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Post-freeze recovery of peripheral nerve function in the freeze-tolerant wood frog, *Rana sylvatica*

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Abstract. We investigated the restoration of peripheral nerve function and simple neurobehavioral reflexes in the freeze-tolerant wood frog (*Rana sylvatica*). Thirty-two specimens, allowed to freeze for 39 h and ultimately cooled to −2.2° C, were sampled at various time intervals up to 60 h after thawing at 5° C was initiated. The sciatic nerves of treated frogs were initially unresponsive to stimulation, but usually regained excitability within 5 h. Except for a slight reduction in nerve excitability, characteristics of the compound action potentials of treated frogs were indistinguishable from those of control frogs. Recovery times for the hindlimb retraction and righting reflexes were 8 h and 14 h, respectively. Concentrations of the cryoprotectant glucose increased 8.2-fold in the sciatic nerve and 10.5-fold in the underlying semimembranosis muscle of treated frogs, and remained elevated for at least 60 h after thawing was initiated. These organs lost 47.2% and 15.9%, respectively, of their water during freezing, but were rehydrated within 2 h of the onset of thawing. The accumulation of glucose and the withdrawal of tissue water apparently are cryoprotective responses which enable this species to survive freezing.

Key words: Freeze tolerance – Sciatic nerve – Osmotic stress – Cryoprotection – Frog, *Rana sylvatica*

Introduction

Freeze tolerance, the ability of an organism to survive the freezing of its body fluids under ecologically relevant conditions, is known in five Nearctic species of terrestrially hibernating anurans (Schmid 1982; Storey and Storey 1986; Costanzo et al. 1992a). In the wood frog (*Rana sylvatica*), freeze tolerance is promoted by several integrated responses to the formation of ice within tissues (Costanzo et al. 1993a; Lee and Costanzo 1993). One principal adaptation is the mobilization of the cryoprotectant glucose, from liver glycogen, and its distribution to tissues throughout the body (Storey and Storey 1986, 1988; Costanzo and Lee 1993). Additionally, water vacates organs during freezing and accumulates innocuously within the coelomic and lymphatic spaces, thus limiting the mechanical injury caused when ice forms within tissues (Lee et al. 1992; Costanzo et al. 1993a).

The physiological mechanisms of recovery from freezing in freeze-tolerant vertebrates are poorly known. Limited data suggest that critical physiological functions, such as autonomic cardiac contraction, rhythmic pulmonary breathing, and tissue perfusion, are progressively restored in the wood frog over several hours after recovery is initiated (Layne and First 1991). However, the actual time-course for recovery also is governed by the severity of the freezing episode (Lee and Costanzo 1993). In some systems, such as the contractile strength of skeletal muscle, recovery from short-term (e.g., ≤2 days) freezing occurs virtually coincident with thawing (Layne 1992), whereas in others, such as tissue metabolite levels and energy status, full recovery is attained only after many days (Storey and Storey 1988).

The nervous system is highly susceptible to freezing and thawing stresses, such as ionic perturbation (Hillman 1988) and anoxia (Pérez-Pinzón et al. 1992). In the present investigation, we focused on the freezing recovery of the wood frog sciatic nerve, a compound peripheral nerve that participates in several distinctive neurobehavioral reflexes. Our primary objective was to determine the time-course for restoration of nerve function, as assessed by examining the characteristics of compound action potentials and simple neurobehavioral reflexes. Secondly, we determined whether glucose accumulation and tissue dehydration may promote the freeze tolerance of the peripheral nervous system.

Materials and methods

Adult male wood frogs (*Rana sylvatica*) weighing 13.8±0.4 g (mean±SEM; $n=41$) were collected near breeding ponds in Adams County, Ohio, USA, during early March, 1992. Freeze tolerance of

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frogs from this population was initially documented by Layne and Lee (1987). To maintain their winter cold hardiness, all frogs were fasted and exposed to 5° C in darkness for 2–6 weeks prior to testing.

Thirty-two frogs were cooled individually in plastic centrifuge tubes (50-ml) submerged in a refrigerated bath. A thermocouple probe, placed against each frog's abdomen, was used in conjunction with a multichannel data logger to record body temperature during cooling. After the frogs had supercooled for 1.2 ± 0.2 h, they were induced to freeze by placing a small ice crystal on their skin. Ice nucleation was confirmed by the appearance of an exotherm, the release of the latent heat of crystallization. We used this inoculation procedure to control both the crystallization temperature (-1.4 ± 0.1° C) and the timing of the onset of freezing. Frogs were allowed to freeze for 38.9 ± 1.2 h during which time they cooled to -2.2 ± 0.03° C. They remained in thermoequilibrium at this temperature for 5.3 ± 0.5 h prior to being used in the experiments.

Experimental design. At the end of the freezing episode, frogs were sampled immediately (0 h) or at discrete intervals (2, 5, 8, 14, 24, 36, 48 and 60 h) after their transfer from the cold bath to humid plastic cages kept at 5° C. Neuromuscular coordination was assessed by sequentially determining whether each frog could retract its manually extended hindlimb (hindlimb reflex) and right itself when placed on its dorsum (righting reflex). Frogs were allowed a single 2-s trial to meet each criterion, after which they were killed by double-pithing. Both sciatic nerves were surgically isolated from each frog; the left nerve was used to study the characteristics of action potentials, whereas the right nerve was used in measurements of glucose and water contents. Tissue analyses were also made on a representative sample of semimembranosis, the skeletal muscle that directly underlies the sciatic nerve. All dissections and tissue handling were performed at 5° C by one investigator and completed within 4–6 min of pithing. A second investigator simultaneously made general observations regarding the amount and distribution of ice within the body. Nine additional frogs, taken (unfrozen) from their cages at 5° C, served as controls.

Measurement of action potentials. In vitro tests of nerve function were conducted using the main trunk of the sciatic nerve, a section (25–34 mm) extending from the nerve cord plexus to the divergence of the peroneal and tibial branches at the knee. Nerves were tested on platinum electrodes in a humidified nerve chamber with a glass lid sealed with mineral oil. All nerves were placed in the chamber ca. 10 min prior to experimentation. The environmental temperature during testing (nominally, 5° C) was controlled by placing the chamber inside a jacketed beaker cooled by a refrigerated circulator. The actual temperature of the nerve (4.9 ± 0.4° C, $n = 36$) was estimated from the recordings of a 36-gauge thermocouple placed on a narrow strip of moistened filter paper inside the chamber. Nerves were stimulated with biphasic shocks (SD9D, Grass Instruments) of uniform delay (0.4 ms) and duration (0.6 ms). Compound action potentials were recorded and analyzed using a software-driven bioamplifier and data acquisition system (AD Instruments). Testing was completed within 2 h of pithing the frog; preliminary work indicated that the impulse characteristics studied were stable during this time interval.

We measured the following parameters: threshold excitation voltage, maximal stimulus voltage and amplitude, conduction velocity, impulse duration, and refractory index. Conduction velocity was calculated from the delay in impulse peaks, initiated by one-half maximal stimulation, arriving at two recording electrodes positioned 5.0 mm apart. Impulse duration (at maximal stimulation) was measured in total, but also partitioned by depolarization and repolarization phases. Refractory index was determined by applying pairs of stimuli (at one-half maximal voltage) spaced at intervals of 10, 20, 35, 50, 80, 100 or 120 ms and calculating the amplitude of the second impulse as a proportion of the first.

Tissue analyses. The untested sciatic nerve and a sample of adjacent semimembranosis muscle were isolated and bisected, with a portion of each used to determine water contents and glucose concentrations. Water contents of the samples were determined from the mass lost during oven drying at 65° C, and expressed as a percentage of fresh tissue mass. Differences in water contents of tissues among groups were calculated from group means, with units expressed as milligrams water per gram of dry tissue. Following Costanzo et al. (1993a), the remaining samples were homogenized in perchloric acid, centrifuged to remove the protein, and analyzed for glucose concentration using an enzymatic, spectrophotometric method (no. 510, Sigma). Because the water content of tissues varied substantially among the experimental groups, glucose concentration was expressed as $mmol \cdot l^{-1}$ tissue water, rather than a per tissue-weight basis.

Statistical analysis. Owing to unequal variances, values from different experimental groups were compared using non-parametric Kruskal-Wallis tests. Where appropriate, subsequent, paired-comparison tests (experimental group versus control group) were made using the non-parametric procedure given by Zar (1984). Percentage data were transformed (square root-arcsine) before use in the analyses. The relationships between nerve and muscle glucose concentration, and nerve and muscle water content, were evaluated using Spearman's rank correlation.

Results

Recovery of neurobehavioral function after freezing

Fully frozen frogs (0-h group) were rigid, inanimate, apneic, and generally as described by Layne and Lee (1987). Ice was present on the external surfaces, within the coelomic and lymphatic spaces, and throughout the organs and tissues. These frogs were completely unresponsive to mechanical stimulation (Table 1). Unfortunately, their sciatic nerves could not be isolated undamaged and so were not tested in the nerve chamber.

Frogs sampled 2 h after thawing was initiated contained comparatively less ice, indicating that some melting had occurred. However, none exhibited neurobehavioral reflexes. Sciatic nerves isolated from these frogs were unresponsive to electrical stimulation of up to 20.0 V (Table 1).

Frogs in the 5-h group, whose body ice had almost completely melted, were flaccid and edematous, and

Table 1. Restoration of nerve function in freezing-exposed wood frogs (*Rana sylvatica*) sampled at various times after thawing was initiated, as indicated by the exhibition of neuromuscular reflexes in intact specimens and the excitability of isolated sciatic nerves

Time after freezing (h)	n	Number of frogs meeting criterion		
		Nerve excitability	Hindlimb reflex	Righting reflex
0	5	n.d.	0	0
2	5	0	0	0
5	5	3	0	0
8	3	3	3	0
14	7	7	5	4
24–60	7	7	6	5

Nine control frogs, which were not frozen, met all criteria
n.d. = not determined

Table 2. Action potential characteristics of isolated sciatic nerves from freezing-exposed wood frogs (*Rana sylvatica*) sampled at various times after thawing was initiated

	Control	Time after freezing (h)				H	P^a
		5	8	14	24–60		
Threshold stimulus (V)	0.4±0.1	0.8±0.1	1.2±0.3	0.6±0.1	1.2±0.5	8.8	0.067
Maximal stimulus (V)	6.8±1.5	4.0±0.0	6.8±0.4	5.1±1.2	14.6±4.3	6.7	0.156
Conduction velocity ($m \cdot s^{-1}$)	17.1±4.6	7.8±2.0	9.6±4.1	12.8±6.0[d]	9.3±2.3	3.2	0.525
Maximum amplitude (mV)	14.7±2.4	17.2±2.4	10.3±6.6	12.4±2.2	9.7±2.0	4.2	0.377
Action potential duration (ms)							
total	4.2±0.5	3.7±0.3	4.3±0.5	4.9±0.7	4.7±0.7	1.4	0.852
depolarization	1.6±0.2	1.7±0.1	1.4±0.1	1.7±0.2	1.6±0.2	1.7	0.782
repolarization	2.6±0.4	2.0±0.3	2.9±0.4	3.2±0.5	3.3±0.6	4.2	0.376
Refractory index (%)[b]							
10 ms interval	29.9±5.6	21.4±4.9	39.0±15.2	31.1±8.9	40.7±11.4	1.6	0.800
50 ms interval	85.5±5.1	79.4±8.4	86.1±5.9	85.3±3.5	83.3±3.0	0.8	0.935
120 ms interval	98.3±1.8	91.0±1.2[c]	96.1±2.3	98.8±1.7	93.0±2.4	7.1	0.132
(N)	(9)	(3)	(3)	(7)	(7)		

Control frogs were not frozen
Means (±1 SEM) are based on the number of frogs given in parenthesis
[a] Probability that differences among the means within the row were significant
[b] Data are shown for three representative intervals
[c] $n=2$
[d] $n=4$

Table 3. Glucose and water contents of sciatic nerve and semimembranosis muscle from freezing-exposed wood frogs (*Rana sylvatica*) sampled at various times after thawing was initiated

	Control	Time after freezing (h)						H	P^a
		0	2	5	8	14	24–60		
Glucose ($mmol \cdot l^{-1}$)									
Nerve	3.6±1.2	19.7±7.8[b]	29.6±9.9[b]	7.4±3.2	18.4±6.3[b]	9.8±3.4[b]	5.3±1.4	16.4	0.012
Muscle	1.1±0.1	8.9±0.7[b]	9.7±0.8[b]	8.1±2.0[b]	11.6±0.7[b]	8.6±1.4[b]	6.9±1.4[b]	24.5	<0.001
Water (% fresh mass)									
Nerve	72.7±0.5	58.4±1.0[b]	71.8±1.1	72.0±1.7	73.4±0.6	72.0±1.1	74.3±1.4	15.8	0.015
Muscle	80.7±0.3	77.7±1.1[b]	80.3±1.0	81.1±0.8	80.4±0.3	79.2±0.5	80.9±0.4	12.0	0.063
(N)	(9)	(5)	(5)	(5)	(3)	(7)	(7)		

Control frogs were not frozen
Means (±1 SEM) are based on the number of frogs given in parenthesis
[a] Probability that differences among the means within the row were significant
[b] Treatment mean differed significantly from the control mean

lacked even simple hindlimb reflexes. Nevertheless, the isolated nerves from three of these five frogs were responsive to electrical stimuli in the nerve chamber tests (Table 1). Analyses of impulse characteristics for this group were thus based on only three nerves.

The hindlimb reflex was exhibited by all frogs sampled 8 h after thawing was initiated, whereas the righting reflex, a neurologically more complex response, was first observed in the 14-h group (Table 1). Because most frogs apparently had recovered by 24 h, data from specimens sampled at latter intervals (36, 48 and 60 h) were combined with the 24-h group for analysis. The frogs in the control and experimental groups were of similar body mass ($H=6.6$, $df=6$; $P>0.3$).

Characteristics of action potentials in isolated sciatic nerves

Generally, freezing resulted in little detectable change in the impulse characteristics of isolated sciatic nerves (Table 2). However, we considered the differences in threshold excitation voltage among control and experimental groups marginally significant ($P=0.067$). A post-hoc analysis, in which data from all experimental groups were pooled and compared against the control set, showed that freezing indeed increased threshold excitation voltage (Mann-Whitney U-test; $U=32$; $P=0.006$). In contrast, maximal stimulus voltage and amplitude, conduction velocity, impulse duration (including depo-

larization and repolarization phases), and refractory index of nerves from the experimental frogs were comparable to those of control frogs (Table 2).

Tissue glucose and water contents

The mean nerve and muscle glucose concentrations in the experimental frogs were up to 8.2-fold and 10.5-fold higher, respectively, than in control frogs, and generally remained elevated throughout the recovery period (Table 3). Nerve and muscle concentrations of glucose were strongly correlated in the experimental frogs ($r_s=0.57$, $n=32$, $P=0.002$), but not in the control frogs ($r_s=0.13$, $n=9$, $P>0.7$).

Freezing decreased the water content of nerve and muscle by 47.2% and 15.9%, respectively (Table 3). However, both organs had fully rehydrated within 2 h after thawing was initiated. Nerve and muscle water contents were strongly correlated in the experimental frogs ($r_s=0.55$, $n=32$, $P=0.002$), but not in the control frogs ($r_s=-0.43$, $n=9$, $P>0.2$).

Discussion

The nervous tissue of wood frogs must tolerate stresses associated with freezing, such as osmotic and ionic imbalance, cellular dehydration, tissue ischemia and anoxia, and hyperglycemia. Low temperature per se is apparently innocuous, since the nerves of supercooled wood frogs readily function at $-5°$ C (Miller and Dehlinger 1969). Interestingly, subzero temperatures are also tolerated by the peripheral nerves of birds (Chatfield et al. 1953) and mammals (Miller 1965, 1967). Nevertheless, our study showed that sciatic nerve of the freeze-tolerant wood frog temporarily was impaired by freezing, since excitability was not restored until ca. 5 h after thawing was initiated.

The transient dysfunction of sciatic nerves from the experimental frogs may be explained on the basis of general cryobiological principles. First, the accumulation of ice in extracellular spaces concentrates solutes in the unfrozen fluid fraction and subjects cells to a high osmotic gradient [review: Mazur (1984)]. Associated shifts in the concentration of critical ions, such as Ca^{2+}, Na^+, and K^+, also may disrupt nerve function (Hillman 1988). Furthermore, the problem of ion imbalance may be compounded by the ischemia that occurs during freezing, since anoxia apparently debilitates the ATP-dependent ion transport mechanism (Strupp et al. 1991; Pèrez-Pinzón et al. 1992).

The mechanisms of recovery from freezing in the wood frog must reestablish water and solute balance (Storey and Storey 1988; Lee et al. 1992) and repair cell and tissue injury (Costanzo et al. 1993b; Lee and Costanzo 1993). Depending on the system, the recovery period may range from hours to days (Storey and Storey 1988; Layne and First 1991; Layne 1992), but ultimately is determined by the severity of the freezing episode (Lee and Costanzo 1993). In the present study, wood frogs required ca. 5 h of post-freeze recovery before their sciatic nerves regained excitability.

Relative to the control frogs, nerve excitability was slightly depressed in the experimental groups. Otherwise, characteristics of these compound action potentials were indistinguishable among control and experimental frogs, and comparable to those reported for the closely-related *R. pipiens* (Schoepfle and Erlanger 1941; Miller and Dehlinger 1969; Meyer and Hegmann 1971). Thus, freezing apparently invokes no lasting impairment of peripheral nerve function.

Recovery of neurobehavioral reflexes after freezing

The sensitivity of a given reflex action to a stressor, such as freezing, partly depends on the number of synapses participating in the specific motor activity. Thus, relatively complex responses typically are more susceptible than more simple responses. Accordingly, in our wood frogs the hindlimb retraction reflex was restored 8 h, whereas the righting reflex was restored 14 h, after thawing was initiated.

The refractory period for neurobehavioral reflexes is ultimately determined by the most sensitive component of the reflex arc, which likely is neither the peripheral nerve (since the sciatic regained function within ca. 5 h after thawing commenced) or the skeletal muscle [which may exhibit normal function within 1 h of thawing; Layne and First (1991); Layne (1992)]. Rather, the synapse likely is the most susceptible element. Indeed, the neuromuscular junction is highly sensitive to environmental stressors, such as thermal extremes (White 1983) and ion perturbation (Dingledine and Somjen 1981), and thus may require additional time to recover from freezing. Conceivably, freezing-related impairment of the central nervous system may also contribute to the delayed recovery of neurobehavioral activity.

Cryoprotection of the peripheral nervous system

Sciatic nerves clearly accumulated large amounts of glucose during freezing. Presumably this glucose was mobilized from liver glycogen and transported to the nerve (and other organs) via the vasculature (Storey and Storey 1986; Costanzo et al. 1993a). The high inter-individual variability in both nerve and muscle glucose concentrations likely reflect the large differences in liver glycogen reserves typical of this species in late winter (Costanzo and Lee 1993).

Recent evidence (Costanzo et al. 1993b) suggests that glucose limits cryoinjury to wood frog tissues (Canty et al. 1986; Costanzo and Lee 1991) by reducing the amount of ice that forms and increasing the fraction of unfreezable water. Some cryoprotectants also stabilize macromolecules and membranes (Karow 1991), a role which may be especially important for preserving the ion pumps in nervous tissue. Additionally, elevated glucose directly enhances the anoxia tolerance of nerves (Strupp et al. 1991).

Organ dehydration is another primary adaptation promoting freeze tolerance in wood frogs (Costanzo et al.

1992b, 1993b; Lee and Costanzo 1993). During freezing, water is physically withdrawn from the organs and sequestered, as ice, within the coelomic and lymphatic spaces. Tissue rehydration occurs rapidly during thawing. This response apparently limits the mechanical injury to the microvasculature within tissues (Rubinsky and Pegg 1988). Some organs, such as the liver (Lee et al. 1992) and heart (Costanzo et al. 1993b), lose ca. 50% of their water during freezing. Our study revealed that the sciatic nerve dehydrates to a comparable degree.

The high correlation between nerve and muscle water contents within individuals implies that a common (as yet unidentified) mechanism promotes dehydration in these organs. However, the markedly disparate levels of dehydration occurring in nerve and muscle (47% versus 16%) also suggests that such mechanism is subordinate to regulation at the organ or tissue level. Our present results for sciatic nerve support an earlier suggestion (Lee et al. 1992; Costanzo et al. 1992b) that the skeletal muscle of limbs dehydrates much less than visceral organs owing to its peripheral location. Additional research is needed to identify the specific mechanism promoting organ dehydration during freezing.

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