less active form of the enzyme in the estivated state. Thus, enzyme affinity for ATP was reduced during estivation; the $S_{0.5}$ ATP increased by 1.4-fold in both foot muscle and hepatopancreas Na$^+$/K$^+$-ATPase, compared with the enzyme from active snails. Similarly, foot muscle from estivated snails showed a 1.8-fold increase in $K_m$ Na$^+$ and a 1.6-fold increase in $K_a$ Mg$^{2+}$ as compared with the enzyme from active snails. In hepatopancreas, the $I_{50}$ $K^+$ was 1.7-fold higher in estivating versus active snails.

Affinity for ATP generally increased during estivation: the $S_{0.5}$ ATP increased by 1.4-fold in estivation for both foot muscle and hepatopancreas Na$^+$/K$^+$-ATPase, compared with the enzyme from active snails. Similarly, foot muscle from estivated snails showed a 1.8-fold increase in $K_m$ Na$^+$ and a 1.6-fold increase in $K_a$ Mg$^{2+}$ as compared with the enzyme from active snails. In hepatopancreas, the $I_{50}$ $K^+$ was 1.7-fold higher in estivating versus active snails.

Na$^+$/K$^+$-ATPase affinity for ATP was also studied as a function of temperature (Fig. 3). Affinity for ATP generally increased ($S_{0.5}$ decreased) with increasing temperature for the enzyme from active snails, with highest affinity measured at 30°C. However, the enzyme from estivated snails did not show a significant change in affinity for ATP with temperature. In both tissues the enzyme from estivated snails had significantly higher $S_{0.5}$ ATP values at each of higher assay temperatures (22°C, 30°C, 40°C), as compared with the corresponding values for active snails (Student’s t-test, $P<0.01$).

Kinetic parameters determined at optimal concentrations of other ions and cosubstrates.

Table 1. Kinetic parameters for Na$^+$/K$^+$-ATPase from foot muscle and hepatopancreas of active and 10-day estivated Otala lactea

<table>
<thead>
<tr>
<th></th>
<th>Foot muscle</th>
<th>Hepatopancreas</th>
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<tbody>
<tr>
<td></td>
<td>Active</td>
<td>Estivated</td>
</tr>
<tr>
<td>$V_{max}$ (mU mg$^{-1}$ soluble protein)</td>
<td>31.0±1.6</td>
<td>20.4±1.6*</td>
</tr>
<tr>
<td>$S_{0.5}$ Mg.ATP (mmol l$^{-1}$)</td>
<td>0.75±0.05</td>
<td>1.07±0.06*</td>
</tr>
<tr>
<td>$K_m$ Na$^+$ (mmol l$^{-1}$)</td>
<td>38±4</td>
<td>67±7*</td>
</tr>
<tr>
<td>$K_m$ K$^+$ (mmol l$^{-1}$)</td>
<td>11±2</td>
<td>16±3</td>
</tr>
<tr>
<td>$I_{50}$ Na$^+$ (mmol l$^{-1}$)</td>
<td>1.7±0.3</td>
<td>2.7±0.3*</td>
</tr>
<tr>
<td>$I_{50}$ Mg$^{2+}$ (mmol l$^{-1}$)</td>
<td>258±33</td>
<td>325±57</td>
</tr>
<tr>
<td>$I_{50}$ K$^+$ (mmol l$^{-1}$)</td>
<td>82±11</td>
<td>167±32</td>
</tr>
<tr>
<td>$I_{50}$ Mg$^{2+}$ (mmol l$^{-1}$)</td>
<td>32±5</td>
<td>45±11</td>
</tr>
<tr>
<td>$I_{50}$ Urea (mol l$^{-1}$)</td>
<td>3.3±0.4</td>
<td>4.4±0.4*</td>
</tr>
<tr>
<td>$C_m$ Urea (mol l$^{-1}$)</td>
<td>3.9±0.5</td>
<td>3.9±0.4</td>
</tr>
<tr>
<td>$E_A$ (kJ mol$^{-1}$)</td>
<td>18.6±2.9</td>
<td>27.1±3.1*</td>
</tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Active</td>
<td>Estivated</td>
</tr>
<tr>
<td>$I_{50}$ Urea (mol l$^{-1}$)</td>
<td>3.9±0.4</td>
<td>3.4±0.7</td>
</tr>
<tr>
<td>$E_A$ (kJ mol$^{-1}$)</td>
<td>22.2±3.5</td>
<td>31.9±3.1*</td>
</tr>
</tbody>
</table>

Assays were conducted at 22°C and data are mean ± s.e.m. ($N=4$), except for foot muscle $V_{max}$ and $K_m$ (Mg$^{2+}$-ATP) ($N=9$) and for $I_{50}$ (Urea) and $C_m$ (Urea) ($N=3$).

$S_{0.5}$ and $K_m$ are substrate concentrations that produce half-maximal velocity for sigmoidal vs hyperbolic relationships, respectively. $K_a$ is the activator concentration that produces half-maximal activation, $I_{50}$ is the concentration of inhibitor that reduces enzyme activity by 50%, and $C_m$ is the denaturant concentration required to unfold 50% of the protein. $E_A$ is the activation energy calculated from Arrhenius plots.

Kinetic parameters were determined at optimal concentrations of other ions and cosubstrates.

*Significantly different from the corresponding value for active snails, $P<0.05$.

Fig. 2. Na$^+$/K$^+$-ATPase activity in O. lactea (A) foot muscle (mean ± s.e.m., $N=9$) and (B) hepatopancreas (mean ± s.e.m., $N=4$) as a function of Mg.ATP concentration.

In vitro incubations to stimulate endogenous kinases and phosphatases on Na$^+$/K$^+$-ATPase

The significant differences in kinetic properties of Na$^+$/K$^+$-ATPase between active and estivated states could be due to an estivation-mediated covalent modification of the enzyme, predictably as a result of reversible protein phosphorylation.
To test this, the enzyme was incubated \textit{in vitro} under conditions that stimulated the activities of endogenous protein kinases or protein phosphatases. The effects of these incubations on \textit{Na}^+/\textit{K}^+-\text{ATPase} activity in foot muscle extracts from active and estivated snails is shown in Fig. 4. Incubation of foot muscle \textit{Na}^+/\textit{K}^+-\text{ATPase} from active snails under conditions that stimulated protein kinases A, C or G resulted in significant decreases in enzyme $V_{\max}$ activity to values that were 51\%, 42\% and 46\% of the corresponding value in control incubations (Fig. 4A). By contrast, conditions that stimulated AMPK or CaMK did not alter \textit{Na}^+/\textit{K}^+-\text{ATPase} activity. The same pattern of results was seen when extracts of estivated foot muscle were incubated; \textit{Na}^+/\textit{K}^+-\text{ATPase} activity was reduced to 61\%, 67\% and 65\% of the corresponding value in control incubations by stimulation of PKA, PKC and PKG, respectively. Fig. 4B shows the effects of incubations that promoted the activity of endogenous phosphatases. In no case did stimulation of phosphatases have an effect on \textit{Na}^+/\textit{K}^+-\text{ATPase} activity from active snails. However, stimulation of phosphatases elevated the activity of \textit{Na}^+/\textit{K}^+-\text{ATPase} from estivated snails under conditions that (a) stimulated total phosphatase activity, (b) stimulated PP1-type activity, or (c) stimulated PP1/PP2A-type activity.

In each of these cases, \textit{Na}^+/\textit{K}^+-\text{ATPase} activity increased by ~1.7-fold over activity in control incubations. Similarly, incubation with calf intestinal alkaline phosphatase (AP) also increased activity, by 1.8-fold as compared with the control incubation.

Similar results were seen in incubation studies with hepatopancreas extracts from active and 10-day estivated snails (Fig. 5). Stimulation of endogenous protein kinases decreased \textit{Na}^+/\textit{K}^+-\text{ATPase} maximal activity in three out of five cases. Stimulation of PKA, PKC and PKG decreased activity to 41\%, 42\% and 45\% of the \textit{Na}^+/\textit{K}^+-\text{ATPase} activity seen in control incubations whereas stimulation of AMPK or CaMK again had no effect (Fig. 5A). However, unlike the situation with foot muscle extracts, \textit{in vitro} incubation of hepatopancreas extracts from estivated snails under conditions that stimulated protein kinases did not alter enzyme activity. Incubations promoting endogenous phosphatase activity again had no effect on \textit{Na}^+/\textit{K}^+-\text{ATPase} $V_{\max}$ in extracts from active
Na+/K+-ATPase in estivating snails (Fig. 5B). However, stimulation of several classes of phosphatases significantly increased Na+/K+-ATPase activity in extracts from estivated snails. This included stimulation of (a) total phosphatase activity, (b) PP1-type activity, (c) PP1/PP2A-type activity, and (d) total phosphatase activity minus PP1/PP2A-type activity, all of which produced ~1.8-fold increases in Na+/K+-ATPase activity. Furthermore, stimulation of PP2C-type activity and incubation with AP led to 1.4-fold and 1.7-fold increases in enzyme activity, respectively, for Na+/K+-ATPase from estivated snails.

In mammalian systems PKA- and PKC-mediated phosphorylation can either stimulate or inhibit Na+/K+-ATPase activity, often dependent on calcium concentration (Cheng et al., 1999). To determine if calcium influenced the effect of phosphorylation on Na+/K+-ATPase activity in extracts from estivated snails. This included stimulation of (a) total phosphatase activity, (b) PP1-type activity, (c) PP1/PP2A-type activity, and (d) total phosphatase activity minus PP1/PP2A-type activity, all of which produced ~1.8-fold increases in Na+/K+-ATPase activity. Furthermore, stimulation of PP2C-type activity and incubation with AP led to 1.4-fold and 1.7-fold increases in activity, respectively, for Na+/K+-ATPase from estivated snails.

Arrhenius plots

The effects of temperature on Na+/K+-ATPase activity are shown as Arrhenius plots in Fig. 7 for both the foot muscle (Fig. 7A) and hepatopancreas (Fig. 7B) enzymes. The plots were linear between 4–33°C in both cases. However, activity did not continue to increase at higher temperatures, suggesting that some high temperature degradation was occurring. Activation energies calculated from the linear parts of the relationship revealed a 1.45-fold higher activation energy for the enzyme from estivated snails, compared with active snails, in both tissues (Table 1).

Na+/K+-ATPase protein content

To determine if the change in Na+/K+-ATPase activity seen during estivation was associated with a change in the amount of Na+/K+-ATPase protein in tissues, western blotting was used with polyclonal antibodies raised against the alpha subunit of Na+/K+-ATPase. The mouse anti-chicken Na+/K+-ATPase antibody recognized an ~100 kDa band from both tissues, consistent with the known size of the alpha subunit. Fig. 8 shows that the amount of Na+/K+-ATPase protein did not differ between active and estivated states in either tissue.

Na+/K+-ATPase protein stability

To determine if there are differences in the structural stability of snail Na+/K+-ATPase between active and estivating states, the
technique of pulse proteolysis was used. Following incubation of the enzyme with multiple concentrations of urea to achieve different degrees of protein unfolding, the enzyme was given a short pulse of proteolysis with thermolysin and then the amount of protein remaining was measured by western blotting. The results revealed that there was no significant difference in resistance of Na⁺/K⁺-ATPase to unfolding/denaturation by urea between active and estivating states in either foot muscle (Fig. 9) or hepatopancreas (not shown). The $C_m$ value (the amount of urea required to unfold 50% of the protein, rendering it susceptible to thermolysin proteolysis) did not differ between active and estivating states in either tissue (Fig. 9A; Table 1). However, the $I_{50}$ value (the amount of urea required to inhibit enzyme activity by 50%) was 1.3-fold higher for Na⁺/K⁺-ATPase from foot muscle of estivating snails (Fig. 9B, Table 1). Inhibition of hepatopancreas Na⁺/K⁺-ATPase by urea was similar in active and estivating states (Table 1).

**Discussion**

Na⁺/K⁺-ATPase harnesses the energy from the hydrolysis of one molecule of ATP to fuel one cycle of transport in which three K⁺ ions are pumped into the cell interior while two Na⁺ ions are actively transported out (Humphrey et al., 2002). Maintenance of membrane potential difference is essential for nerve and muscle cell excitability, for the proper functioning of renal tissue, and volume regulation, pH balance and other functions characteristic of healthy activity in all living cells (Mobasheri et al., 2000). Species that can reversibly enter a hypometabolic state must maintain all of these functions during dormancy but must do so with a much lower net turnover of ATP. Activities of a multitude of cellular functions are turned down in a coordinated manner (Storey and Storey, 2004) with particular attention to highly energy expensive functions such as the ATP costs of maintaining membrane potential difference. Hence, for example, a reduction in facilitated transmembrane ion flow through channels (channel arrest) must be co-ordinated with the suppression of active ion transport by ion pumps to facilitate long term survival in a

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**Fig. 7.** Arrhenius plots showing Na⁺/K⁺-ATPase maximal activity as a function of temperature (Kelvin) for (A) foot muscle and (B) hepatopancreas from *O. lactea*. Values are means ± s.e.m., N=4 independent preparations of enzyme. Assay mixtures were pre-incubated for 5 min in water baths set to specified temperatures prior to initiating reactions by the addition of Mg$$^2$$-ATP. Assays were conducted at maximal substrate concentrations. Arrhenius activation energy ($E_A$) was calculated from the linear part of the graphs.

**Fig. 8.** Western blots showing Na⁺/K⁺-ATPase protein content in (A) foot muscle and (B) hepatopancreas of active and 10-day estivated snails. Representative blots are shown for four independent samples. Band densities were normalized against the combined measured densities of three protein bands stained strongly with Coomassie Blue that did not change between control and estivated states, and histograms show the mean normalized band densities (± s.e.m., N=10 for foot muscle, N=8 for hepatopancreas). The band shown corresponds to ~100 kDa, as determined from a standard curve developed using Kaleidoscope protein size markers, and is consistent with the expected alpha subunit size of Na⁺/K⁺-ATPase.
hypometabolic state. A key mechanism of metabolic rate depression across phylogeny has proved to be reversible protein phosphorylation; the activities of multiple enzymes and functional proteins are suppressed in a coordinated manner by the addition (or removal) of covalently bound phosphate groups that produce major changes in activity states (Storey and Storey, 2004).

Previous studies have documented an overall change in the phosphorylation pattern of cellular proteins during estivation in O. lactea (Brooks and Storey, 1995) as well as specific phosphorylation-mediated suppression of the activities of selected enzymes of carbohydrate catabolism (Whitwam and Storey, 1990, 1991; Brooks and Storey, 1992, 1997). The present study provides evidence that reversible phosphorylation control during O. lactea estivation also extends to Na+/K+-ATPase, an enzyme that is one of the greatest energy consumers in cells. The maximal activity of Na+/K+-ATPase in muscle and hepatopancreas of estivating snails was reduced by about one-third as compared with active O. lactea. A suppression of Na+/K+-ATPase activity by a similar magnitude also occurred for Na+/K+-ATPase in another hypometabolic system, the hibernating ground squirrel Spermophilus lateralis (MacDonald and Storey, 1999). Known mechanisms for the acute regulation of the Na+/K+ pump activity can include increased availability of Na+ and K+ at the membrane, increased affinity for intracellular Na+ (as a result of stimulation by hormone or pharmacological agents), differential distribution of the enzyme between sarcolemma and endosome fractions, and increased turnover number of the enzyme (Claussen, 2003). In the longer term, changes in protein synthesis or degradation also influence the net amount of Na+/K+-ATPase protein. Some of these mechanisms cannot be evaluated in a soluble enzyme system. The present study evaluated two aspects of Na+/K+-ATPase in estivation: (a) the amount of enzyme protein present, and (b) stable modification of the enzyme. Western immunoblotting tested the first possibility and revealed that the intensity of the ~100 kDa band representing the alpha subunit of Na+/K+-ATPase was the same in active and estivating snails. Hence, this indicates that the amount of Na+/K+-ATPase protein did not change in O. lactea tissues during estivation and supports the idea that a stable modification of the enzyme protein may be responsible for the change in activity state during estivation.

Stable changes to several kinetic parameters of Na+/K+-ATPase occurred between active and estivated states. For Na+/K+-ATPase from estivated snails, as compared with active animals, these included reduced affinity for ATP in both foot muscle and hepatopancreas, reduced affinity for Na+ and Mg2+ by the foot muscle enzyme, and increased Arrhenius activation energy for the enzyme from both tissues. The reduced affinities for substrates (ATP, Na+) and co-substrates (Mg2+) suggest that the enzyme in estivating snails is a less active form which is consistent with metabolic rate depression. Notably, Arrhenius analysis revealed a linear relationship up to 35–38°C, a considerably higher break point in the temperature–activity relationship when compared to vertebrate Na+/K+-ATPase, which has been studied as the soluble enzyme or the enzyme in membrane preparations (Esmann and Skou, 1988) or as a partially purified enzyme in microsomes (Matsuda and Iwata, 1985). The difference between the Arrhenius profile in this study and previous Na+/K+-ATPase studies might be attributed to the fact that O. lactea encounters a much wider range of environmental temperatures in nature and needs an enzyme that functions optimally across a broad range of temperatures.

Stable changes in the kinetic properties of an enzyme between two physiological states are often indicative of a post-translational modification of the protein. Vertebrate Na+/K+-ATPase is subject to reversible phosphorylation of the catalytic alpha-subunit by several protein kinases (PKA, PKC, PKG, tyrosine kinase) (Beguin et al., 1994; Ewart and Klip, 1995; Bertorello and Katz, 1995; Lopina, 2001). Phosphorylation by PKA and PKC has been described both in vitro and in vivo as generally having an inhibitory affect on the rate of ATP hydrolysis (Tung et al., 1990; Bertorello et al., 1991; MacDonald and Storey, 1999; Feschenko et al., 2000; Khundmirl et al., 2005). However, in some vertebrate studies,
PKA or PKC activation led to an increase in Na⁺/K⁺-ATPase activity (Vasilets et al., 1992; Breton et al., 1994; Sampson et al., 1994; Delamere et al., 1997). Other research has shown that stimulation of pump activity by phosphorylation is dependent on calcium concentration (Gao et al., 1992; Cheng et al., 1999) or oxygen supply (Kirovtcheva et al., 1999). The β-subunit does not appear to be a target for phosphorylation (Geering, 2001) but the newly recognized γ-subunit (FXYD protein) as well as other proteins that can bind to Na⁺/K⁺-ATPase can also be susceptible to phosphorylation control (Lopina, 2001; Geering et al., 2003).

We adapted an in vitro experimental design used previously (Storey, 1994) to assess the protein kinases and protein phosphatases that might act on O. lactea Na⁺/K⁺-ATPase. The in vitro studies showed that (a) Na⁺/K⁺-ATPase activity in extracts from active snails decreased after incubation under conditions that promoted the activities of selected endogenous serine/threonine specific kinases; (b) promoting PKC activity inhibited enzyme activity at all Ca²⁺ concentrations tested; and (c) PKA- and PKG-mediated reductions in enzyme activity was independent of calcium. Incubations that stimulated PKA, PKC or PKG reduced Na⁺/K⁺-ATPase activity in both foot muscle and hepatopancreas extracts to levels that were very similar to the activities found in tissue extracts from estivating snails. This implicates phosphorylation by one or more of these protein kinases in the natural mode of suppression of Na⁺/K⁺-ATPase activity during estivation and indicates that the less active form found during estivation is probably a phosphoprotein. In addition to modifying enzyme kinetics, a change in phosphorylation state can confer a change in conformational stability, as has been observed in glucose-6-phosphate dehydrogenase, another enzyme regulated by reversible phosphorylation in this animal (Ramnanan and Storey, 2006). However, the modification of Na⁺/K⁺-ATPase activity observed in estivation was not accompanied by a change in enzyme structural stability, since both the active and estivated forms of the enzyme showed similar urea denaturation kinetics in both tissues.

Interestingly, Na⁺/K⁺-ATPase activity in foot muscle (but not hepatopancreas) extracts from estivating snails could also be further suppressed after in vitro incubation under conditions that stimulated PKA, PKC or PKG. This could occur for one of two reasons: (a) Na⁺/K⁺-ATPase activity in foot muscle of estivating snails is not fully suppressed to its lowest possible activity state, as it is in hepatopancreas, or (b) the effects of different types of protein kinases on foot muscle Na⁺/K⁺-ATPase are additive. With respect to the latter, only one protein kinase may be responsible for the change in enzyme activity in vivo during estivation but stimulation of other kinases in vitro could have an additive effect to further reduce activity. It has been established that Na⁺/K⁺-ATPase can be phosphorylated in vivo and in vitro by several protein kinases (Lopina, 2001). PKG is of particular interest to us because it has been implicated in regulating metabolic responses to anoxia (another situation of metabolic rate depression) in marine molluscs including the marine whelkBusycon canaliculatum (Brooks and Storey, 1990), the mussel Mytilus edulis (Michaelidis and Storey, 1990, 1991), and the marine snailLittorina littorea (Larade and Storey, 2002). Phosphorylation of PKF and PK in O. lactea hepatopancreas extracts was also stimulated by incubation with Mg-ATP plus the second messengers of PKA, PKG or PKC (Whitwam and Storey, 1990; Whitwam and Storey, 1991) but further studies showed that changes in PKA activity and levels of cAMP and inositol 1,4,5-trisphosphate in O. lactea hepatopancreas were not consistent with either PKA or PKC being the kinases involved in mediating estivation-induced phosphorylation of PK (Brooks and Storey, 1994). This suggested that PKG is probably the protein kinase that controls PK in vivo in O. lactea during estivation and this may also be true of Na⁺/K⁺-ATPase. PKG control of O. lactea Na⁺/K⁺-ATPase could provide the estivation-specific regulation of the enzyme during metabolic rate depression, leaving PKA or PKC to regulate the enzyme in response to other metabolic signals.

The probable role of reversible phosphorylation in the control of O. lactea Na⁺/K⁺-ATPase during estivation was further supported by the responses of the enzyme to incubations with protein phosphatases. Stimulation of serine/threonine protein phosphatases did not affect the activity of the enzyme from active snails but significantly raised Na⁺/K⁺-ATPase activity in extracts from estivated snails, typically returning the enzyme to activity levels similar to those seen in extracts from active snails. Using specific activators and inhibitors, it appeared that stimulation of a phosphatase with characteristic PP1/PP2A-type behavior was responsible for this effect in both tissues. PP1 can dephosphorylate and activate Na⁺/K⁺-ATPase in rat kidney (Li et al., 1995) and rat choroid plexus (Fisone et al., 1998). Hence, it is possible that PP1 may also be involved in changing Na⁺/K⁺-ATPase activity when snails arise from estivation.

Reversible phosphorylation control of mammalian Na⁺/K⁺-ATPase activity can also come from activating the phosphoproteins DARPP-32 (a 32 kDa dopamine and cAMP regulated phosphoprotein) and inhibitor-1 (I-1), both of which are well-known to be potent inhibitors of PP1 activity when phosphorylated (Higuchi et al., 2000). Hence, by inhibiting PP1, these proteins promote the conversion of Na⁺/K⁺-ATPase to the phosphorylated, less active form. The role of these proteins in controlling Na⁺/K⁺-ATPase during hypometabolism remains to be determined. Dephosphorylation of Na⁺/K⁺-ATPase in human eye lens by protein tyrosine phosphatase-1B was recently reported (Bozulic et al., 2004) but our data suggest that tyrosine-specific phosphatases are probably not involved in O. lactea Na⁺/K⁺-ATPase control since, in the presence of NaF, which potently inhibits all serine/threonine specific phosphatases, no change in enzyme activity was observed. In hepatopancreas extracts, an increase in Na⁺/K⁺-ATPase activity also occurred under conditions that inhibited PP1, but stimulated the activity of a magnesium-activated phosphatase. Thus, a phosphatase activity with PP2C-like characteristics could also be involved in Na⁺/K⁺-ATPase regulation. In general, physiological roles of PP2C...
type enzymes have yet to be firmly categorized, and although PP2C activity is reduced in estivating toads (Cowan et al., 2000), PP2C has yet to be characterized in any estivating invertebrate.

In summary, Na+/K+-ATPase activity was significantly reduced in both foot muscle and hepatopancreas in O. lactea during estivation, suggesting that suppression of the activity of this ATP-expensive membrane ion pump has a key role to play in the transition to the hypometabolic state. This provides evidence that invertebrate systems of hypometabolism also target Na+/K+-ATPase for regulation, similar to previous reports of Na+/K+-ATPase control in hibernating ground squirrels and anoxia-tolerant turtles (Hochachka et al., 1996; MacDonald and Storey, 1999). Suppression of Na+/K+-ATPase during estivation was not a function of changes in the amount of the catalytic α subunit of the protein but appeared to be linked to posttranslational modification of the enzyme. The enzyme from estivating snails was characterized by reduced maximal velocity, increased $K_m$ ATP, and elevated activation energy in both tissues, as compared with active snails, as well as by decreased affinity for sodium and magnesium in foot muscle. In vitro incubations that manipulated endogenous kinase and phosphatase activities implicated PKA, PKC or PKG action in Na+/K+-ATPase suppression during estivation whereas PP1 or PP2A phosphatases may mediate the reactivation of the enzyme during arousal from estivation.

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