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FACTORS AFFECTING MEMBRANE PERMEABILITY AND IONIC HOMEOSTASIS IN THE COLD-SUBMERGED FROG

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Summary

Frogs (*Rana temporaria*) were submerged at 3 °C in either normoxic (P_{O_2}=155 mmHg, P_{O_2}=20 kPa) or hypoxic (P_{O_2}=60 mmHg; P_{O_2}=8 kPa) water for up to 16 weeks, and denied air access, to mimic the conditions of an ice-covered pond during the winter. The activity of the skeletal muscle Na^+/K^+ pump over the first 2 months of hibernation, measured by ouabain-inhibitable $^{22}Na^+$ efflux, was reduced by 30 % during normoxia and by up to 50 % during hypoxia. The reduction in Na^+/K^+ pump activity was accompanied by reductions in passive $^{22}Na^+$ influx and $^{86}Rb^+$ efflux (effectively K^+ efflux) across the sarcolemma. This may be due to a decreased Na^+ permeability of the sarcolemma and a 75 % reduction in K^+ leak mediated by ATP-sensitive K^+ channels (‘K_{ATP}’ channels). The lowered rates of $^{22}Na^+$ and $^{86}Rb^+$ flux are coincident with lowered transmembrane ion gradients for [Na^+] and [K^+], which may also lower Na^+/K^+ pump activity. The dilution of extracellular [Na^+] and intracellular [K^+] may be partially explained by increased water retention by the whole animal, although measurements of skeletal muscle fluid compartments using ^{3}H-labelled inulin suggested that the reduced ion gradients represented a new steady state for skeletal muscle. Conversely, intracellular ion homeostasis within ventricular muscle was maintained at pre-submergence levels, despite a significant increase in tissue water content, with the exception of the hypoxic frogs following 4 months of submergence. Both ventricular muscles and skeletal muscles maintained resting membrane potential at pre-submergence levels throughout the entire period of hibernation. The ability of the skeletal muscle to maintain its resting membrane potential, coincident with decreased Na^+/K^+ pump activity and lowered membrane permeability, provided evidence of functional channel arrest as an energy-sparing strategy during hibernation in the cold-submerged frog.

Key words: hypometabolism, skeletal muscle, cardiac muscle, ion channel arrest, hibernation, frog, *Rana temporaria*, Na^+/K^+-ATPase, homeostasis.

Introduction

When frogs (*Rana temporaria*) are denied access to air at 3 °C, to mimic the conditions of an ice-covered pond in nature, the submerged animals respond by progressively suppressing their aerobic metabolic rates by up to 70 % of those seen in the resting air-breathing animal at 3 °C (Donohoe et al., 1998; Donohoe and Boutilier, 1998). A metabolic rate depression of this magnitude enables the frog to remain largely aerobic even when confronted with an oxygen-limited environment. Clearly, it is advantageous for such animals to avoid fuelling ATP demands using the relatively inefficient anaerobic pathways because this will allow the hibernating animal to conserve fuel reserves and avoid self-pollution by the toxic end-products of anaerobiosis. A prevailing hypothesis is that the major proportion of the reduction in whole-animal metabolic rate is a consequence of hypoperfusion (i.e. oxygen limitation) of the oxyconforming skeletal muscle mass (Donohoe and Boutilier, 1999). However, the cellular basis for metabolic rate depression by the skeletal muscle is poorly understood. The ion channel arrest hypothesis (Hochachka, 1986) proposes that O_2 lack at the cellular level induces decreases in cell membrane permeability in hypoxia-tolerant tissues, thereby reducing the amount of energy required to translocate ions when ATP supplies become limiting. The aim of this paper was to test the hypothesis that decreased Na^+/K^+ pump activity in the skeletal muscle of the hibernating cold-submerged frog could occur as part of an energy-sparing response to hypothermia and hypoxia. Inhibition of Na^+/K^+ pump activity would result in significant reductions in metabolic cost but, in the long term, this inhibition could have deleterious effects upon cells unless combined with a decreased requirement to pump ions; i.e. a less leaky membrane would facilitate reduced leakage of Na^+ into the cell.

Ion leakage across cell membranes occurs as a result of both intracellular and extracellular ions drifting towards their electrochemical equilibrium. The maintenance of a homeostatic intracellular environment requires the

redistribution of these ions through the use of ATP-dependent pumping systems such as Na^+/K^+-ATPase, the energy requirements of which can constitute 20–80 % of the cell's resting metabolic rate (Buck and Hochachka, 1993; Edwards et al., 1989; Priebe et al., 1996) depending on the extent of electrical activity of the tissue (e.g. muscle *versus* brain). In hypoxia-sensitive tissues such as brain, O_2 lack interrupts the normally continuous and high rates of ATP production. Under these conditions, anaerobic generation of ATP is insufficient to supply the demands of the ATP-dependent transporters, resulting in a dissipation of ionic gradients across the cell membrane. This hypoxia-induced membrane failure, as ions drift towards their electrochemical equilibrium, eventually leads to catastrophic cell swelling and death. In contrast, ectothermic facultative anaerobes maintain pre-anoxic ionic gradients (Sick et al., 1982) at the same time as having a lowered rate of ATP turnover (Perez-Pinzon et al., 1992b). Protein synthesis and degradation are generally considered to account for between 60 and 70 % of the ATP production expected from the oxygen consumption of normoxic non-neuronal tissues (Hochachka et al., 1996). However, when hypoxia-tolerant cells (e.g. turtle hepatocytes) are exposed to anoxia, the ATP demand of protein turnover can drop to less than 10 % of the total ATP production (Buck and Hochachka, 1993). At such times, the ATP concentrations of the cells remain constant or may even rise (Donohoe and Boutilier, 1998; Donohoe et al., 1998; Kelly and Storey, 1988), while total normoxic ATP turnover rates decrease by approximately 90 % (Buck and Hochachka, 1993); all this occurs while the resting potential of the membrane is maintained in both neurones and hepatocytes (Doll et al., 1991; Perez-Pinzon et al., 1992a).

In the present study, the flux of Na^+ and K^+ across the sarcolemma was determined isotopically to investigate both the passive membrane permeabilities to Na^+ and K^+ and the Na^+/K^+ pump activity during hypothermia and hypoxia. Furthermore, intracellular and extracellular $[Cl^-]$ was measured to determine whether cellular membrane potential (calculated using the Nernst equation) was maintained in the face of decreased Na^+/K^+ pump activity, as this would provide evidence in support of the ion channel arrest hypothesis (Hochachka, 1986). It was also possible to determine whether there were different responses between the largely inactive skeletal muscle and the actively contracting ventricular muscle.

Materials and methods

All animals used in these experiments were adult male *Rana temporaria* (L.) (25–30 g) collected by commercial suppliers (Blades Biological Co., UK) from a wild population in England.

In vitro *experiments*

Eight groups of six frogs (N=48) were acclimated in water at 3 °C over a 4 week period during which they had direct access to air. The animals were weighed and then submerged in a water-filled Perspex box maintained at 3 °C. Twenty-four of the frogs were supplied with a constant flow of normoxic water (P_{O_2}=155 mmHg=20 kPa) at $1\,l\,min^{-1}$, while the other 24 were supplied with a constant flow of hypoxic water (P_{O_2}=60 mmHg=8 kPa) at $1\,l\,min^{-1}$. At intervals of 1 week, 1 month and 2 months, six 'normoxic' and six 'hypoxic' animals were individually removed from the chamber, weighed and rapidly decapitated prior to surgery. The animals were quiescent throughout the transfer and surgical procedure, which lasted approximately 20 s. The chest cavity was then opened and the heart ventricle removed. A 200 µl blood sample was taken by inserting a capillary tube into the systemic arch. The sample was centrifuged at 15 800 ***g***, and the resultant plasma was frozen in liquid nitrogen and subsequently stored at −80 °C pending analysis for Na^+, K^+ and Cl^- concentrations. For measurements of intracellular ion concentrations, the heart ventricle and gastrocnemius muscles were carefully dissected out under a microscope, trimmed of any extraneous tissue, washed in isotonic sucrose solution, blotted on filter paper, weighed and dried to constant mass at 80 °C. They were then placed in scintillation vials containing $5.5\,mmol\,l^{-1}$ HNO_3 and agitated for 24 h to destroy the cells. $[Na^+]$ and $[K^+]$ were determined for thawed plasma and digested gastrocnemius extracts using a Corning model 410 flame photometer, and $[Cl^-]$ was measured on the same extracts using a Jenway PCLM chloride meter.

Paired sartorius muscles were also taken from each animal at day 0 and at 1 week, 1 month and 2 months of hibernation and prepared for *in vitro* ion flux experiments. Rubidium efflux (effectively K^+ efflux) from sartorius muscle was measured using $^{86}Rb^+$. Sartorius muscles were loaded with $^{86}Rb^+$ by bathing them in Ringer's solution maintained at 3 °C containing ($mmol\,l^{-1}$): NaCl, 106; $NaHCO_3$, 20; KCl, 3.2; NaH_2PO_4, 3.1; $MgSO_4$, 1.4; glucose, 5.0; $CaCl_2$, 1.3; CH_3COONa, 10.0; buffered with 2 % bovine serum albumin, pH 8.0, containing the isotope ($185\,kBq\,ml^{-1}$) for 5 h. The preparations were then pre-washed for 30 min in unlabelled Ringer's solution, and the radioactivity released from the muscles was discarded. Subsequently, the preparations were washed in a series of scintillation vials, containing either 5 ml of Ringer's solution or 5 ml of Ringer's solution plus $1\,mmol\,l^{-1}$ glibenclamide (a K_{ATP} channel inhibitor) at intervals of 5 min. At the end of the experiment, the muscles were digested in glacial acetic acid. The wash-out samples and digested muscles were assayed for Cerenkov radioactivity in a Packard 1600 TR scintillation counter. Internal standards were used to determine quenching; the counting efficiency was 45 %. The uptake of $^{86}Rb^+$ was determined (a non-specific measure of Na^+ pump activity), and the difference between the total (non-specific) and glibenclamide-inhibited $^{86}Rb^+$ efflux provided a measure of $^{86}Rb^+$ efflux (effectively K^+ efflux) mediated by the K_{ATP} channel.

Na^+ efflux in the paired sartorius muscle was measured using a modification of the above procedure. At each sampling period (day 0 and at 1 week, 1 month and 2 months of hibernation), paired sartorius muscles were excised from animals and exposed for 7 h to a Ringer's solution maintained at 3 °C

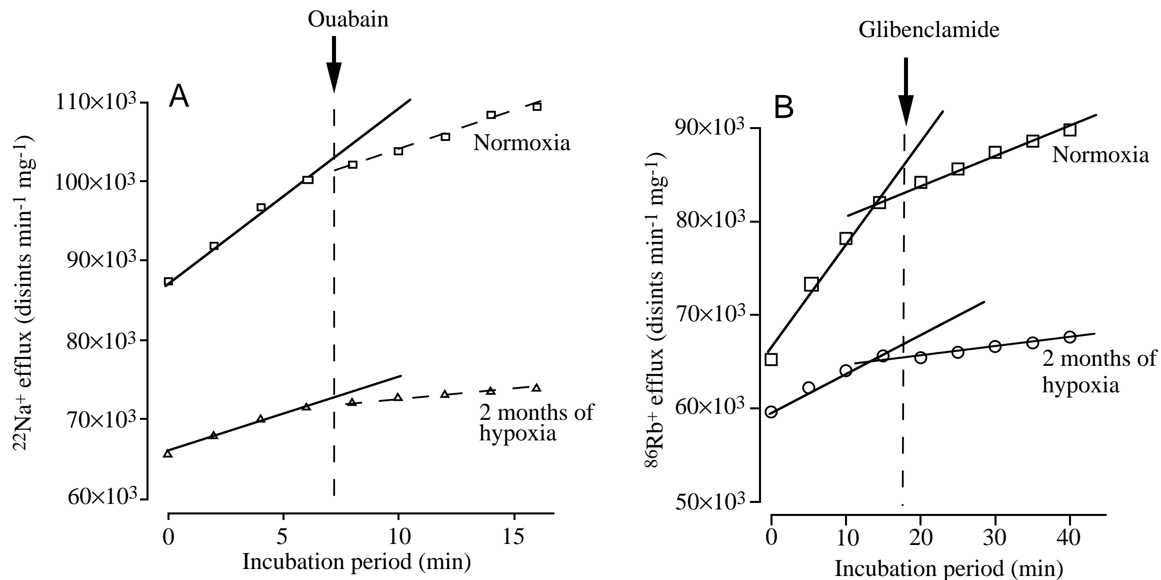

Fig. 1. Representative traces demonstrating the effect of (A) ouabain (a specific inhibitor of the Na^+/K^+ pump) on $^{22}Na^+$ efflux rates and (B) glibenclamide (a specific blocker of the K_{ATP} channel) on $^{86}Rb^+$ (a congener for K^+) efflux rates by sartorius muscles of *Rana temporaria* pre-incubated in anoxic and hyperoxic (P_{O_2}=740 mmHg=98.6 kPa) Ringer's solution containing 185 kBq ml^{-1} $^{22}Na^+$ for 7 h or 185 kBq ml^{-1} $^{86}Rb^+$ for 5 h, respectively. Glibenclamide-inhibitable $^{86}Rb^+$ efflux was calculated as the difference between the rates of $^{86}Rb^+$ efflux measured in samples prior to and after the addition of glibenclamide to the bathing solution. Glibenclamide-inhibitable $^{86}Rb^+$ efflux was taken to reflect the contribution of the K_{ATP} channel to the K^+ leak of the sarcolemma. Ouabain-inhibitable $^{22}Na^+$ efflux was calculated as the difference between rates of $^{22}Na^+$ efflux measured in samples prior to and after the addition of ouabain to the bathing solution. Ouabain-inhibitable $^{22}Na^+$ efflux was taken to reflect the contribution of the Na^+/K^+ pump to active transport of $^{22}Na^+$ across the sarcolemma. Squares are for normoxic cold-submerged frogs; triangles and circles are for hypoxic cold-submerged frogs.

buffered with 2 % bovine serum albumin, pH 8.0, containing the isotope $^{22}Na^+$ (185 kBq ml^{-1}). The preparation was pre-washed for 20 min in unlabelled Ringer's solution, and the released radioactivity was discarded. Subsequently, the preparations were washed in a series of scintillation vials containing 5 ml of Ringer's solution or 5 ml of Ringer's solution plus 1 mmol l^{-1} ouabain maintained at 3 °C at intervals of 2 min. A 1 ml sample was removed from each scintillation vial and, following addition of 15 ml of scintillation cocktail, the digested muscles and samples from the wash-out vials were sampled for radioactivity using a Packard 1600 TR scintillation counter. In this way, the uptake of $^{22}Na^+$ (a measure of membrane permeability to Na^+) can be determined. The difference between the total (non-specific) and ouabain-inhibited $^{22}Na^+$ efflux provides an estimate of Na^+ pump activity.

The paired sartorius muscles from pre-submergence control frogs and those exposed to normoxic submergence were incubated in scintillation vials containing Ringer's solution bubbled with 98 % O_2 and 2 % CO_2 housed within a 98 % O_2/2 % CO_2 atmosphere in a hooded, temperature-controlled (3 °C) shaking water bath (Grant Instruments). Muscles from hypoxia-exposed frogs were similarly incubated and washed in Ringer's solution except that they were equilibrated with 98 % N_2 and 2 % CO_2 in a 98 % N_2/2 % CO_2 atmosphere to mimic the more severe hypoxic conditions *in vivo*.

Six pre-submergence control animals were sampled at day 0, and their blood and tissues were prepared and analysed in exactly the same manner as detailed above.

The effectiveness of the blockers glibenclamide and ouabain on $^{86}Rb^+$ and $^{22}Na^+$ efflux rates was tested in a separate series of experiments on sartorius muscles taken and processed as above throughout the course of the study. In all experiments, rates of $^{22}Na^+$ and $^{86}Rb^+$ efflux remained constant for at least 20 min and 60 min, respectively. Addition of ouabain (Fig. 1A) or glibenclamide (Fig. 1B) at any time in the respective efflux curve led to a marked reduction in the rate of $^{22}Na^+$ and $^{86}Rb^+$ movements. Apart from demonstrating the effectiveness of the two blockers, the data show how the initial rates of efflux for both $^{22}Na^+$ and $^{86}Rb^+$ are markedly reduced after the animals have been hibernating for 2 months in hypoxic water.

In vivo *experiments*

In a parallel experiment, the relative volumes of the tissue water compartments of muscle and ventricle were determined by injecting three groups of six frogs (control, submerged for 4 months of normoxia and submerged for 4 months of hypoxia, P_{O_2}=60 mmHg) intraperitoneally with 1.1 MBq of ^{3}H-labelled inulin for 16 h before decapitation and subsequent withdrawal of blood. Each sample of blood was immediately centrifuged (15 800 g), and the radioactivity of the plasma was measured using a scintillation counter. The ratio of the extracellular fluid volume of the muscle (V_{em}) to the total muscle water (V_m), obtained from the concentration of ^{3}H-labelled inulin in muscle water and plasma, respectively, was taken to represent the fractional extracellular space (Q_{em}). Intracellular fluid volumes

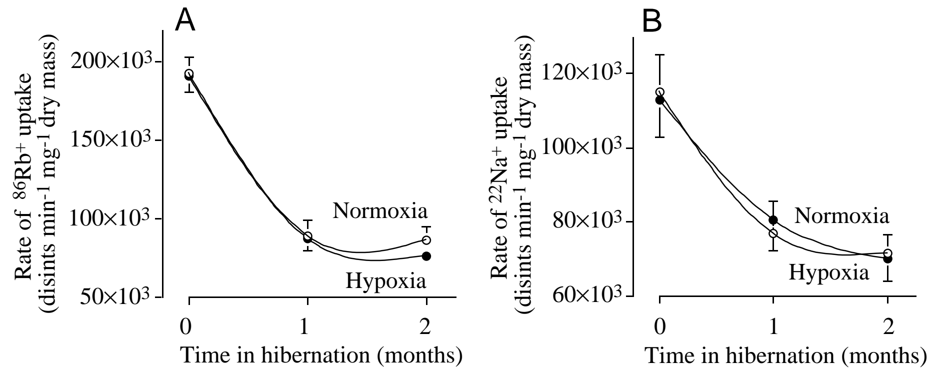

Fig. 2. Rates of uptake of (A) $^{86}Rb^{+}$ and (B) $^{22}Na^{+}$ by paired sartorius muscles of *Rana temporaria* pre-incubated in anoxic and hyperoxic (P_{O_2}=740 mmHg=98.6 kPa) Ringer's solution containing 185 kBq ml^{-1} $^{86}Rb^{+}$ for 5 h or 185 kBq ml^{-1} $^{22}Na^{+}$ for 7 h, respectively. Values are presented as means ±1 S.E.M. (N=6). Open symbols represent values obtained from normoxic cold-submerged frogs and filled symbols represent values obtained from hypoxic cold-submerged frogs.

V_i were taken as V_i=V_m−V_{em}. Intracellular Na^{+} concentrations ($[Na^{+}]_i$) were calculated according to the equation:

$$[Na^{+}]_i = ([Na^{+}]_m - [Na^{+}]_e Q_{em})/(1 - Q_{em}),$$

where $[Na^{+}]_m$ and $[Na^{+}]_e$ represent the concentrations of Na^{+} in the total muscle water and plasma, respectively (Boutilier et al., 1986a). This equation was applied equally to all other intracellular ion concentrations reported.

Comparisons against control values were made using one-way analysis of variance (ANOVA) and Dunnett's multiple-comparison tests. Comparisons between hypoxic and normoxic time pairs were made using one-way ANOVA and Tukey's test. All results were considered statistically significant at $P \leqslant 0.05$ and are presented as mean ± one standard error of the mean (S.E.M.).

Results

The rates of $^{86}Rb^{+}$ and $^{22}Na^{+}$ uptake of sartorius muscles taken from control and hibernating animals are shown in Fig. 2A,B. The uptake of $^{86}Rb^{+}$ is taken to represent the active transport of K^{+} against its electrochemical gradient and, consequently, provides an indirect estimate of Na^{+}/K^{+} pump activity. Over the course of normoxic hibernation for 2 months, the $^{86}Rb^{+}$ uptake by the muscles decreases by approximately 60 % (Fig. 2A). The reduction in apparent Na^{+}/K^{+} pump activity, suggested by the lesser accumulation of $^{86}Rb^{+}$ in the submerged frogs, is supported by the ouabain-inhibitable $^{22}Na^{+}$ efflux rate (Fig. 3B) which falls by 30–50 %, indicative of a similar reduction in Na^{+}/K^{+} pump activity. The uptake of $^{22}Na^{+}$ by the muscles was used to estimate the relative permeability of the sarcolemma to Na^{+}. Fig. 2B illustrates a greater than 30 % fall in Na^{+} uptake by the muscles, which is representative of a down-regulation of Na^{+} countertransport across the membrane or a decreased membrane permeability or a combination of both these processes. The 50–80 % reduction in glibenclamide-inhibitable $^{86}Rb^{+}$ efflux (i.e. mediated by K_{ATP} channels; Fig. 3A) suggests that these channels are down-regulated during long-term submergence. These results suggest that Na^{+}/K^{+}-ATPase activity decreased by more than 50 %, coincident with a 30 % decrease in membrane permeability to Na^{+}. The only significant differences observed in ion flux rates between 'hypoxic'- and 'normoxic'-exposed muscles from 'hypoxic'- and 'normoxic'-submerged animals were those for $^{22}Na^{+}$ efflux (Fig. 3B); efflux decreased by 30 % in normoxic muscle and by 50 % during hypoxic muscle.

Ventricular intracellular Na^{+} and extracellular K^{+} concentrations remained constant at pre-submergence values throughout all stages of hibernation in both normoxic and hypoxic conditions (Table 1). However, intracellular $[K^{+}]$ decreased by approximately one-third in the gastrocnemius

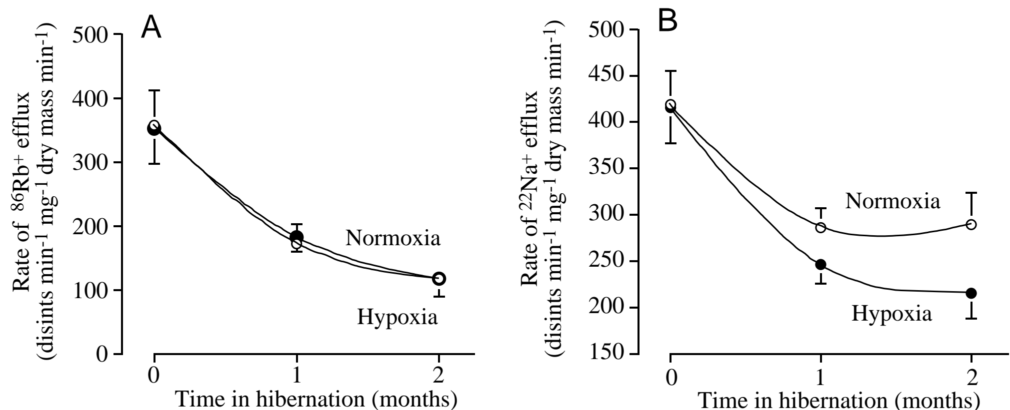

Fig. 3. (A) Glibenclamide-inhibitable $^{86}Rb^{+}$ efflux rate and (B) ouabain-inhibitable $^{22}Na^{+}$ efflux rate by paired sartorius muscles of *Rana temporaria* pre-incubated in anoxic and hyperoxic (P_{O_2}= 740 mmHg=98.6 kPa) Ringer's solution containing 185 kBq ml^{-1} $^{86}Rb^{+}$ for 5 h or 185 kBq ml^{-1} $^{22}Na^{+}$ for 7 h, respectively. Values are presented as means ±1 S.E.M. (N=6). Open symbols represent values obtained from normoxic cold-submerged frogs and filled symbols represent values obtained from hypoxic cold-submerged frogs.

Table 1. *Ion concentrations and calculated Nernst equilibrium potentials in the gastrocnemius muscle, ventricular muscle and plasma of submerged, hibernating* Rana temporaria *exposed to normoxia and hypoxia* (P_{O_2}*=60 mmHg) at 3 °C*

	Control (*N*=6)	1 week (*N*=6)	1 month (*N*=6)	2 months (*N*=6)	4 months (*N*=4)
Gastrocnemius muscle					
$[K^+]_i$ (μmol g^{-1} tissue)					
Normoxic	130.00±4.06	112.59±4.12*	81.50±3.90*	91.87±3.62*	82.97±5.28*
Hypoxic	130.00±4.06	86.85±3.29*,‡	87.58±2.49*	83.51±2.55*	64.63±4.76*
E_K (mV)					
Normoxic	−93.02±1.49	−87.03±3.81	−85.82±2.99	−83.64±1.74	−80.04±2.89*
Hypoxic	−93.02±1.49	−82.63±0.85	−79.66±2.62	−75.10±4.32*	−73.67±3.37*
$[Na^+]_i$ (μmol g^{-1} tissue)					
Normoxic	13.39±1.27	15.24±1.72	13.62±2.16	8.67±2.07	9.73±2.28
Hypoxic	13.39±1.27	9.08±1.83	8.93±1.20	11.18±1.83	9.54±1.28
E_{Na} (mV)					
Normoxic	57.88±2.81	43.88±3.34	46.42±4.37	58.48±5.71	50.10±4.26
Hypoxic	57.88±2.81	49.88±3.95	53.32±4.14	47.95±4.14	52.82±5.58
$[Cl^-]_i$ (μmol g^{-1} tissue)					
Normoxic	4.41±0.45	5.52±0.93	6.85±0.45	4.06±0.71	4.19±0.45
Hypoxic	4.41±0.45	3.64±0.65	4.85±0.55	4.94±0.73	4.88±0.96
E_{Cl} (mV)					
Normoxic	−65.08±2.82	−59.93±4.58	−57.28±1.58	−70.44±4.06	−67.89±2.56
Hypoxic	−65.08±2.82	−68.17±3.72	−63.80±2.33	−63.99±3.56	−66.57±6.22
Ventricular muscle					
$[K^+]_i$ (μmol g^{-1} tissue)					
Normoxic	136.24±4.11	122.55±4.97	134.70±4.55	128.36±4.67	130.28±5.71
Hypoxic	136.24±4.11	122.50±4.85	131.31±4.11	133.38±4.14	110.99±5.11*
E_K (mV)					
Normoxic	−93.31±1.61	−90.81±1.42	−89.88±2.77	−85.31±4.60	−90.48±2.97
Hypoxic	−93.31±1.61	−89.02±3.94	−95.98±2.19	−92.27±1.84	−87.06±1.42
$[Na^+]_i$ (μmol g^{-1} tissue)					
Normoxic	10.57±0.85	8.65±1.12	7.46±0.82	9.36±1.09	9.45±1.02
Hypoxic	10.57±0.85	8.77±0.75	10.04±0.65	8.13±0.44	7.99±0.53
E_{Na} (mV)					
Normoxic	58.09±2.00	57.77±3.74	61.01±4.19	64.01±3.32	55.41±2.82
Hypoxic	58.09±2.00	55.87±1.49	52.26±1.56	56.97±2.80	56.05±1.31
$[Cl^-]_i$ (μmol g^{-1} tissue)					
Normoxic	4.43± 0.26	4.40±0.38	5.68±0.34	6.31±0.67	5.60±0.61
Hypoxic	4.43± 0.26	3.76±0.46	4.94±0.54	5.76±0.90	5.53±0.54
E_{Cl} (mV)					
Normoxic	−64.25±1.93	−63.69±2.14	−61.69±1.47	−58.95±3.07	−61.12±2.59
Hypoxic	−64.25±1.93	−66.53±2.61	− 63.29±2.56	−60.29±3.86	−61.42±2.34
Plasma					
$[Na^+]_e$ (μmol g^{-1} tissue)					
Normoxic	130.83±3.91	94.18±2.87*	91.76±6.09*	93.18±7.64*	98.16±12.80*
Hypoxic	130.83±3.91	90.53±4.01*	90.05±4.80*	90.14±7.53*	84.60±6.12*
$[K^+]_e$ (μmol g^{-1} tissue)					
Normoxic	2.63±0.14	2.69±0.10	3.13±0.29	3.68±0.59	2.94±0.31
Hypoxic	2.63±0.14	3.03±0.47	2.25±0.24	2.75±0.20	2.89±0.25
$[Cl^-]_e$ (μmol g^{-1} tissue)					
Normoxic	66.50±1.84	62.83±2.23	75.33±1.36*	73.00±1.03*	72.00±1.78
Hypoxic	66.50±1.84	59.67±0.88	68.67±1.87	68.33±2.14	72.00±1.47

*Significantly different with respect to the control ($P\leqslant0.05$); ‡significantly different with respect to the time pair ($P\leqslant0.05$).
Data are presented as means ± 1 S.E.M.

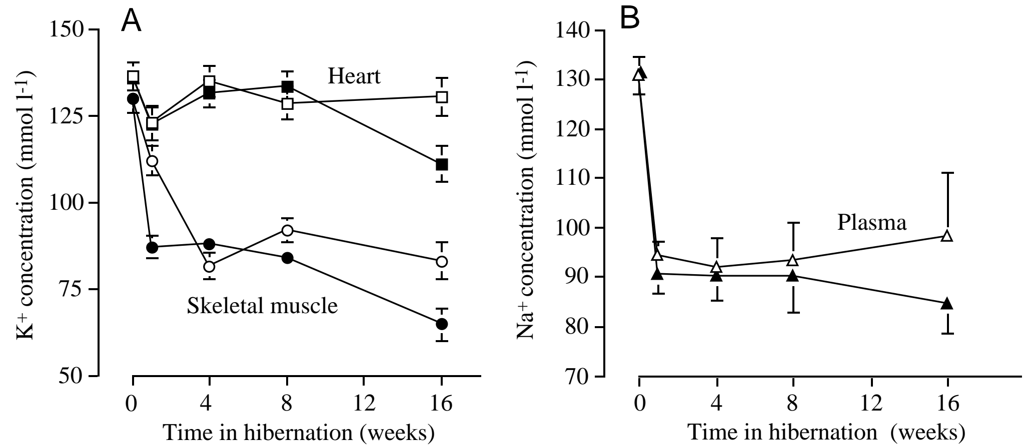

Fig. 4. (A) Intracellular K^+ concentrations in the ventricular and gastrocnemius muscles of *Rana temporaria* and (B) plasma Na^+ concentrations in *R. temporaria* over the course of 16 weeks of submergence in normoxic (P_{O_2}=155 mmHg=20 kPa) or hypoxic water (P_{O_2}=60 mmHg=8 kPa) at 3 °C. Values are means ±1 S.E.M. (N=6). Open symbols represent values obtained from normoxic cold-submerged frogs and filled symbols represent values obtained from hypoxic cold-submerged frogs.

muscles of both normoxic and hypoxic groups of submerged frogs during the first 2 months of submergence. Whereas the $[K^+]_i$ of normoxic animals remained at this new set point, the $[K^+]_i$ of hypoxic frogs continued to decrease, reaching less than half the control values by 4 months of hibernation (Fig. 4A). A 33 % fall in $[Na^+]_e$ occurred over the first 1–4 weeks of submergence in normoxic or hypoxic water, and $[Na^+]_e$ remained at this new level for the duration of hibernation (Fig. 4B). In contrast, ventricular $[K^+]_i$ remained stable over the entire course of the experiment, with the caveat that a significant decrease in $[K^+]_i$ was observed in the hearts of the hypoxic frogs following 4 months of submergence.

The effects of the changes in extracellular and intracellular Na^+ concentrations upon the equilibrium potentials for Na^+ (E_{Na}) in both ventricular and skeletal muscle were calculated according to the Nernst equation (which was also applied to all other ion concentrations reported; Table 1). The equilibrium potentials for Na^+ and Cl^- remained stable in both the normoxic and hypoxic skeletal and ventricular muscles throughout the experiment. Similarly, the equilibrium potential for K^+ (E_K) in the ventricular muscles of both groups of frogs was maintained; however, the hypoxic-submerged frogs exhibited a significant and maintained decrease in E_K by the gastrocnemius muscle by the second month of submergence. A significant reduction in skeletal muscle E_K was also evident in the normoxic frogs by 4 months of submergence.

The effect of 4 months of submergence upon both normoxic and hypoxic frogs was an 11 % increase in total body mass (Table 2). The increase in body mass must represent water uptake by the frog because it has no opportunity to feed over this period and, if anything, should be losing body mass as a result of starvation. The increase in total body water content of

Table 2. *Determinations of water distribution in the whole animal and in gastrocnemius and ventricular muscles made on* in vivo *samples taken from submerged, hibernating* Rana temporaria *exposed to normoxia and hypoxia* (P_{O_2}=60 mmHg) *at 3 °C*

	Control (air access)	4 months of submergence (P_{O_2}=155 mmHg)	4 months of submergence (P_{O_2}=60 mmHg)
Whole animal			
Percentage increase in total body mass	–	11.48±2.62*	10.94±0.79*
%ECV	21.28±1.25	21.51±1.41	21.97±1.69
Ventricular muscle water content (ml O_2 g^{-1} dry mass)			
Total water, V_m	3.59±0.15	4.14±0.17*	4.29±0.06*
Extracellular water, V_{em}	0.50±0.02	0.71±0.08	0.77±0.19
Intracellular water, V_i	3.09±0.17	3.43±0.16	3.51±0.20
Gastrocnemius muscle water content (ml O_2 g^{-1} dry mass)			
Total water, V_m	3.98±0.15	4.68±0.16*	3.60±0.19‡
Extracellular water, V_{em}	0.70±0.08	1.03±0.03*	0.81±0.07
Intracellular water, V_i	3.28±0.11	3.65±0.14	2.79±0.13*,‡

*Significantly different with respect to the control ($P\leq0.05$); ‡significantly different with respect to the time pair ($P\leq0.05$).

V_m, V_{em} and V_i are presented as means ± 1 S.E.M. (N=6).

The intracellular (V_i) and extracellular (V_e) fluid volumes of the gastrocnemius and ventricular muscles are standardised to 1 g dry mass to allow for comparisons to be made between groups.

ECV, extracellular fluid volume.

the cold-submerged frogs appears to be equally partitioned between the extracellular and intracellular fluid compartments of the whole animal: there is no significant increase in the ratio of extracellular fluid to total body mass (%ECV; Table 2). The total water content (V_m) of the ventricular muscle in both sets of frogs increases significantly, after 4 months, as a result of small increases in both V_i and V_{em}. However, while the gastrocnemius muscles from the normoxic submerged frogs also increased their V_m, this was combined with a significant increase in V_{em}; i.e. the extracellular compartment became oedematous. The converse was true for the skeletal muscle from the hypoxic-submerged frogs: there was no significant change in V_m, while the volume of the intracellular fluid compartment V_i decreased (Table 2).

Discussion

The ability of the frog *R. temporaria* to down-regulate its metabolic rate to a new steady-state value is the key to its survival during prolonged periods of cold-submergence and/or hypoxia. The reduction in whole-animal metabolic rate is thought to be brought about by hypoperfusion of an oxyconforming skeletal muscle mass that makes up 35–40 % of the total mass of the animal (Donohoe and Boutilier, 1998, 1999; Donohoe et al., 1998). One of the cellular mechanisms proposed to lead to a reduction in the metabolic rate of muscle tissue is ion channel suppression (Hochachka, 1986). This may occur as a result of a generalised decrease in cell membrane permeability to ions or through a decline in the electrochemical gradient for maintaining ionic homeostasis. Both these processes would reduce the demand for Na^+/K^+-ATPase activity and could, therefore, be construed as energy-sparing responses. In this light, the hibernation-induced change in intracellular [K^+] and decreases in Na^+ and K^+ flux rates of skeletal muscle suggest energy-sparing responses that bring about the hypometabolic response to cold-submergence.

Plasma [Na^+] falls to a new set point over the first week of cold-submergence that is one-third below the levels seen in air-breathing control animals at 3 °C (Fig. 4B). This decrease sets up the possibility of energetic savings both at the cutaneous interface with the environment and at the sarcolemmal level for the cold-submerged frog. The amphibian skin serves as a major osmoregulatory organ which, along with the kidney, is responsible for maintaining ionic and osmotic homeostasis. However, the skin of the cold-submerged frog has another, potentially conflicting, role as the sole organ of gas exchange, in that it responds to reduced oxygen availability by increasing its functional surface area through capillary recruitment (i.e. hyperperfusion; Boutilier et al., 1986b; Pinder, 1987). While an increased functional surface area facilitates gas exchange under O_2-limiting conditions (Boutilier et al., 1992), it has the disadvantage of increasing the passive loss of ions from the animal to the environment. Thus, to maintain water and electrolyte balance at minimum cost, an 'osmoregulatory compromise' must be reached between increasing the surface area required for gas exchange and minimising the surface area available for ion loss to the environment. It is well known that exposure to cold temperatures (<5 °C) disrupts normal ionic and osmotic homeostasis in frogs. Even though the rate of osmotic water influx declines, a cessation of urine production leads to marked retention of water, with a consequent decrease in extracellular osmolality (Bradford, 1983). It has also been reported that water retention is greater during hypoxic than during normoxic submergence (Christiansen and Penney, 1973), indicating some hypoxia-induced breakdown in osmoregulatory function. However, the hypoxic frogs in the present experiment became no more oedematous on a whole-animal level than the normoxic frogs (Table 2). The observed reduction in plasma [Na^+] (Fig. 4B) may well be a consequence of the above dilution effect. However, the lowered plasma [Na^+] may represent an actively altered set-point in osmotic balance that places the animal in an energetically more favourable position; i.e. a more dilute extracellular fluid could combine the advantages of reducing the osmotic flux of water into the animal with the ability to maintain ionic balance against a smaller transcutaneous [Na^+] gradient. The dilution of extracellular fluid is partly achieved by a 10 % increase in frog mass over the course of the 4 months of submergence, an increase that appears to be evenly distributed between the intracellular and extracellular compartments because the extracellular volume (ECV) remains a constant fraction of total body mass (Table 2). Intriguingly, the gastrocnemius muscles from the hypoxic-submerged frogs maintained their total water content at control levels, although the intracellular fluid volume decreased, while the normoxic frogs increased the total water content of the gastrocnemius as a result of an increase in extracellular fluid volume (Table 2).

The 33 % fall in extracellular [Na^+] is mirrored intracellularly by a 33 % decrease in the [K^+] of gastrocnemius muscle (Fig. 4A). The fall in [K^+]$_i$ may be partially explained by the increased water content of the intracellular compartment in the normoxic frogs. However, the extent of dilution by this route alone can be calculated to be only a small percentage of the overall decline in [K^+]$_i$. The progressive fall in intracellular [K^+] has the effect of gradually lowering the electrochemical gradient for K^+ by approximately 20 mV to within 10 mV of the equilibrium or reversal potential for K^+, E_K (Table 1). Thermodynamics dictates that the movement of charge for a species of ion is zero at its equilibrium potential (Hille, 1992). This means that there is a lowered electromotive force 'drawing' K^+ out of the cell, and this may confer energetic savings in two ways. First, the maintenance of a lowered concentration gradient across the sarcolemma may offer energetic savings associated with maintaining ionic homeostasis against a smaller transmembrane [K^+] gradient. Second, there would be a lowered requirement for reuptake by the Na^+/K^+-ATPase of K^+ lost across the sarcolemma because there would be a lowered K^+ efflux as a result of the lowered electromotive force.

In contrast to the large ionic changes seen in skeletal muscle, [K^+]$_i$ of heart is maintained relatively constant throughout the long periods of cold-submergence (Fig. 4A) despite a

significant increase in total fluid volume (Fig. 2; Table 2). Indeed, only after 4 months of hypoxic submergence was there any significant fall in $[K^+]_i$ of heart muscle (Fig. 4A; Table 1). The constancy of the intracellular $[K^+]$ is undoubtedly related to the requirement of the heart to pump blood to and from the respiratory exchange site to ensure that oxygenated blood is delivered to the hypoxia-sensitive core tissues such as brain (Wegener and Krause, 1993). Both the skeletal and ventricular muscles of *R. temporaria* maintain ATP homeostasis at a constant level when confronted with long-term hypoxic and normoxic submergence at 3 °C (Donohoe and Boutilier, 1998; Donohoe et al., 1998). One way of achieving this in hypoxic skeletal muscle might be to reduce ATP requirements by exploiting the oxyconforming response of this tissue; i.e. redistributing oxygenated blood away from the hypoxia-tolerant skeletal muscle mass and towards the hypoxia-sensitive core organs (Donohoe and Boutilier, 1999). It seems entirely feasible that the skeletal muscle demand for ATP could be reduced by lowering the electrochemical gradient across the sarcolemma, since there is a much reduced requirement for locomotion in the hibernating frog. However, the frog must maintain its cardiovascular capacity in order to perfuse the cutaneous vasculature and distribute the returning oxygenated blood to the cerebral circulation. Therefore, ionic gradients must be preserved, because they are integral to the contractility of the ventricular muscle. It is noteworthy in this respect that conservation of ventricular $[K^+]_i$ in the face of skeletal muscle $[K^+]$ depletion has also been reported in the hypoxia-tolerant ferret when fed on a low-K^+ diet (Ng, 1993). Moreover, there would be little advantage gained, in terms of energy savings, by reducing the electrochemical gradient of ventricular tissue since the heart represents such a small fraction of body mass (<0.5 %) compared with the 35–40 % of body mass that can be attributed to the skeletal muscle mass (Putnam, 1979).

Unlike the loss of intracellular K^+, the fall in plasma $[Na^+]$ does not result in a significant reduction in the equilibrium potential for Na^+ (E_{Na}) that drives Na^+ into the cell down its electrochemical gradient (Table 1). Since E_{Na} remains more than 100 mV above the resting potential of the skeletal muscle cell, it seems more likely that the observed 30 % reduction in Na^+ leak into the cell (Fig. 2B) is achieved by changes in membrane permeability, rather than by a reduction in the electromotive force for Na^+. A reduction in the permeability of the sarcolemmal membrane would almost certainly result in energetic savings because Na^+/K^+-ATPase activity is closely linked to intracellular $[Na^+]$ (Blaustein, 1993) and, consequently, can be thought of as being driven by the leak of Na^+ into the cell. A reduced Na^+ leak into the cell would necessarily result in a lowered requirement for the Na^+/K^+-ATPase to cycle and, thereby, lower the rate of ATP consumption by this active transport mechanism.

The results indicate that the sarcolemma, as well as having a reduced permeability to Na^+, also becomes less permeable to K^+. Indeed, the leakage of K^+ from the cell that can be attributed to K_{ATP} channels is reduced by 75 % (Fig. 3A). If the muscle fibre becomes less leaky to K^+, then this would also result in energetic savings by decreasing Na^+/K^+-ATPase activity because there would be a reduced requirement to replace K^+ lost extracellularly. K_{ATP} channels are one of the most common K^+ channels located in the sarcolemma of adult frog skeletal muscle (Spruce et al., 1985), which suggests that they have an important physiological role. The precise role of K_{ATP} channels is unclear, but opening of the K_{ATP} channels has been implicated in the catastrophic K^+ efflux observed during anoxic and ischaemic insults on mammalian heart muscle and brain (Noma and Shibasaki, 1983; Hansen, 1985; Spruce et al., 1985). Previous studies have shown that frog skeletal muscle ATP homeostasis is maintained throughout normoxic and severely hypoxic (P_{O_2}=30 mmHg) submergence (Donohoe and Boutilier, 1998, 1999; Donohoe et al., 1998), and it seems likely that on a gross organ level K_{ATP} channels would not be expected to open. It has been suggested that the 'K_{ATP} channels open in response to a lowering of submembrane ATP levels by ATPase activity in the membrane' (Spruce et al., 1987). However, the opening of K_{ATP} channels would increase the need to translocate K^+ using the Na^+/K^+-ATPase, which would increase energy demand rather than contribute to the sustained hypometabolic state observed in the overwintering frog (Donohoe and Boutilier, 1998; Donohoe et al., 1998). In view of the discussion above, it would probably assist the frog to lower its metabolic rate by reducing the K^+ efflux mediated by the high-density K_{ATP} channels.

The measured fall in active transport of $^{22}Na^+$ and $^{86}Rb^+$ (effectively K^+ efflux; Spruce et al., 1987) supports the premise that Na^+/K^+-ATPase activity is down-regulated by approximately 30 and 50 % in response to normoxic and hypoxic submergence respectively (Figs 2A, 3B). How much of a metabolic saving in terms of whole-animal metabolic rate this represents is open to debate. In liver hepatocytes of the anoxia-tolerant turtle, the major ATP-consuming pathways during normoxia are Na^+/K^+-ATPase cycling, protein synthesis and protein breakdown, which represent 29 %, 36 % and 17 % of total ATP demand respectively. However, during anoxia, protein degradation and synthesis are both reduced by more than 90 % such that Na^+/K^+-ATPase cycling represents 75 % of ATP consumption in the metabolically suppressed hepatocyte (Buck and Hochachka, 1993). Similarly, while it seems probable that protein metabolism may represent the majority of ATP turnover in the non-hibernating animal, protein metabolism would become suppressed because it could be argued that there is little impetus for large amounts of protein synthesis in a starving, hibernating animal. Thus, under conditions of reduced protein synthesis, the importance of the Na^+ pump to whole-animal metabolic rate would increase greatly. Further, any mechanism that lowers skeletal muscle Na^+/K^+-ATPase activity will have a large effect on metabolic rate since skeletal muscle constitutes 35–40 % of total body mass.

Calculations of resting membrane potential, using the passively distributed chloride ion, indicate that the membrane potential remains stable throughout hibernation (Table 1). This

occurs while the cell's ability to generate a transmembrane ion gradient is progressively reduced as a result of the observed decrease in Na^+/K^+ pump activity (Fig. 3B). However, the down-regulation of the Na^+ pump occurs coincident with a decreased Na^+ leak into and K^+ leak out of the cell, as well as a decrease in the electrochemical gradient for K^+ across the sarcolemmal membrane and for Na^+ across the skin. Taken together, it would appear that a coordinated channel arrest and new set point for ionoregulation may combine to lower the energetic demands of the cold-submerged frog.

Another potentially confounding problem for overwintering animals is that the ion transport processes involved in maintaining the electrochemical equilibrium of ions across the sarcolemma may become altered by cold exposure. For example, it is well known that the relative proportion of polyunsaturated phospholipids increases in the plasma membranes of cold-acclimated ectotherms, so as to maintain membrane fluidity. And it is the high content of polyunsaturates in both the plasma and mitochondrial membranes of mammals that is thought to be responsible for their greater leakiness to ions compared with those of ectotherms (Hulbert and Else, 1989; Brand et al., 1994). This suggests that, if the proportion of polyunsaturated phospholipids were to increase progressively in cold-acclimated frogs in the same way as it does in other ectotherms, then the plasma membranes of overwintering frogs may become increasingly leakier as the acclimation period increases. Indeed, loss of cellular K^+ leads to death in hypothermic mammals (Willis, 1979). Although we also found that the K^+ concentrations of muscle decreased over the first months of hibernation, this loss of cellular K^+ subsided as (Rb^+) K^+ efflux decreased. Hulbert and Else (1989) have suggested that plasma and mitochondrial membranes may act as metabolic pacemakers. The ectotherms so far studied (fish, etc.) that acclimate to cold temperatures maintain membrane fluidity in order that their excitable tissues can continue to function and remain responsive to changes in their environmental temperature. This is achieved, at the cost of increasing ion flux across so-called pacemaker membranes, by increasing the proportion of polyunsaturated lipids forming the lipid bilayer. However, there is no evidence from our study that frogs increase membrane ion flux in a manner that is consistent with homeoviscous adaptation. An alternative explanation might be that preservation of saturated lipids in the membranes leads to hibernation, i.e. reduced ion flux, reduced responsiveness to the environment and a profound reduction in metabolic rate due to decreased membrane leakiness across the pacemaker membranes.

To summarize, during long-term submergence in either normoxic or hypoxic water, it has been shown that the activity of the skeletal muscle Na^+ pump is significantly depressed. The data suggest that there are three discrete ways in which the muscle manages to down-regulate Na^+/K^+ pump activity, all of which reduce ion leakage across the sarcolemma. First, K^+ leakage from the cell is reduced by the progressive lowering of the reversal potential for K^+ (E_K). The fall in intracellular [K^+] has the effect of lowering the electrochemical gradient for K^+ by approximately 20 mV to within 10 mV of E_K. Intracellular [K^+] and extracellular [Na^+] also fall to new set points, which are one-third below the pre-submergence levels, over the first month of the experiment. Second, Na^+ leakage into the cell is reduced by 25 %. The data suggest that E_{Na} remains 100 mV above the resting potential of the cell over the entire course of the experiment despite the large fall in extracellular [Na^+]. In view of the large electrochemical gradient, it seems likely that the reduction in the Na^+ leak into the cell is achieved by changes in membrane permeability. Third, the leakage of K^+ from the cell that can be attributed to K_{ATP} channels is reduced by 75 %. The results suggest that membrane potential is regulated coincident with decreased Na^+ pump activity and lowered rates of Na^+ and K^+ flux across the plasma membrane. Conversely, ion homeostasis in the heart shows no large decrease in intracellular [K^+] over the entire experiment apart from the measurement during the fourth month of hypoxia, although this has no significant effect upon ventricular E_K. This is almost certainly related to the continued contractile functioning of the ventricle during hibernation. The observation that resting membrane potential remains constant in both ventricular and skeletal muscle throughout the experiment, concomitant with decreased Na^+/K^+ pump activity and ion channel down-regulation in the skeletal muscle, provides evidence of functional channel arrest as an energy-sparing response during hibernation in the cold-submerged frog.

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