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CHANGES IN MOTONEURON MEMBRANE POTENTIAL AND REFLEX ACTIVITY INDUCED BY SUDDEN COOLING OF ISOLATED SPINAL CORDS: DIFFERENCES AMONG COLD-SENSITIVE, COLD-RESISTANT AND FREEZE-TOLERANT AMPHIBIAN SPECIES

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Summary

The effects of sudden cooling of the spinal cord were studied in three species of amphibians – a cold-sensitive tropical toad (*Bufo marinus*), a cold-resistant, aquatic, hibernating frog (*Rana pipiens*, northern leopard frog) and a freeze-tolerant frog (*Rana sylvatica*, wood frog). Ventral root (motoneuron) potentials were recorded from isolated, hemisected spinal cords of each species mounted in a sucrose-gap recording apparatus and superfused with HCO_3^--buffered Ringer's solution at room temperature (21 °C).

In the toad, sudden cooling to 6–8 °C produced large, sustained motoneuron depolarizations that returned slowly to baseline levels and were accompanied by extensive paroxysmal activity. Larger, but shorter-lasting, motoneuron depolarizations associated with only a limited amount of paroxysmal activity were generated by rapid cooling of the leopard frog spinal cord. Small, brief motoneuron depolarizations followed by a hyperpolarization, or hyperpolarizations not preceded by depolarizations, were seen in cooled wood frog spinal cords. The wood frog displayed a large amount of spontaneous motoneuron activity, but little paroxysmal activity in response to sudden cooling. Following prolonged cooling, rewarming the spinal cords of all three species resulted in motoneuron hyperpolarizations that slowly decayed towards the baseline value. The amplitude of the rewarming-induced response was larger and longer in toad motoneurons than in leopard frog and wood frog motoneurons. At room temperature, a single supramaximal dorsal root stimulus evoked a depolarizing ventral root potential in toad and leopard frog motoneurons that was decreased in amplitude and prolonged when the spinal cords were cooled to 8 °C or below. In contrast, at room temperature, the ventral root reflex in the wood frog was followed by a distinct hyperpolarization. Cooling the wood frog spinal cord only slightly reduced the amplitude of the ventral root potential. In contrast, the evoked hyperpolarization was blocked by sudden cooling and also by the addition of dihydro-ouabain to the Ringer's solution.

The motoneuron hyperpolarizations induced by sudden cooling in the wood frog were converted to depolarizations when Cl^- in the superfusate was replaced with isethionate. The depolarizations elicited by sudden cooling were reduced by the addition of kynurenate in all three species. A dose–response curve generated by short applications of L-glutamate demonstrated that wood frog motoneurons were less sensitive than leopard frog motoneurons to L-glutamate.

In summary, three species of amphibians, differing in their adaptations to the temperature of their environments, vary in their responses to sudden reductions in temperature. The relationship of these responses to their environmental adaptations remains to be determined.

Key words: amphibians, spinal cord, cooling, excitatory amino acids, *Rana pipiens*, *Rana sylvatica*, *Bufo marinus*.

Introduction

The consequences of spinal cord cooling have been the focus of investigation for many years (Ozório de Almeida, 1939, 1943; Ozório de Almeida *et al.* 1943; Kolmodin and Skoglund, 1953; Brooks *et al.* 1955; Tebecis and Phillis, 1968). In

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particular, sudden cooling of the isolated spinal cord of South American amphibians has been shown to induce epileptiform activity that is easily observed in attached muscles (Ozório de Almeida, 1939, 1943; Ozório de Almeida *et al.* 1943). Spinal cord cooling does not produce such activity in North American or European amphibians. The precise reasons for these geographic differences are unclear. However, because some North American amphibians normally survive temperatures near freezing point, the resistance of their spinal cords to cold-induced seizures may result from the development of adaptive strategies that allow some amphibians to sustain large variations in ambient temperature. In contrast, cooling the spinal cord of homeothermic animals only a few degrees below normal enhances motoneuron reflex discharges. Moreover, repetitive motoneuron firing occurs in the cat when the temperature is reduced to 25 °C or below (Grundfest, 1941; Brooks *et al.* 1955).

In the present study, the effects of sudden spinal cord cooling were compared in three species of amphibians – a cold-sensitive tropical toad (*Bufo marinus*), a cold-resistant, aquatic, hibernating frog (*Rana pipiens*, northern leopard frog) and a freeze-tolerant frog (*Rana sylvatica*, wood frog). Use was made of the isolated, superfused spinal cord to clarify the effects of cold on motoneuron membrane potential and reflex activity. Spinal cord cooling affected motoneuron and reflex responses differently in the three species. Some indications were obtained with regard to the mechanisms of some of these motoneuron and reflex responses, but the association of neuronal alterations to climatic adaptations remains unclear. A preliminary account of some of this work has been reported (Daló *et al.* 1993).

Materials and methods

Experiments were performed on adult tropical toads (*Bufo marinus*, 90–180 g) purchased from Lemberger Co., Oshkosh, Wisconsin, USA, in September–October, northern leopard frogs (*Rana pipiens*, 30–55 g) purchased from Kons Scientific Co., Germantown, Wisconsin, USA, in the summer and housed in an amphibian facility maintained at 21 °C, and wood frogs (*Rana sylvatica*, 4–13 g) collected in Ottawa, Canada, in September–October and kept acclimatized in a cold room at 5 °C. As a control, three leopard frogs were also kept acclimatized in a cold room at 5 °C. The responses of this group of frogs did not differ from those kept at room temperature, and the data from the two groups were pooled.

All animals were anesthetized by cooling on crushed ice to the point of unresponsiveness. Toads were pretreated with urethane by injection into the ventral lymph sac (0.5 g kg^{-1}) 30 min before being placed on ice to prevent induction of the seizure activity that is produced by exposure to cold (see Piña-Crespo and Daló, 1992). After decapitation and laminectomy, the spinal cord was removed, hemisected sagittally and one half-cord with attached ninth and tenth ventral (VR) and dorsal (DR) roots was placed in a sucrose-gap apparatus (Davidoff and Hackman, 1980). The spinal cord and the intramedullary portion of motoneurons was superfused at a rate of 10 ml min^{-1} with HCO_3^--buffered amphibian Ringer's solution with the following composition (mmol l^{-1}): NaCl, 114; $CaCl_2$, 1.9; KCl, 2.0; $NaHCO_3$, 10; glucose, 5.5. Equilibration with 95 % O_2/5 % CO_2 maintained the Ringer's solution at pH 7.4.

The ninth VR was placed across a 3 mm sucrose gap to record the membrane potential of motoneurons which is electrotonically conducted along their axons contained in the root. Differential d.c. recordings were obtained between the spinal cord bath and the distal end of the VR with calomel electrodes connected *via* agar–Ringer bridges to each recording site. The preparation was left ungrounded. The signals were amplified and recorded on a rectilinear pen recorder.

The fibers contained in the ninth DR were stimulated with supramaximal rectangular pulses (1.0 ms) delivered *via* bipolar silver/silver chloride electrodes applied to the sciatic nerve that had been left in continuity with the DR.

All spinal cords were stabilized in Ringer's solution for at least 1 h at room temperature (21–22 °C). Wood frog spinal cords usually demonstrated excessive spontaneous DR and VR activity and sometimes required 3 h or more to establish a stable baseline. Wood frog spinal cords were usually used for 2 days and stored overnight in a refrigerator at 4.5 °C. Reflex activity did not change after overnight storage.

Solution changes were accomplished with the aid of a solenoid valve assembly that quickly admitted different solutions to the chamber containing the spinal cord. Immediate cooling was achieved by switching a solenoid valve changing from Ringer's solution at room temperature to cold Ringer's solution. The latter was produced by connecting the reservoir of medium to a 2 m long coil of polypropylene tubing submerged in crushed ice. Cords were usually exposed to cold Ringer's solution for 15–30 min before being warmed to room temperature. The temperature inside the spinal cord bath was constantly monitored using a 29 gauge needle microprobe (model MT-29/3, Physitemp Instruments Inc., Clifton, NJ, USA).

All data are expressed as mean ± S.E.M. Differences between species were examined using analysis of variance (ANOVA) and *post-hoc* non-paired Student's *t*-tests. $P<0.05$ was considered significant.

Drugs and amino acids were dissolved in Ringer's solution shortly before use to minimize chemical degradation. The pH was adjusted when necessary. Tetrodotoxin (TTX) was purchased from Calbiochem and sodium isethionate, kynurenate and dihydro-ouabain from Sigma.

Results

Rapid decreases in the temperature of the Ringer's solution used to superfuse the spinal cord produced changes in the membrane potential of motoneurons, increased spontaneous motoneuron activity and modified evoked reflex responses in all three species. However, the amplitude, duration and direction of the changes varied among species.

Changes in motoneuron membrane potential evoked by cooling

Cooling of toad and leopard frog spinal cords produced motoneuron depolarizations in all preparations. As seen in Fig. 1A, abrupt lowering of the temperature of the Ringer's solution superfusing the toad spinal cord from room temperature to between 6 and 8 °C produced substantial (6.2±1.0 mV; *N*=19 experiments), rapid VR depolarizations (Table 1). The motoneuron membrane potential returned slowly to baseline despite continued cooling, but the process required between 4 and 56 min (26.2±4.2 min) in different experiments. The magnitude and duration of the depolarizations depended upon the amount of cooling. Superfusion with cold Ringer's solution at a temperature of 10–12 °C produced smaller (3.0, 3.4 mV; *N*=2), shorter-lasting (10, 14 min) depolarizations. In contrast to the findings observed in toad spinal cords, larger (10.7±1.2 mV, *N*=20), but shorter-lasting (5.5±0.6 min), VR depolarizations were elicited by cooling the spinal cords of leopard frogs to between 6 and 8 °C (Figs 1B, 6B; Table 1).

Sectioning the VR in leopard frogs at the point where the root exited from the spinal cord separates motoneuron somata and initial axonal segments from the remainder of the motoneuron axon. Preparing the cord in this way (*N*=2) eliminated depolarizations evoked by cold. This leads to the conclusion that changes in VR membrane potential reflect changes in the motoneuron cell body and/or initial segments of the VR axon.

Continued cooling after the depolarizations had returned to baseline levels resulted in a small motoneuron hyperpolarization in approximately 50 % of leopard frog cords (0.5±0.2 mV, *N*=8) (not illustrated). In contrast, continued cooling did not hyperpolarize toad motoneurons.

Unlike the findings in toad and leopard frog, rapid cooling of wood frog spinal cords to between 6 and 8 °C produced motoneuron depolarizations in only six of eight preparations tested. The depolarizations seen in these six frogs were small (2.9±0.8 mV, *N*=6) and brief (1.0±0.2 min) **(**Fig. 1C; Table 1). With continued cooling, the motoneuron membrane potential hyperpolarized (1.6±0.5 mV). These levels were below the baseline levels recorded when the cords were maintained at room temperature. The motoneuron hyperpolarizations remained for the duration of cooling. In the other two preparations, only hyperpolarizing potential changes (without preceding depolarizations) were recorded from the VR after rapid cooling (4.2, 1.2 mV, *N*=2) (Figs 1D, 5A).

Changes in motoneuron membrane potential produced by rewarming

Motoneuron hyperpolarizations were induced in all three species when the temperature of the spinal cords was raised by replacing cold Ringer's solution with medium at room

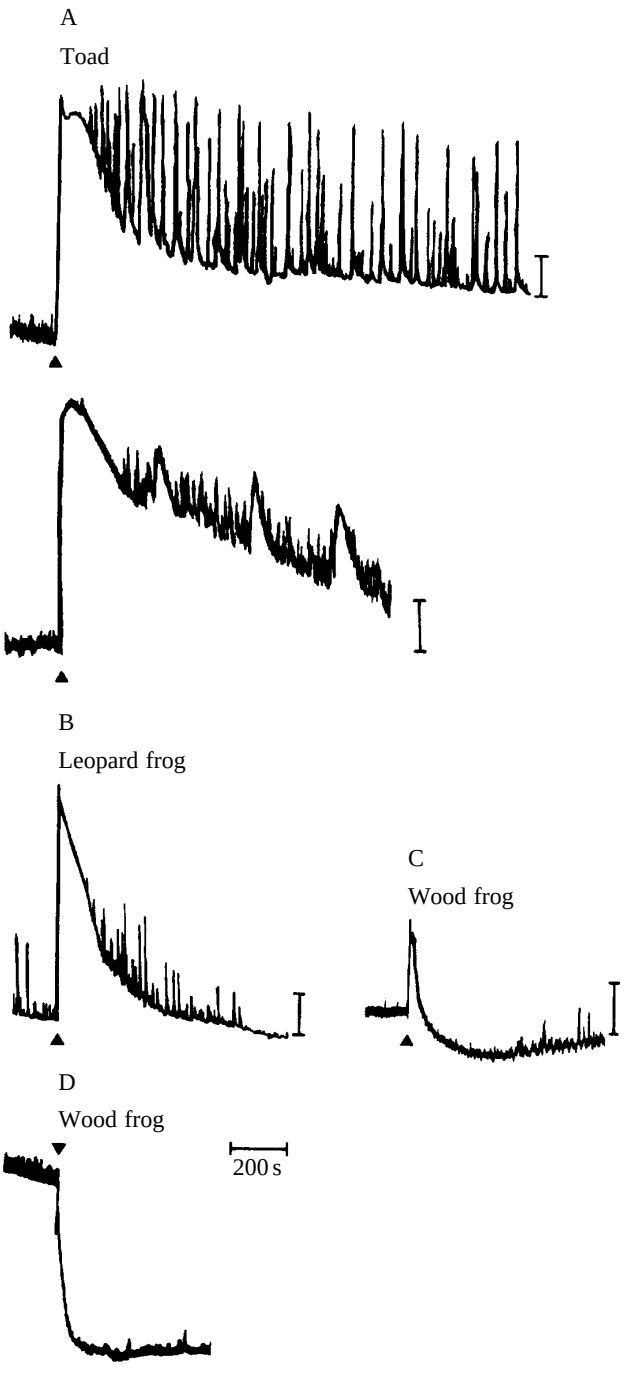

Fig. 1. Some effects of sudden cooling of isolated toad, leopard frog and wood frog spinal cords. In this and in all subsequent figures, an upward movement of the tracing represents motoneuron depolarization. Cords were cooled from room temperature (21–22 °C) to 6–8 °C. The onset of cooling is indicated by arrowheads. (A) Large cold-induced toad motoneuron depolarizations in two different cords. Both cords were cooled to 7 °C. The slow declining phases of the cold-induced depolarizations were accompanied by paroxysmal, slow motoneuron depolarizations. (B) Large, shorter-lasting depolarization evoked in leopard frog motoneurons by cooling (7 °C). Only small amounts of spontaneous motoneuron activity are seen both before and during cooling. (C) Small cold-induced (8 °C) motoneuron depolarization in wood frog spinal cord. The cord hyperpolarized while cooling was maintained. (D) Motoneuron hyperpolarization induced in wood frog spinal cord by cooling (6 °C). No preceding depolarization occurred. Vertical calibrations: A, B and C, 1.0 mV; D, 0.5 mV.

Table 1. *Motoneuron depolarizations induced by sudden cooling*

	Bufo marinus (*N*=19)	*Rana pipiens* (*N*=20)	*Rana sylvatica* (*N*=8)
Amplitude (mV)	6.2±1.0	10.7±1.2	2.2±0.8
Duration (min)	26.2±4.2	5.9±0.6	1.0±0.2

The number of experiments is shown in parentheses. All cords were cooled to between 6 and 8 °C.

ANOVA reveals that there is a significant difference among species for both amplitude ($F_{2,42}$=7.9, P<0.01) and duration ($F_{2,42}$=17.2, P<0.0001) of responses.

Post hoc t-tests revealed that *all* species were significantly different from each other with regard to both amplitude and duration.

Table 2. *Motoneuron hyperpolarizations produced by rewarming to room temperature*

	Bufo marinus (*N*=13)	*Rana pipiens* (*N*=20)	*Rana sylvatica* (*N*=8)
Amplitude (mV)	4.4±0.4	3.2±0.2	2.4±0.5
Duration (min)	26.4±3.3	3.0±0.4	0.8±0.2

The number of experiments is shown in parentheses. All spinal cords were rewarmed from 6–8 °C to 21–22 °C.

ANOVA reveals that there is a significant difference among species for both amplitude ($F_{2,38}$=6.4, P<0.01) and duration ($F_{2,38}$=55.2, P<0.00001) of responses.

Post hoc t-tests revealed that *all* species were significantly different from each other with regard to both amplitude and duration.

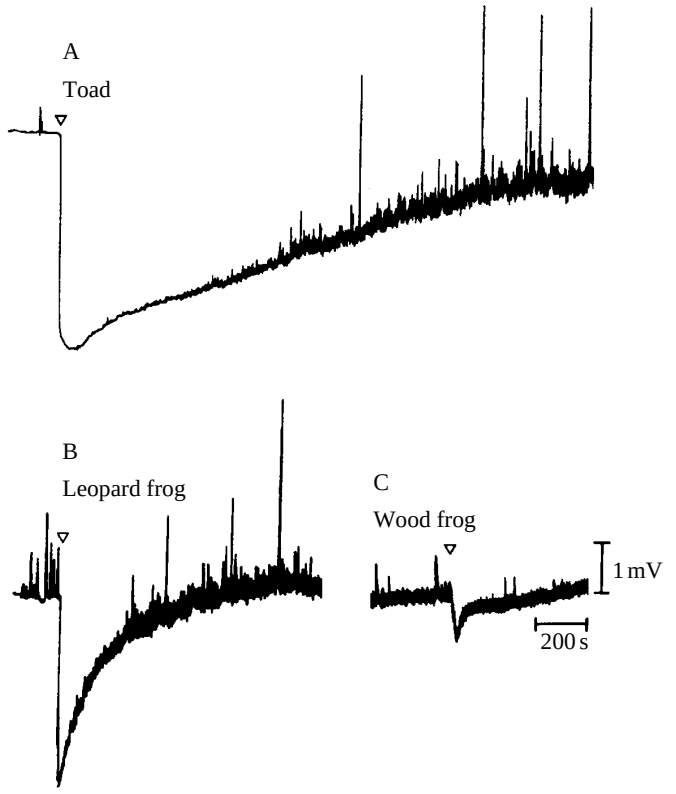

Fig. 2. Rapid rewarming to room temperature produced hyperpolarizations of toad, leopard frog and wood frog motoneurons. (A) Substantial, long-lasting hyperpolarization of toad motoneurons induced by warming from 7 to 21 ˚C. (B) A less dramatic hyperpolarization produced in leopard frog motoneurons by warming from 7 to 21 ˚C. (C) Small-amplitude, transient wood frog motoneuron hyperpolarization as a result of warming (from 7 to 21 ˚C). Arrowheads indicate the point of temperature change.

temperature. However, clear differences among species were noted (Fig. 2; Table 2). In toad spinal cords, the hyperpolarizations induced by rewarming averaged 4.4±0.4 mV (*N*=13) in amplitude. The motoneuron membrane potential required 13–30 min (26.4±3.3 min) to return to baseline levels (Fig. 2A). In contrast, motoneuron hyperpolarizations generated by rewarming the leopard frog spinal cord were slightly smaller in amplitude (3.2±0.2 mV, *N*=20) and returned to pre-cooling resting levels in only 2–10 min (3.0±0.4 min) (Fig. 2B). In the wood frog spinal cord, motoneuron hyperpolarizations produced by rewarming were small (2.4±0.5 mV, *N*=8) in amplitude and brief, returning to room temperature levels between 30 s and 2 min after rewarming (0.8±0.2 min) (Fig. 2C).

Rapid cooling and spontaneous motoneuron potentials

Extensive paroxysmal activity was recorded from the VRs of all toad spinal cords when cooled to between 6 and 8 ˚C (Fig. 1A). This activity consisted of repetitive, slow, negative potential changes of varying duration that were sometimes accompanied by superimposed action potentials. The activity developed approximately 100–250 s after the commencement of cooling. The amplitude of the paroxysmal ventral root potentials varied, but in 62.5 % of preparations was 2.0 mV or more (2.2±0.2 mV, *N*=16). Lower-amplitude (approximately 1.0–1.5 mV) paroxysmal activity was noted in 77 % of the leopard frog preparations after cooling to the same temperatures (0.9±0.2 mV, *N*=10) (Figs 1B, 6B). In addition, the cold-induced paroxysmal activity in the leopard frog VR was usually short-lived. In contrast, 75 % of wood frog cords exhibited very high rates of spontaneous motoneuron activity even after several hours of superfusion with Ringer's solution at room temperature (cf. Figs 3A, 1C, 5A). In two spinal cords with low rates of activity, cooling (to below 8 ˚C) produced spontaneous motoneuron depolarizations, but the depolarizations were of low amplitude (approximately 0.5 mV) and short duration.

As expected (Ryan *et al.* 1984), tetrodotoxin (TTX) rapidly abolished all cold-evoked spontaneous motoneuronal activity in the spinal cords of toads (not illustrated; *N*=6), leopard frogs (not illustrated; *N*=6) and wood frogs (Fig. 3B; *N*=2). TTX reduced cold-induced depolarizations in leopard frog motoneurons (not illustrated; 47±4 % of control amplitude, *N*=2), but did not alter the cold-induced hyperpolarizations in wood frog spinal cords (Fig. 3B; *N*=2). TTX was not tested in toad or wood frog spinal cords that depolarized after exposure to cold.

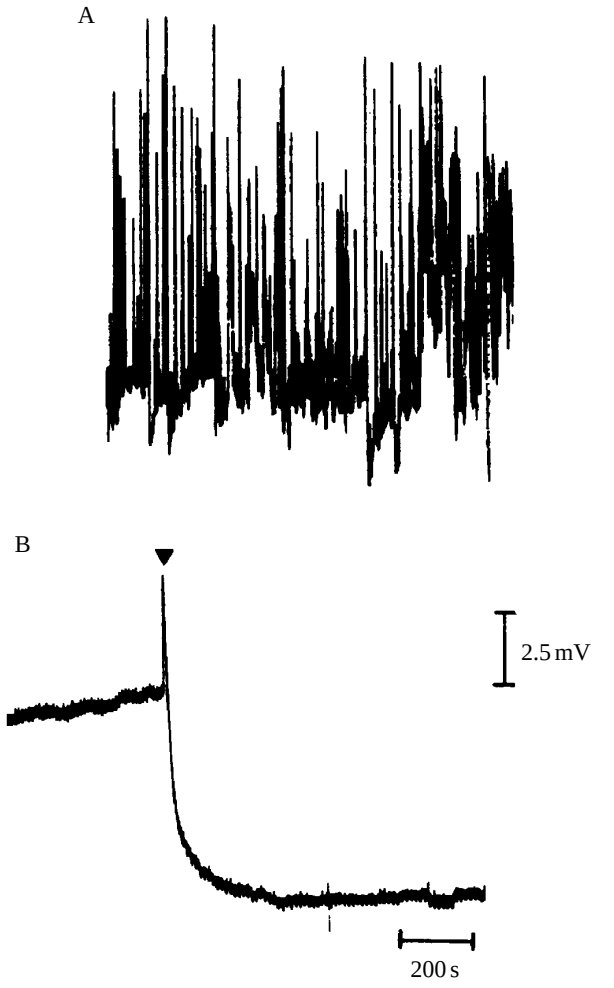

Fig. 3. Spontaneous motoneuron activity in wood frog spinal cords. (A) Spontaneous ventral root potentials in a wood frog spinal cord superfused with Ringer's solution at room temperature (21 °C) for 2 h. (B) Exposure of the cord to tetrodotoxin (0.78 μmol l^{-1}; 15 min) eliminated spontaneous ventral root potentials, but did not eliminate the brief depolarization followed by hyperpolarization produced by cooling (6 °C; the arrowhead marks the onset of cooling).

The effects of cold on evoked VR reflex potentials

The VR potentials (VRPs) evoked by supramaximal stimulation of the afferent fibers contained in the DR (DR-VRPs) consist of two components: a slow component produced by the electronic transmission of postsynaptic potentials in motoneurons and fast components produced by motoneuron action potentials conducted by VR axons. Cooling of the toad spinal cord to between 10 and 12 °C increased the amplitude of the slow component of DR-VRPs (130, 136 % increase, *N*=2; Fig. 4A) (Kolmodin and Skoglund, 1953; Tebecis and Phillis, 1968). When the cord was returned to room temperature, the enhancement of reflex activity was only partially reversible (Fig. 4A) (Kolmodin and Skoglund, 1953). In contrast, cooling of the spinal cords of toad and leopard frog to 8 °C or below decreased the amplitude of the early components of DR-VRPs (toad 39±5.0 % decrease, *N*=6; leopard frog 38±3.0 % decrease, *N*=6) and dramatically prolonged the polysynaptic components (toad 218±115 % increase, *N*=6; leopard frog 170±40 % increase, *N*=6) (Fig. 4B,C). As can be seen in Fig. 4B, increased motoneuron firing was produced in the cooled toad spinal cord despite the decreased amplitude of the slow potentials.

DR-VRPs evoked in the wood frog spinal cord at room temperature substantially differed from those evoked in toad and leopard frog spinal cords. In the wood frog, a single stimulus applied to the DR evoked a VR depolarization that was always followed by a substantial hyperpolarization (1.4±0.4 mV, *N*=8; Fig. 4Di). We have previously shown in the leopard frog that such after-hyperpolarizations are not produced by single DR stimuli, but require a DR tetanus (Shope *et al.* 1993). Exposure to cold reduced the after-hyperpolarizations produced in all wood frog motoneurons (Fig. 4Dii). The DR-VRP itself was usually reduced in amplitude (25±4 %, *N*=3), but not prolonged, under the same conditions. The after-hyperpolarization was also sensitive to a cardiac glycoside (dihydro-ouabain 10 μmol l^{-1}) (Fig. 4Diii). The sensitivity to cold and to dihydro-ouabain is compatible with the idea that the after-hyperpolarizations were caused by activation of an electrogenic Na^+ pump.

Cl^-- and cooling-induced hyperpolarization in wood frog spinal cord

The motoneuron hyperpolarizations induced by sudden cooling recorded in wood frog spinal cords may be produced by a change in ionic permeability. The possibility that the motoneuron hyperpolarizations were caused by an increase in Cl^- conductance was evaluated by experiments in which the concentration of Cl^- was reduced. This was accomplished by replacing 114 mmol l^{-1} NaCl in the Ringer's solution with an equimolar amount of sodium isethionate. Excessive amounts of paroxysmal motoneuron activity, presumably produced by the elimination of both pre- and postsynaptic inhibition, were prevented by application of kynurenate (2.0 mmol l^{-1}), a broad-spectrum antagonist of amino-acid-induced excitations in the spinal cord (Elmslie and Yoshikami, 1985; Jahr and Yoshioka, 1986). In these experiments, kynurenate did not interfere with the ability of cold to hyperpolarize wood frog motoneurons (Fig. 5B) but, in medium in which most of the Cl^- had been replaced with isethionate, cooling the cord induced motoneuron depolarizations instead of the expected hyperpolarizations (Fig. 5C).

Sensitivity to excitatory amino acids

The motoneuron depolarizations produced by cooling the spinal cords of all three amphibian species were sensitive to application of kynurenate. As seen in Fig. 6A, application of kynurenate (2.0 mmol l^{-1}) blocked the brief, cold-induced motoneuron depolarizations in wood frog spinal cords and the larger, longer-lasting depolarizations seen in leopard frog spinal cords (Fig. 6B; 66±4 % of control amplitude, *N*=7). Similar observations concerning the actions of kynurenate have been made in the toad spinal cord (Daló *et al.* 1993).

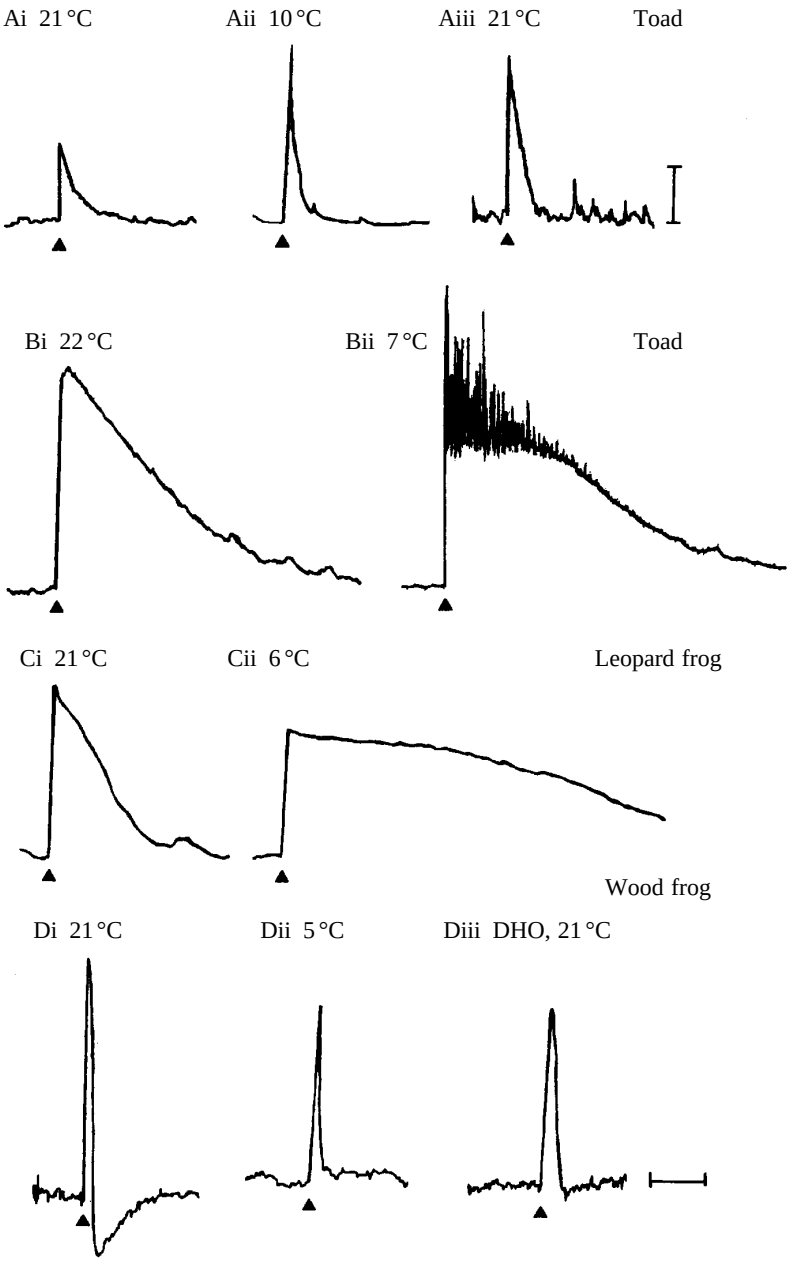

Fig. 4. Evoked ventral root potentials (DR-VRPs) recorded at room temperature (21 °C, control DR-VRPs) and during superfusion with cold Ringer's solution (starting at the arrowheads). In all experiments, the fibers contained in the dorsal root (DR) were supramaximally stimulated by electrodes applied to the sciatic nerve, which had been left in continuity with the DR. (A) Toad spinal cord. (Ai) Control DR-VRP. (Aii) The amplitude of the DR-VRP increased during cooling (10 °C, 40 min). (Aiii) Partial recovery of the DR-VRP after return to room temperature (21 °C, 23 min). (B) Toad spinal cord. (Bi) Control DR-VRP. (Bii) DR-VRP demonstrating increased duration of polysynaptic activity in cold medium (7 °C, 20 min). (C) Leopard frog spinal cord. (Ci) Control DR-VRP. (Cii) Marked augmentation of the duration of the DR-VRP in cold Ringer's solution (6 °C, 11 min). (D) Wood frog spinal cord. (Di) Control DR-VRP evoked by single DR stimulus at room temperature. Note the after-hyperpolarization following the VRP. (Dii) DR-VRP recorded during exposure to cold Ringer's solution (5 °C, 30 min). (Diii) Evoked VRP recorded after return to room temperature. Dihydro-ouabain (DHO, 10 μmol l^{-1}, 30 min) had been added to the superfusate. Note the block of the after-hyperpolarization by both cold and dihydro-ouabain. Vertical calibration: A, C, 2.5 mV; B, 1.0 mV; D, 0.5 mV. Horizontal calibration: A, D, 20 s; B, C, 2.0 s.

Cold-induced hyperpolarizations in the wood frog were unaffected by kynurenate (Figs 5B, 6A; *N*=2).

To determine whether there were differences in motoneuron sensitivity to the putative spinal excitatory transmitter L-glutamate in the spinal cords of leopard and wood frogs, we tested the ability of brief (10 s) applications of L-glutamate to produce VR depolarizations in the two species. The dose-dependency of the L-glutamate-induced effects is shown in Fig. 7.

Discussion

In the course of evolution, amphibians have adapted to an extended range of thermal conditions. Although the effects of temperature on amphibian spinal cords have been extensively studied (e.g. Ozório de Almeida, 1939, 1943; Grundfest, 1941; Kolmodin and Skoglund, 1953; Tebecis and Phillis, 1968; Hackman and Davidoff, 1991), the electrophysiological changes produced by cooling vary among different reports. Presumably some of these differences reflect the use of diverse species of amphibians, but the electrophysiological differences that characterize amphibians differing in their sensitivity to temperature changes have not been systematically investigated. In the present study, sucrose-gap recordings from the ventral roots of amphibian spinal cords have demonstrated that sudden cooling of the spinal cord results in marked changes in the membrane potential of motoneurons, changes in spontaneous motoneuron activity and altered evoked reflex responses. Furthermore, the changes in spinal cord function produced by cold differ substantially among three species of

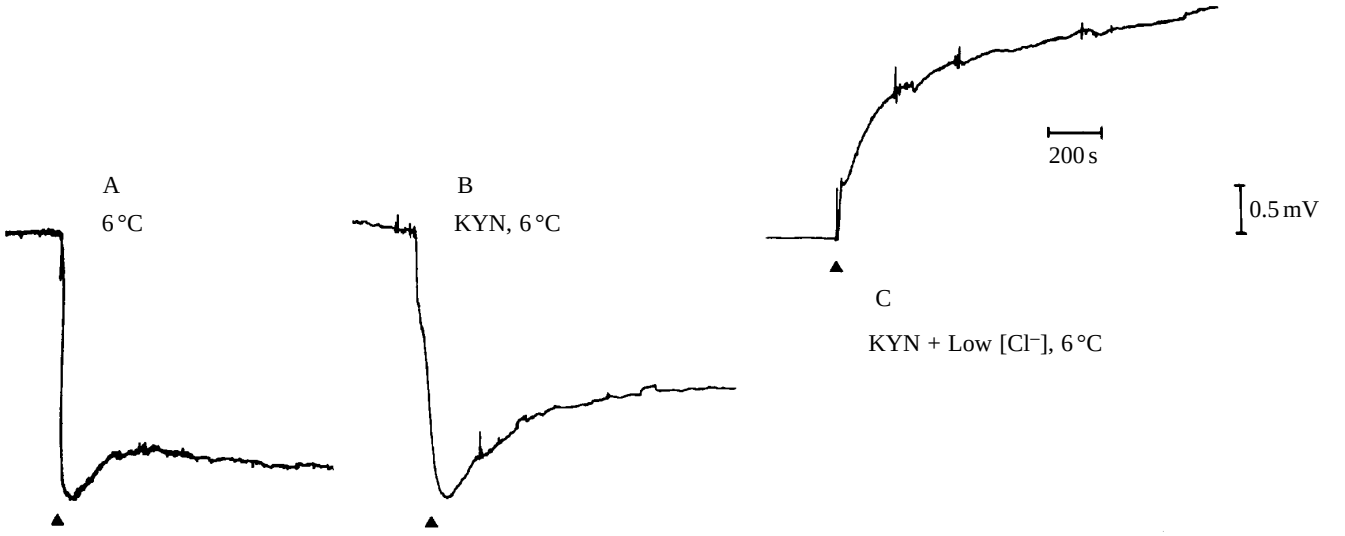

Fig. 5. The replacement of NaCl with sodium isethionate blocked the hyperpolarization induced by sudden cooling in the wood frog spinal cord. The onset of cooling (to 6 °C) is indicated by an arrowhead. (A) Control motoneuron hyperpolarization produced by exposure to cold Ringer's solution. (B) Repeat exposure to cold Ringer's solution after addition of kynurenate (KYN, 2.0 mmol l^{-1}; 30 min). (C) Recording obtained during exposure to cold medium after replacing 114 mmol l^{-1} NaCl with an equivalent amount of sodium isethionate.

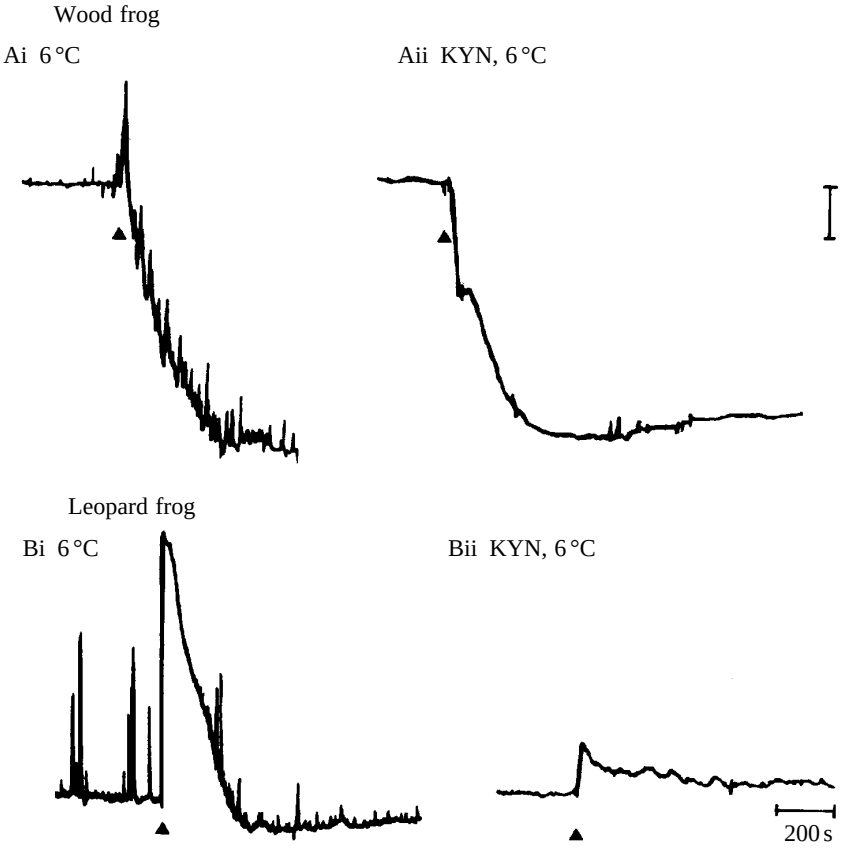

Fig. 6. Kynurenate blocks cold-induced motoneuron depolarization. (A) Wood frog. (Ai) Control exposure to cold Ringer's solution (6 °C; onset of cooling at the arrowhead). (Aii) Similar cooling after addition of kynurenate (KYN, 2.0 mmol l^{-1}, 30 min) to the medium. (B) Leopard frog. (Bi) Control exposure to cold medium (6 °C). (Bii) Repeat exposure after addition of kynurenate (KYN, 1.0 mmol l^{-1}, 30 min). Vertical calibration: A, 0.5 mV; B: 2.5 mV.

amphibians that vary in their climatic adaptations and in their tolerance to cold temperatures.

Large and prolonged VR depolarizations were produced in leopard frog and toad spinal cords by rapid cooling. In contrast, VR depolarizations were either small and short-lived or were not seen in wood frog spinal cords. Moreover, when VR depolarizations were noted in wood frog spinal cords, they were always followed by a large hyperpolarization. In a minority of wood frog spinal cords, no depolarization was recorded from the VR; only a hyperpolarization was noted. Slow potential changes recorded from the VR represent potentials electrotonically conducted from motoneurons whose

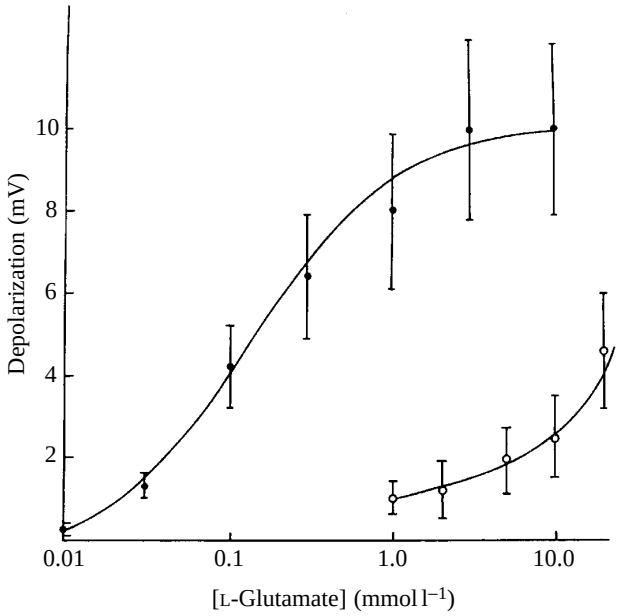

Fig. 7. Mean dose–response curves for L-glutamate-evoked motoneuron depolarizations in wood and leopard frog spinal cords. Ordinate: amplitude of changes in VR potential produced by 10 s applications of L-glutamate. Abscissa: concentration (mmol l^{-1} of L-glutamate in superfusate). (○) Wood frog. (●) Leopard frog. Each point represents the mean ± S.E.M. from six preparations. Note the substantial difference in sensitivity to applied L-glutamate between the two species.

axons exit from the spinal cord in the VR and they therefore represent changes in the membrane potential of the population of motoneurons contained in a spinal segment. Such cold-induced depolarizing changes in the potential of motoneurons are presumably caused both by indirect synaptic effects exerted *via* interneurons and by direct actions on motoneuron membranes.

It is presumed that much of the excitatory synaptic transmission in the amphibian spinal cord is mediated by the synaptic release of L-glutamate and/or related amino acids from afferent terminals and interneurons and by the subsequent activation of excitatory amino acid receptors on interneurons and motoneurons (Davies *et al.* 1982). Reduction of the cold-induced depolarizations by kynurenate, a broad-spectrum antagonist of amino-acid-induced excitations in the spinal cord (Elmslie and Yoshikami, 1985; Jahr and Yoshioka, 1986), suggests that the cold-induced depolarizations were caused, at least in part, by ionic shifts of Na^+ through excitatory amino acid receptor-regulated ion channels. Moreover, the size of the cold-induced depolarizations appeared to correlate with the sensitivity of motoneurons to exogenous L-glutamate. Thus, in leopard frogs the amplitude of the cold-induced motoneuron depolarizations was large, as was the ability of L-glutamate to depolarize motoneurons, but in wood frogs, where the cold-induced depolarizations were small or absent, motoneurons were relatively insensitive to applications of the excitatory amino acid. Our results, however, do not indicate whether the increased excitatory amino-acid-mediated synaptic transmission produced by exposure to cold temperatures was caused by an increased release or by a decreased uptake of excitatory amino acids, or by a low-temperature-stimulated conformational change in the postsynaptic excitatory amino acid receptors. Multiple factors were presumably involved. For example, L-glutamate is released from spinal cord slices of toads, but not leopard frogs, as a result of suddenly lowering the temperature of the medium (Daló and Larson, 1991). Moreover, low temperature has been shown to interfere with the L-glutamate re-uptake mechanism (Davidoff and Adair, 1975).

Other processes presumably contribute to the development of depolarizations produced by spinal cord cooling. For example, the behavior of membranes, enzymatic reaction rates and ion channel kinetics are dependent upon neuronal temperature (Pringle and Chapman, 1981). In particular, in some cells, the electrogenic Na^+ pump generates a net outward transmembrane current which contributes directly to their resting membrane potential (Kerkut and York, 1971; Koketsu, 1971). The electrogenic Na^+ pump is reported to depend upon temperature in some invertebrates (Carpenter and Alving, 1968; Gorman and Marmor, 1970). It is thus possible that inhibition of the Na^+ pump by cold may contribute to the depolarizations observed in our experiments.

As in other neurons, the resting transmembrane potential of amphibian motoneurons can presumably be determined by the Nernst equation. In its general form, the Nernst equation indicates that the membrane potential is proportional to the absolute temperature (K) and that a decrease in temperature would result in motoneuron depolarization. Assuming that a rapid change in temperature does not alter passive ion permeabilities, a shift in temperature from 21 to 6 °C (from 294 to 279 K) would reduce the motoneuron membrane potential from −90 mV (Barrett and Barrett, 1976) to approximately −85.4 mV. But VR sucrose-gap measurements record only a variable proportion of the changes in motoneuron membrane potential (Stämpfli, 1954). We estimate that about one-third of the actual changes in motoneuron membrane potential are reflected in sucrose-gap VR recordings (J. C. Hackman, unpublished observations). We therefore believe that only a fraction of the large cold-induced depolarizations recorded from leopard frog and toad spinal cords are caused by changes in Nernstian kinetics.

The motoneuron hyperpolarizations generated by rapid cooling of wood frog spinal cords appear to be produced by an increment in Cl^- conductance of motoneuron membranes because the hyperpolarizations were converted to depolarizations in medium in which the Cl^- concentration was reduced. It is possible that the increased Cl^- conductance is the result of augmented release of putative inhibitory amino acid neurotransmitters such as glycine and γ-aminobutyric acid, but we have not used inhibitory amino acid antagonists to pursue this hypothesis.

Several reports have appeared regarding the effects of temperature on anuran reflexes (Winterstein and Terzioglu,

1942; Kolmodin and Skoglund, 1953; Tebecis and Phillis, 1968), but the results of these investigations are contradictory. In part, these differences reflect the use of different recording techniques and, in particular, upon differences in amplifier coupling. DR-VRPs recorded using the sucrose-gap technique from amphibian spinal cords are considerably larger and longer than those recorded in the conventional manner with wire electrodes placed on two points on a VR because (1) the sucrose-gap technique allows recording of stable high-resolution potential changes, which correspond more closely to the size and duration of the exact intracellular events underlying the VRP; and (2) a.c.-coupled recording with a long time constant, conventionally used to record reflexes, can artifactually and substantially shorten the record by filtering out the slower components of the potential. In our experiments, the slower components appear to be very sensitive to cold.

The increased duration of DR-VRPs in the toad and leopard frog produced by cold is not unexpected. Even at low temperatures, amphibian spinal cords maintain chemically mediated transmission (Czéh and Dezso, 1982). Cold would be expected to have facilitatory effects on the evoked release of synaptic transmitters because of the well-characterized ability of low temperatures to prolong action potentials (Klee *et al.* 1974). A broadening of action potentials would most probably augment evoked transmitter release. In addition, afferent axons in the frog branch repeatedly after they enter the spinal cord and before they make synaptic contacts (Grantyn *et al.* 1984). Increased spike duration in afferent fibers produced by cooling would be expected to facilitate propagation of impulses through these branched axons (Cruzblanca and Alvarez-Leefmans, 1989).

The spinal cord isolated from the brain is capable of convulsive activity very similar to that observed in intact animals (Somjen *et al.* 1978). Ozório de Almeida (1943) first observed that rapid cooling of the South American frog evoked epileptiform seizures in the hind limbs. It is now realized that sudden cooling of the isolated spinal cords of many species of South American frogs and toads to below 10 °C is sufficient to generate spinal seizure activity (Daló and Larson, 1991; Novis, 1948). In contrast, in North American and European frogs, animals naturally exposed to cold weather, spinal epileptiform activity is seen only if spinal cords are subjected to temperatures near freezing point (Ozório de Almeida, 1939, 1943).

In the present experiments, large, long-duration paroxysmal motoneuron depolarizations were seen in the cold-sensitive tropical toad spinal cord when the temperature of the superfusate was reduced to between 6 and 8 °C. By contrast, in the leopard frog, a cold-resistant animal, sudden cooling to these temperatures induced only short-duration, short-lived, low-amplitude paroxysmal activity resembling the spontaneous motoneuron potentials seen in frog spinal cord at higher temperatures *in vitro* (Ryan *et al.* 1984). In the freeze-resistant wood frog, high rates of spontaneous motoneuron activity were recorded from the VRs of most spinal cords at room temperature. In those cords without spontaneous discharges, cooling produced spontaneous motoneuron depolarizations, but the depolarizations were of low amplitude and short duration. We presume that the large, long-duration paroxysmal motoneuron depolarizations seen in the toad spinal cord are the electrophysiological basis for the epileptiform activity seen in the hind limbs of cooled South American toads.

Frogs show a wide range of adaptations to nearly all kinds of living conditions, but the differences in the central nervous systems among species differing in their temperature sensitivities have not been investigated. This is the first report of central nervous system responses to sudden cooling among amphibian species that are normally exposed to different environmental temperatures and that vary in their behavioral responses to cold. We have shown that three species of amphibians, a cold-sensitive tropical toad (*Bufo marinus*) that usually dies when cooled to 5 °C or below, a cold-resistant aquatic hibernating frog (*Rana pipiens*, northern leopard frog) that survives at temperatures of 2–5 °C and a freeze-tolerant frog (*Rana sylvatica*, wood frog) that endures sustained freezing at −2.5 °C without apparent detrimental effects (Storey and Storey, 1988), also vary in their electrophysiological responses to cold. We have some clues as to the mechanisms of some of these changes, but the relationship of these responses to climatic adaptations remains to be determined.

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