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Characterization of sarcolemma and sarcoplasmic reticulum isolated from skeletal muscle of the freeze tolerant wood frog, *Rana sylvatica*: the β_2-adrenergic receptor and calcium transport systems in control, frozen and thawed states

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In freeze tolerant wood frog *Rana sylvatica*, the freeze-induced liberation of glucose plays a critical role in survival in response to sub-zero temperature exposure. We have shown that the glycaemic response is linked to selective changes in the expression of hepatic adrenergic receptors through which catecholamines act to produce their hepatic glycogenolytic effects. The purpose of the present study was to determine if skeletal muscle, another catecholamine-sensitive tissue with glycogenolytic potential, displayed similar or different changes. In order to achieve these objectives, skeletal muscle derived from *Rana sylvatica* was studied in control, frozen and thawed states. In isolated sarcolemmal fractions, freezing effected an 88% decrease in β_2-adrenergic receptor expression but was without effect on the calcium pump; while thawing resulted in a recovery of the β_2-adrenergic receptor to 60% of control levels and a 2.4-fold increase in calcium transport. In isolated sarcoplasmic reticular fractions, freezing effected a 52% decrease in calcium binding and a 92% decrease in oxalate-stimulated calcium uptake; while thawing elicited partial normalization to control levels to 70% with respect to calcium binding and to 47% with respect to calcium uptake. Freezing and thawing were associated with increases and decreases, receptively, in blood glucose levels but were without effect on skeletal muscle glycogen content. Thus these muscle changes in *Rana sylvatica* in freezing and thawing are not linked to glycogen breakdown, are different from those previously seen in liver, and may provide a role in recovery of muscle function during thawing by protecting glycogen stores for contraction and maximizing extracellular calcium for excitation–contraction coupling in the frozen state. The involvement of thyroid hormone in triggering these muscle changes is discussed. Copyright © 2001 John Wiley & Sons, Ltd.

KEY WORDS — *Rana sylvatica*; freeze tolerance; skeletal muscle; sarcolemma; sarcoplasmic reticulum; β_2-adrenergic receptor; calcium pump

INTRODUCTION

It is well established that the wood frog, *Rana sylvatica*, is freeze-tolerant.[1] The range of this North American frog extends as far north as the Arctic circle where animals are exposed to subzero temperatures for much of the winter.[2] As temperatures drop below zero, adaptive responses are elicited that allow the frog to withstand the otherwise lethal effects of freezing and subsequent thawing. These responses include: ice-nucleating proteins that induce and regulate ice formation; circulatory alterations with blood flow diverted to critical organs such as the heart, liver and brain, which sustains their functional integrity; and an hepatic glycaemic response that produces massive amounts of glucose, which serves as a cellular cryoprotectant.[3,4]

While it is recognized that the formation of ice crystals on the skin of the frog elicits a signal eventuating in hepatic glycogenolysis and glucose output,[5] and that this event is critical in effecting freeze-tolerance,

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the nature of the signal is presently unknown. Activation of the sympathetic nervous system has been considered, based on the rapidity of the response and consideration that in mammals, hepatic glycogenolysis is strongly stimulated by catecholamines released upon activation of the sympathetic nervous system.[6] Additionally, the catecholamine, epinephrine, which serves as both a hormone and a neurotransmitter in most frog species, is a strong activator of the hepatic glycogenolytic process.[7] Earlier studies failed to show increments in catecholamine levels in *R. sylvatica* in response to subzero exposure, apparently ruling out a simple increase in the outflow from sympathetic nerves (cited in reference 3 and 4). However, our recent studies have demonstrated that a freeze-induced alteration occurs in the expression of hepatic adrenergic receptors, through which catecholamines act to produce their hepatic glycogenolytic effects, consistent with an increased sensitization of the liver to the actions of catecholamines.[8] As to the identity of the sensitizing influence responsible for effecting hepatic adrenergic receptor changes, we have postulated it to be a freeze-induced alteration in thyroid hormone levels.[8]

Modulation of hepatic adrenergic receptor expression by thyroid hormone is well established in mammals.[9] In amphibians, the thyroid gland is physically and functionally well developed[10] and thyroid hormones are physiologically significant.[11] In *R. sylvatica*, thyroid hormone levels change significantly on freezing and thawing.[8,12] We considered that, in addition to effects on hepatic metabolism, a freeze-induced alteration in thyroid hormone levels might also have widespread effects on adrenergic receptor expression in other tissues, with physiological consequences that might contribute to freeze-tolerance. We chose to examine adrenergic expression in *R. sylvatica* skeletal muscle, since skeletal muscle, in frogs as well as mammals, is known to express the β_2 adrenergic receptor which is present on the sarcolemmal surface.[13] Activation of this receptor in skeletal muscle has been shown to modulate calcium-induced excitation-contraction coupling by altering the inwardly directed movement of calcium through calcium channels.[14] Activation of this receptor also leads to a stimulation of muscle glycogen catabolism to fuel glycolytic ATP production to support muscle contraction.[15]

In this study, we have looked at the impact of freezing and thawing of wood frogs on the skeletal muscle β_2-adrenergic receptor. We have also determined the status of the sarcolemmal and sarcoplasmic reticular calcium pumps as a means of assessing calcium movements, reflecting alterations in excitation–contraction coupling and/or the contractile state. Studies on skeletal muscle from a number of species, including the frog, have shown that the sarcolemmal calcium pump controls the outward movement of calcium[16] whereas the sarcoplasmic reticular calcium pump controls the removal of cytoplasmic calcium.[17] The relative activities of both pumps determine the final intracellular calcium concentration which ultimately determines whether the muscle is in a contracted or relaxed state.[18]

In our studies, the approach taken was to isolate sarcolemma and sarcoplasmic reticulum from skeletal muscle of control, frozen and thawed *R. sylvatica* and to establish the status of the sarcolemmal β_2-adrenergic receptor and calcium pump and the sarcoplasmic reticular calcium pump by radioligand binding techniques.

MATERIALS AND METHODS

Chemicals and radiochemicals

Chemicals used were of analytical or reagent grade and were obtained from either Sigma Chemical Company, St. Louis, MO, USA, or Fisher Scientific Company, Edmonton, Alta, Canada. The radioligand [^{125}I]-Pindolol ($2200 \cdot \mathrm{Ci} \cdot \mathrm{mmole}^{-1}$) and the radiochemical [^{45}Ca]-$CaCl_2$ ($34.12 \cdot \mathrm{mCi} \cdot \mathrm{mg}^{-1}$) were purchased from Dupont–New England Nuclear, Boston, MA, USA.

Animals

Guidelines established by the Canadian Council for Animal Care were followed and animal protocols were approved by the University of Saskatchewan Animal Care Committee.

Male spring-emerged wood frogs, 5–7 g body weight, were collected in the wild from breeding ponds around Ottawa, Ontario in late April. After washing in a tetracycline bath, frogs were maintained in pond water at 4°C. Frogs were then divided into three groups: controls (CON) were maintained in pond water at 4°C; frozen (FRZ) frogs were transferred to -2.5°C for 24 h; and thawed (THAW) frogs were given 24 h freezing followed by 18 h thawing at 4°C. Freezing at -2.5°C was carried out as previously described[8,12] by placing frogs in freezing compartments that were immersed in a salt–water–ice slurry maintained at -2.5°C. For thawing, frogs were transferred back to dishes containing a small amount of pond water at 4°C

Control, frozen and thawed frogs were double pithed and blood was removed using an anticoagulant-coated microcapillary pipette from the region of, or above, the heart and plasma prepared, as described previously.[8,12] The hind legs were stripped of skin to expose the skeletal muscle which was quickly removed from the thigh and plunged into liquid nitrogen. Plasma and quick-frozen muscle were maintained at ultracold temperatures, packed in dry ice and shipped to Saskatoon. On receipt, materials were transferred to an ultracold freezer at $-80°C$.

Preparation of skeletal muscle fractions

Frozen skeletal muscle was thawed in 0.9% saline, trimmed of any adhering skin, nerves, fat or connective tissue, and weighed.

Skeletal muscle sarcolemma. The isolation procedure followed was that described for the rapid isolation of sarcolemma from small amounts of frog tissue, detailed in the preceding paper[19] starting with 0.5–2.0-g portions of trimmed skeletal muscle for each fraction. Sarcolemma fractions were used immediately for β_2-adrenergic receptor binding and calcium transport assays.

Skeletal muscle sarcoplasmic reticulum. The isolation procedure followed was that described in detail in the preceding paper[19] starting with 0.5–1.0-g portions of trimmed skeletal muscle for each fraction. Calcium transport was determined in freshly prepared skeletal muscle sarcoplasmic reticular fractions.

Skeletal muscle glycogen extraction

The method followed was one we developed for liver.[20] Weighed portions (30–75 mg) of frozen skeletal muscle were dropped into perspex tubes containing 2.0 ml of boiling 30% KOH, set in a boiler. The tissue was dispersed by stirring with a glass rod and boiled for 30 min to effect complete digestion. Tubes were set on ice to cool and a sample was withdrawn for protein determination. A 5.0-ml aliquot of ice-cold 95% ethanol was then added to each tube, the contents were vortexed thoroughly, and the tubes were capped securely and placed at $-20°C$ for 24 h to allow glycogen precipitation. Samples were then removed from the freezer and centrifuged at 2200 *g* at 4°C in an IEC preparative centrifuge for 20 min to pellet the glycogen. The supernatant was poured off and the tubes were inverted on Kleenex to remove any residual traces of ethanol. Each glycogen pellet was dissolved in 4.0 ml of glass distilled water and the glycogen extracts were stored at $-20°C$ for <1 week prior to determining glycogen content.

Assessment of samples

Blood glucose, skeletal muscle protein and skeletal muscle glycogen. Glucose levels were determined in whole blood samples from wood frogs by the method of Raabo and Terkildsen.[21] The protein contents of the isolated skeletal muscle fractions and skeletal muscle digests, were determined by the method of Lowry *et al.*[22] using bovine serum albumin as the standard. Glycogen contents were measured by the phenol sulphuric acid method as previously described,[20] using highly purified crystalline glycogen as the standard. Glycogen results are expressed per mg digested protein as well as per g frozen tissue weight.

Isolated skeletal muscle sarcolemma and sarcoplasmic reticulum.

β_2-adrenergic receptor characterization – β_2-adrenergic receptor binding was assessed in isolated skeletal muscle sarcolemma fractions by radioligand binding using the β_2-adrenergic receptor antagonist, [^{125}I]-Pindolol, following our method and conditions described in detail elsewhere.[8,19] Briefly, 30–50 μg of sarcolemma protein was incubated with 15 nM [^{125}I]-Pindolol ($5–10 \cdot cpm \cdot fmole^{-1}$) for 10 min at 30°C in a 150-μl reaction mixture containing: 10 mM $MgCl_2$, 50 mM Tris-Cl, ± 20 mM propranolol, pH 7. Specific [^{125}I]-Pindolol binding was obtained by taking the difference between total binding, determined in the absence of 20 μM propranolol, and non-specific binding, determined in the presence of 20 μM propranolol. Results are expressed as fmole [^{125}I]-Pindolol bound $\cdot mg^{-1}$ sarcolemmal protein ($fmol \cdot mg^{-1}$).

Calcium transport determination – Calcium transport was determined in the skeletal muscle sarcolemmal and sarcoplasmic reticular fractions by radioligand binding using [^{45}Ca]-$CaCl_2$ using a modification of our previous conditions[19,23] to optimize the assay for skeletal muscle. Briefly, 25–175 μg of sarcolemmal protein or 20–150 μg of skeletal muscle protein were incubated–30 min for sarcolemma; 15 min for sarcoplasmic reticulum–at 37°C in a 500 μl reaction mixture containing 250 mM sucrose, 0.2 mM EGTA, 10 mM $MgCl_2$,+20 mM sodium azide, 10 mM ATP, 0.2 mM $CaCl_2$ containing $8000–12{,}000 \cdot cpm. \cdot nmole^{-1}$, 100 mM potassium oxalate and 50 mM Tris–Cl, pH 6.0. Results are

expressed as nanomoles of calcium transported per mg sarcolemmal or sarcoplasmic reticular protein per total incubation time: $nmol \cdot mg^{-1}$. Results in the absence of oxalate reflect calcium binding; results in the presence of oxalate reflect calcium uptake.

Statistical analysis

A paired *t*-test was performed with values of $p < 0.05$ considered significant.

RESULTS

General characteristics

Exposure of *Rana sylvatica* to $-2.5°C$ for 24 h effected a complete freezing of the frog. These frogs were immobile, covered with ice crystals and exhibited no visible signs of life. Thawing of these frogs for 18 h at 4°C restored the frogs to their original pre-freezing status, physically and behaviourally without deleterious effects. Freezing elicited a glycaemic response in *Rana sylvatica*, with the concentration of blood glucose being: 2–5 mM in the control state; 60–80 mM in the frozen state; and, 20–40 mM in the thawed state (data not shown).

The skeletal muscle obtained from control, frozen and thawed *Rana sylvatica* did not appear to be grossly different. In all cases, the thigh muscle used in our studies was whitish-cream in colour. The concentration of glucose in these muscles, using methods previously described[3–5] was found to be 19-mM (data not shown).

Skeletal muscle glycogen levels

The glycogen content in the skeletal muscle of *R. sylvatica* is presented in Figure 1. Results depicted in panel A represent the glycogen content of the muscle expressed per mg of frozen skeletal muscle. Results depicted in panel B represent the glycogen content of the muscle expressed per mg of skeletal muscle protein. There was a small increase in the glycogen content in the frozen state: 1.3-fold when expressed per mg skeletal muscle (panel A) and 1.5-fold when expressed as skeletal muscle protein (panel B); however, this increase was not statistically significant. There was no difference in

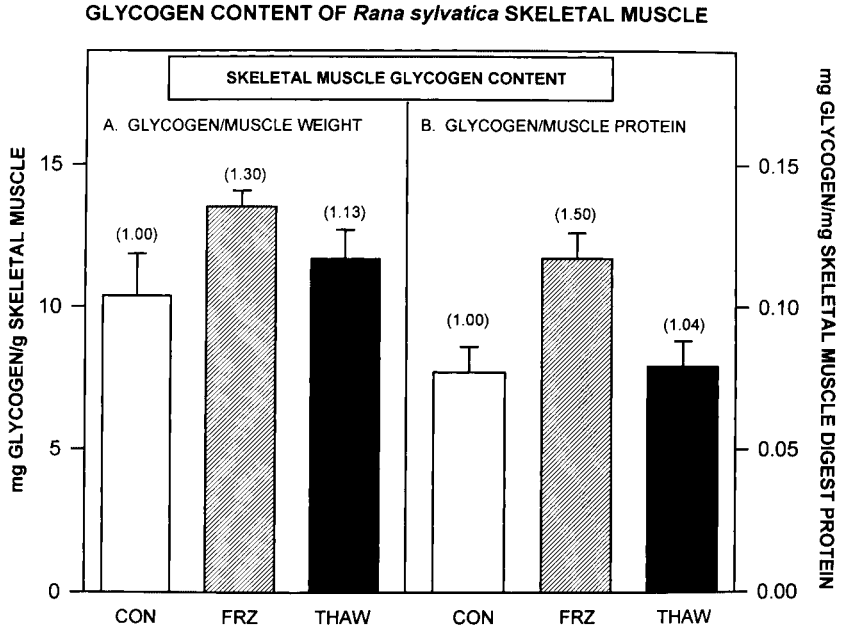

Figure 1. Glycogen was extracted from quick frozen thigh muscle of *Rana sylvatica* in the control state (CON: open bars); after 24 h at $-2.5°C$ in the frozen state (FRZ: hatched bars); or, after freezing for 24 h followed by thawing for 24 h in the thawed state (THAW: solid bars). Results in panel A show skeletal muscle glycogen expressed per g wet weight of muscle. Results in panel B show skeletal muscle glycogen expressed per mg skeletal muscle digest protein. Results are means ± SEM, $n = 4–6$. * Statistical significance set at $p < 0.05$

glycogen content in skeletal muscle in the control and thawed states.

Skeletal muscle sarcolemma

Figure 2, panel A, indicates that β_2-adrenergic receptor binding in sarcolemma isolated from the skeletal muscle of *R. sylvatica* in the control state is comparable to that observed in other species of frogs.[19] After 24 h at $-2.5°C$, in the completely frozen state, β_2-adrenergic receptor binding exhibits a marked, 88%, reduction from the control state. Thawing frozen *R. sylvatica* for 24 h at 4°C, elicits a return to control levels of β_2-adrenergic receptor expression with β_2-adrenergic receptor binding being recovered to 60% control levels.

Figure 2, panel B, indicates that sarcolemmal calcium binding is low in *R. sylvatica* in the control state but in accord with that observed in other frogs.[19] Freezing *R. sylvatica* is without notable effect on sarcolemmal calcium binding while thawing frozen *R. sylvatica* effects a large increase in activity, 2.4-fold greater than the activity in the control state.

Skeletal muscle sarcoplasmic reticulum

As illustrated in Figure 3, sarcoplasmic reticulum isolated from skeletal muscle of *R. sylvatica* displays calcium transport activity comparable to that observed for the sarcoplasmic reticulum isolated from the skeletal muscle of frogs of other species.[19] Calcium binding is low (panel A) and strongly stimulated, 76-fold, by oxalate (panel B).

In the frozen state, calcium binding (panel A) and oxalate-stimulated calcium uptake (panel B) both exhibit a profound decrease from control levels: 52% with respect to the former; 92% with respect to the latter. The consequence of thawing on the sarcoplasmic reticular calcium transport system is an increase from the low levels of the frozen state back towards control levels. Calcium binding (panel A) is increased from 48 to 70% of control levels; calcium uptake (panel B) is increased from 8 to 47% of control levels.

The net effect of these freeze and thaw-induced changes in the calcium transport system in *Rana sylvatica* is to alter the sensitivity to oxalate-induced stimulation from 76-fold in the control state, to

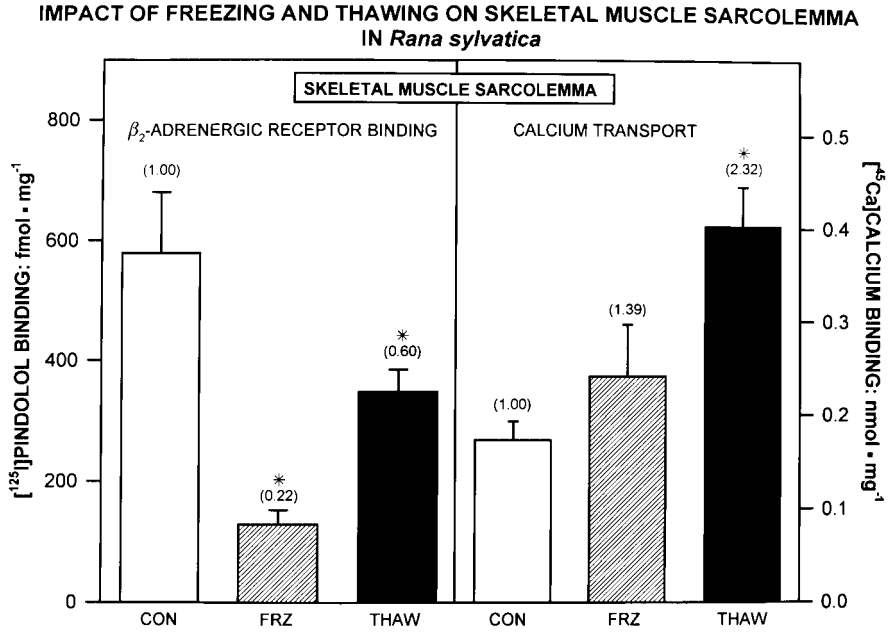

Figure 2. Skeletal muscle sarcolemma was isolated from quick frozen thigh muscle of control (CON: open bars); 24 h frozen at $-2.5°C$ (FRZ: hatched bars); and 24 h thawed at 4°C (THAW: solid bars) frogs. β-Adrenergic receptor binding (panel A) was determined using [^{125}I]-Pindolol as radioligand. Calcium transport (panel B) was determined in the absence of stimulation as calcium binding using [^{45}Ca]-calcium chloride. Results are means ± SEM, $n = 4$–6 fractions, each prepared from muscle pooled from 4–6 thighs. Numbers above the bars reflect fold differences relative to controls, set at 1.0. * Statistical significance set at $p < 0.05$

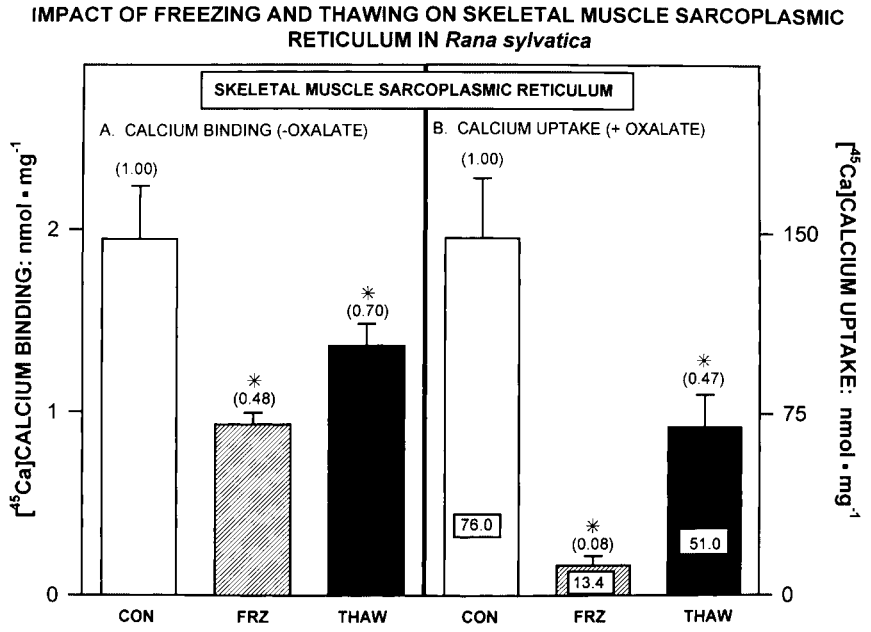

Figure 3. Skeletal muscle sarcoplasmic reticulum was isolated from quick frozen thigh muscle of control (CON: open bars); 24 h frozen at -2.5°C (FRZ: hatched bars), and 24 h thawed at 4°C (THAW: solid bars) frogs. Calcium transport was determined in the absence of oxalate as calcium binding (panel A) and in the presence of oxalate as calcium uptake (panel B). Results are means ± SEM, $n = 4$–6 fractions per group, each prepared from muscle pooled from 4–6 thighs. Numbers above the bars reflect fold differences relative to controls, set at 1.0. Values inset within the bars in panel B reflect fold stimulation by oxalate in the indicated states. * Statistical significance set at $p < 0.05$

13-fold in the frozen state, to 51-fold in the thawed state.

DISCUSSION

The natural freeze tolerance of the wood frog *Rana sylvatica* supports its winter hibernation on land.[1–4] Wood frogs can survive for days or weeks at temperatures as low as -6 to -8°C with up to 65% of their total body water converted to ice. Upon thawing, breathing or circulation resume within a couple of hours and after 12–24 h all faculties have returned to normal. Many aspects of freezing survival have been extensively studied but the full range of adaptations that support this unique ability have by no means been completely elucidated.

It is well established that glucose plays a cryoprotective role in the frogs. Freezing triggers an activation of hepatic glycogenolysis that can raise blood and tissue glucose to as high as 200–300 mM, levels that are 100-fold or more higher than normal.[3–5] Catecholamines are known to be the most potent stimulus for hepatic glycogenolysis in mammals as well as amphibians,[6,7] suggesting that these hormones may mediate the freeze-induced hepatic glycogenolytic response in *Rana sylvatica*. However, catecholamine levels do not rise in *Rana sylvatica* during freezing,[24] ruling out a simple increment in these hormones as the mechanism of action.

We have considered that a change in the adrenergic receptors through which catecholamines act may be involved.[8] Our previous studies have shown that exposure to sub-zero temperatures sets in motion a series of adrenergic receptor shifts in the liver of *R. sylvatica* that are sufficient to alter the hepatic response to catecholamines. These adrenergic receptor shifts occur as freezing commences and as freezing is completed. The former involves a sharpening of the β_2-adrenergic receptor response, by maintaining the expression of the β_2-adrenergic receptor and decreasing the expression of the α_1- and α_2-adrenergic receptors. The latter involves a shift to an α_1-adrenergic receptor response, by increasing the expression of the α_1- and α_2-adrenergic receptors, and virtually eliminating the expression of the decreasing β_2-adrenergic receptor. These, in turn, correlate with the early initial burst, and the later sustained output, of glucose from the liver arising from the breakdown of liver glycogen stores. On thawing, a slow normalization of adrenergic receptor expression parallels, and may be causally related

to, an equally low re-uptake of glucose into the liver and its redisposition as glycogen. Although we proposed[8] that what we observed reflects a liver-specific reaction to freezing related to the production of cryoprotectant glucose, we felt compelled to consider the possibility that the liver response may be part of a general reaction in which freezing alters adrenergic receptor responsiveness in all tissues.

Thus, the present study analyses these systems in skeletal muscle and describes changes in adrenergic receptor expression and select effector system activities in the skeletal muscle of wood frogs during freezing and thawing. Skeletal muscle was chosen because it shares the following characteristics with liver (for a review see reference 25): (i) stores glucose as glycogen; (ii) expresses adrenergic receptors which mediate the response to catecholamines;[13] (iii) expresses calcium pumps that direct intracellular events in skeletal muscle;[16,17] (iv) responds to catecholamines acting through adrenergic receptors with a stimulation of glycogenolysis;[6,7] (v) relies heavily on calcium, and the regulatable activity of calcium pumps, for intracellular responses.[18]

With respect to adrenergic receptors, we focused on the β_2-adrenergic receptor in skeletal muscle. This is the only functionally significant adrenergic receptor in the skeletal muscle of frogs and mammals and is located in the skeletal muscle sarcolemma.[13,15,26,27] Catecholamines, working through this receptor, facilitate skeletal muscle contraction. Since skeletal muscle is not innervated by sympathetic nerves,[26,27] the catecholamines in question are blood-borne, emanating from the adrenal medulla. Contraction is driven by energy in the form of ATP supplied by catecholamine-stimulated breakdown of skeletal muscle glycogen stores. Contraction is achieved at the level of myofibrils by a calcium signal, expressed as a sharp transient increase in intracellular calcium effected by changes in the activities of calcium channels, pumps and release systems across the sarcolemmal and sarcoplasmic reticular membranes (for a review see reference 28). We focused on the calcium pumps in these two membranes, which are responsible for the termination of the calcium signal by removing excess calcium from the cytoplasm either by active efflux across the sarcolemma or by re-uptake into the sarcoplasmic reticulum.[14,17,18,28–30]

We selected our recently developed methods for the isolation of skeletal muscle sarcolemma and sarcoplasmic reticulum[19] as they addressed the special needs in studying *R. sylvatica*.

In *R. sylvatica* used in this study, a freeze-induced glycaemic response was observed in confirmation of previous results.[8,12] This response has been proposed to reflect the activation of hepatic glycogenolysis and the breakdown of the large hepatic glycogen stores.[3–5] Our previous studies have linked the glycaemic response to a shift in hepatic adrenergic receptor expression, a shift we have proposed to be effected by a drop in thyroid hormone levels, which changes the response to catecholamines.[8] Catecholamines in the frog are the most potent activators of glycogenolysis (see references 7 and 8). In this study, we considered the possibility that the glycogen stores in the muscle may be additionally tapped for glucose under the conditions of freezing. It is established in mammals that in the normal state, skeletal muscle glycogen is a storage form but not a releasable form of glucose.[25] It is generally felt that skeletal muscle glycogenolysis proceeds to the generation of lactate.[30] The situation in the frog of a freeze-tolerant species requiring glucose for cryopreservation warranted investigation. We demonstrated, however, that the concentration of glycogen in skeletal muscle of *Rana sylvatica* was unaltered as a consequence of freezing and thawing. Thus, we ruled out that freezing effected a unique alteration in skeletal muscle allowing glucose to be released to add to that released from the liver for the purposes of cryoprotection.

With respect to the status of the β_2-adrenergic receptor in skeletal muscle sarcolemma of *R. sylvatica*, our study showed that this receptor was greatly diminished in the frozen state and had begun to normalize in terms of its level of expression in the thawed state. This pattern of change is not comparable to that which we found in liver.[8] In the liver, the β_2-adrenergic receptor decreased to low levels in the frozen state, but also remained low in the thawed state. Thus, the skeletal muscle loses its sensitivity to catecholamines through this receptor subtype, as does the liver, but reacquires it again on thawing, unlike the liver. Since catecholamines act at the level of the skeletal muscle to modulate contraction which is a functionally significant but energetically expensive process,[18,25] the decrease in the receptor may represent a mechanism to prevent an unnecessary, and energetically wasteful function while the animal is frozen. However, the need for responsiveness to catecholamines in the thawed state with the return to physical function and movement of the limbs driven by muscle contraction and relaxation, would dictate the necessity of returning to a normal level of expression of this important adrenergic receptor subtype in keeping with our observations. As the frog thaws, first the heart begins to beat, then breathing resumes and finally limb movement is observed.[1,3,4]

The dependency of skeletal muscle contraction and relaxation on calcium is well established in both mammalian and amphibian muscle.[18,25,29–30] A calcium signal in skeletal muscle, manifested as a sharp transient increase in intracellular calcium, is initiated largely by release of calcium from the sarcoplasmic reticulum allowing cytoplasmic calcium levels to rise and terminated by the uptake of calcium into the sarcoplasmic reticulum and efflux across the sarcolemma, allowing calcium levels to fall to normal. The termination of the calcium signal in this way is achieved by energy-dependent calcium pumps, enzymatically expressed as calcium ATPases.

Our results on the impact of freezing and thawing on the activities of the calcium pumps in the sarcolemma and sarcoplasmic reticulum of wood frogs yield some interesting points. First, the changes at the plasma membrane, or sarcolemma, differ from those that we have reported for the calcium pump in the liver plasma membrane.[8] Again, this indicates that the regulatable components of muscle and liver are reacting in a tissue specific, as opposed to a general, manner to sub-zero temperature exposure. Second, the activities of the calcium pumps in these two important organelles exhibit quite different changes during freezing and thawing. That of the sarcolemma exhibits no appreciable alteration on freezing but a significant increase on thawing. That of the sarcoplasmic reticulum exhibits a decrease in the frozen state and an incomplete normalization in the thawed state. The sensitivity of the sarcoplasmic reticular calcium pump to stimulation is greatly diminished in response to freezing, whereas it returns to near normal levels in the thawed state.

These results suggest that as the skeletal muscle of wood frogs freezes, it does so with an excessive amount of intracellular calcium dictated by the status of the calcium pumps, with that of the sarcoplasmic reticulum being preferentially affected and playing the most important role. This suggests that as skeletal muscle thaws, it would have a pre-existing intracellular pool of calcium with which to activate contraction and relaxation as opposed to relying on the generation of a new calcium signal. Additionally, in the thawed state, the increased activity of the sarcolemmal calcium pump, coupled with the normalization of the sarcoplasmic reticular pump, suggests that mechanisms are in place to offset a calcium overload, which could have toxic ramifications.[31] To this end, both the sarcolemmal and sarcoplasmic reticular calcium pumps are affected and together play important roles. In times of calcium excess, this implies that the sarcolemmal calcium pump is recruited to assist that of the sarcoplasmic reticulum in the removal of calcium.

In the wood frogs, we have proposed that a freeze-induced decrease in thyroid hormone levels may effect the hepatic alterations in adrenergic receptors and calcium effector systems underscoring the glycaemic response.[8] The frog can, and does, produce thyroid hormones,[32] although levels of these hormones are lower than those in mammals.[8,12,32] These hormones exhibit distinct seasonal patterns in frogs that hibernate, being much lower in winter and much higher in summer.[33] In *R. sylvatica*, changes in these hormones occur in response to freezing and with thawing.[8,12] Thyroid hormone is important functionally in the frog, playing critical roles in metamorphosis,[34] modulating metabolism[35] and controlling the activity of specific enzymes in frogs in general and in freeze-tolerant frogs in particular, as we have proposed.[36]

Skeletal muscle is strongly dependent on thyroid hormone, physically and functionally. Thyroid hormone has been shown to play a pivotal role in the differentiation and maturation of mammalian skeletal muscle in the postnatal period with the simplistic design of fetal muscle giving way, under the influence of thyroid hormone, to cell fusion, terminal differentiation, organelle development and expression of specific components that define the functional state of skeletal muscle in the adult.[37] Amongst components known to be thyroid hormone dependent are: the β_2-adrenergic receptor of the sarcolemma[11,38,39] and the calcium pump of the sarcoplasmic reticulum.[37] In mammals, thyroid hormone deficient states, be they at the early neonatal state or the hypothyroid state, characteristically display a decrease in the β_2-adrenergic receptor and a decrease in the activity of the sarcoplasmic reticular calcium pump, relative to the situation in the euthyroid state. Against this background, a decrease in thyroid hormone levels during freezing in wood frogs and their recovery during thawing, corresponds with the decrease in the β_2-adrenergic receptor levels in muscle and in the activity of the sarcoplasmic reticular calcium pump. This suggests that the latter is a consequence of the former, as is the case in mammals.

The dependency of the sarcolemmal calcium pump in skeletal muscle on thyroid hormone is not known. Our results do not point to a simple relationship between the levels of thyroid hormone and the activity of this pump. Factors other than thyroid hormone may therefore be involved. The increase in the activity of this pump in the thawed state may play a highly specific role in cytoprotection against excess calcium and may therefore be internally regulated by the

intracellular level of calcium itself or externally regulated by such factors as pH, which has been shown to influence the activity of the sarcolemmal calcium pump.[16,17]

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