

## Rapid cold-hardening in larvae of the Antarctic midge *Belgica antarctica*: cellular cold-sensing and a role for calcium

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**Teets NM, Elnitsky MA, Benoit JB, Lopez-Martinez G, Denlinger DL, Lee RE, Jr.** Rapid cold-hardening in larvae of the Antarctic midge *Belgica antarctica*: cellular cold-sensing and a role for calcium. *Am J Physiol Regul Integr Comp Physiol* 294: R1938–R1946, 2008. First published April 16, 2008; doi:10.1152/ajpregu.00459.2007.—In many insects, the rapid cold-hardening (RCH) response significantly enhances cold tolerance in minutes to hours. Larvae of the Antarctic midge, *Belgica antarctica*, exhibit a novel form of RCH, by which they increase their freezing tolerance. In this study, we examined whether cold-sensing and RCH in *B. antarctica* occur in vitro and whether calcium is required to generate RCH. As demonstrated previously, 1 h at  $-5^{\circ}\text{C}$  significantly increased organismal freezing tolerance at both  $-15^{\circ}\text{C}$  and  $-20^{\circ}\text{C}$ . Likewise, RCH enhanced cell survival of fat body, Malpighian tubules, and midgut tissue of larvae frozen at  $-20^{\circ}\text{C}$ . Furthermore, isolated tissues retained the capacity for RCH in vitro, as demonstrated with both a dye exclusion assay and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based viability assay, thus indicating that cold-sensing and RCH in *B. antarctica* occur at the cellular level. Interestingly, there was no difference in survival between tissues that were supercooled at  $-5^{\circ}\text{C}$  and those frozen at  $-5^{\circ}\text{C}$ , suggesting that temperature mediates the RCH response independent of the freezing of body fluids. Finally, we demonstrated that calcium is required for RCH to occur. Removing calcium from the incubating solution slightly decreased cell survival after RCH treatments, while blocking calcium with the intracellular chelator BAPTA-AM significantly reduced survival in the RCH treatments. The calmodulin inhibitor *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) also significantly reduced cell survival in the RCH treatments, thus supporting a role for calcium in RCH. This is the first report implicating calcium as an important second messenger in the RCH response.

insect cold-hardening; rapid acclimation; calcium signaling; freeze-tolerant insects

RAPID COLD-HARDENING (RCH), which allows many insects to significantly enhance their cold tolerance, was first described in freeze-susceptible insects more than 20 years ago (8, 29). While most cold acclimation processes occur over days, weeks, or even months, RCH drastically enhances cold tolerance after only a brief exposure to moderately low temperatures. In addition to protecting insects against chilling injury, RCH also improves courtship and reproduction (50, 52). Furthermore, RCH occurs during ecologically relevant diurnal temperature cycles, allowing insects to track daily changes in temperature (24, 25). Thus it appears that RCH is part of a generalized acclimatory response that allows insects to adjust rapidly to changes in their environment.

While the ecological relevance of the RCH response is established, its underlying mechanism is poorly understood.

For insects living in thermally variable environments, cold shock is a common threat that has long been associated with damage to cell membranes (19, 28, 57). Recently, several studies have shown that RCH protects against cold shock injury at the cellular level. During RCH, the proportion of unsaturated fatty acids in the plasma membrane increases (36, 45), leading to increased membrane fluidity that may protect membranes from subsequent chilling injury (30). In addition, RCH protects against cell death by inhibiting cold-induced apoptosis (68). At the molecular level, a number of genes are upregulated during brief exposure to cold, including ones that encode heat shock proteins, enzymes of cellular metabolism, cytoskeletal proteins, and proteins involved in transcription and translation as well as signal transduction (35, 48). However, in general the function of these changes in gene expression in conferring cold tolerance is unknown.

Isolated tissues can undergo RCH in vitro, indicating that cold-sensing occurs at the cellular level, without regulation from the central nervous system (CNS) and major endocrine glands (67). The signaling events responsible for cellular cold-sensing and the RCH response have not been identified in insects, but one candidate messenger that may be involved in eliciting the RCH response is calcium. In plants, cellular cold-sensing is initiated by calcium flux (38, 39). Here, chilling causes changes in membrane properties that increase calcium flux from both extracellular and subcellular compartments, thereby activating  $\text{Ca}^{2+}$ -dependent protein kinases and transcription factors that regulate cold-specific proteins and genes.

In general, the two strategies for surviving subzero temperatures are freeze avoidance and freeze tolerance, where freeze tolerance refers to the ability to survive extracellular ice formation. While previous research on RCH was confined to freeze-susceptible insects, Lee et al. (31) demonstrated that RCH can increase the freeze tolerance of the Antarctic midge, *Belgica antarctica*, the first report of RCH in a freeze-tolerant insect. In *B. antarctica* larvae, a 1-h exposure at  $-5^{\circ}\text{C}$  dramatically increases larval and tissue-level freeze tolerance. The  $-5^{\circ}\text{C}$  acclimatory temperature used in this study is the lowest known temperature to induce RCH. However, the physiological mechanisms of this novel form of RCH have not been studied, and it is not certain whether cold-sensing and RCH in *B. antarctica* are cellular level responses similar to those described by Yi and Lee (67). Furthermore, while larvae freeze at  $-5^{\circ}\text{C}$  during RCH (31), it is uncertain whether freezing is required for RCH to occur in *B. antarctica*, or whether RCH can occur in both the frozen and supercooled states at  $-5^{\circ}\text{C}$ .

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In the present study, we examined the effect of RCH in *B. antarctica* larvae at both organismal and cellular levels. The objectives of this study were 1) to examine whether cold-sensing and RCH can occur in vitro without input from the CNS and major endocrine glands, 2) to determine whether RCH occurs in both the supercooled and frozen states, and 3) to test whether calcium and calmodulin are involved in cellular cold-sensing and RCH.

## MATERIALS AND METHODS

**Insects.** Larvae of *B. antarctica* were collected on Torgersen and Cormorant Islands near Palmer Station on the Antarctic Peninsula in January 2006 and January 2007. Substrate containing larvae was shipped frozen (approximately  $-5^{\circ}\text{C}$  for 7 days) to Miami University and stored at  $4^{\circ}\text{C}$ . The larvae had been stored for 2 mo or more at  $4^{\circ}\text{C}$  when these experiments were conducted. Just before experimental use, larvae were sorted from the substrate and placed in ice-cold water.

**Organismal survival.** Since the RCH experiments of Lee et al. (31) were conducted on freshly collected larvae, similar experiments were repeated in this study to confirm that RCH occurs in laboratory-stored larvae and to provide context for the in vitro experiments. Groups of 10 larvae maintained at  $4^{\circ}\text{C}$  were placed in capped 1.6-ml microcentrifuge tubes and submerged in  $\sim 50\ \mu\text{l}$  of double-distilled  $\text{H}_2\text{O}$  ( $\text{ddH}_2\text{O}$ ). Larvae were transferred from  $4^{\circ}\text{C}$  and directly exposed to  $-10^{\circ}\text{C}$ ,  $-15^{\circ}\text{C}$ , or  $-20^{\circ}\text{C}$  for 24 h in a programmable cold bath. For the RCH treatment, larvae were held at  $-5^{\circ}\text{C}$  for 1 h before exposure to either  $-15^{\circ}\text{C}$  or  $-20^{\circ}\text{C}$  for 24 h. During treatment, the water in the tubes froze at  $-5^{\circ}\text{C}$ , and the larvae, because of their high susceptibility to inoculative freezing, were also frozen (31). After 24 h of recovery at  $4^{\circ}\text{C}$ , survival was assessed by observing the larva's ability to move either spontaneously or after gentle prodding. Six replicates of 10 larvae were run for each treatment.

**In vivo cell survival.** Groups of 10 larvae were placed in microcentrifuge tubes and submerged in  $\text{ddH}_2\text{O}$  as in the previous experiment. Larvae were divided into three treatment groups: control, frozen, and RCH. The control group was maintained at  $4^{\circ}\text{C}$ , the frozen group was directly exposed to  $-20^{\circ}\text{C}$  for 24 h, and larvae in the RCH group were held frozen at  $-5^{\circ}\text{C}$  for 1 h before exposure to  $-20^{\circ}\text{C}$  for 24 h. After a 24-h recovery period at  $4^{\circ}\text{C}$ , groups of four larvae from each treatment were dissected to assess cell survival. Fat body, midgut, and Malpighian tubule tissues were dissected in ice-cold Coast's solution (10) containing (in mM) 100 NaCl, 8.6 KCl, 4.0  $\text{NaHCO}_3$ , 4.0  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 1.5  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 8.5  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 24 glucose, 25 HEPES, and 56 sucrose. Cell survival was assessed according to Yi and Lee (66) with the LIVE/DEAD sperm viability kit (Molecular Probes, Eugene, OR). Living cells with intact membranes fluoresced green or yellow-green, while dead cells fluoresced red or orange-red. For fat body and midgut mean cell survival was based on the mean of three counts of 100 cells in each of the four replicates, while for the Malpighian tubules all cells were counted in each of the four replicates.

**In vitro cellular survival.** To determine whether isolated tissues undergo RCH, treatments similar to those described above were conducted in vitro. Isolated fat body, midgut, and Malpighian tubule tissues were dissected in Coast's solution and placed in sealed 1.6-ml microcentrifuge tubes containing  $50\ \mu\text{l}$  of Coast's solution. The tissues were then subjected to control ( $4^{\circ}\text{C}$  for 48 h), frozen ( $-20^{\circ}\text{C}$  for 24 h, recovery at  $4^{\circ}\text{C}$  for 24 h), or RCH ( $-5^{\circ}\text{C}$  for 1 h,  $-20^{\circ}\text{C}$  for 24 h, recovery at  $4^{\circ}\text{C}$  for 24 h) treatments. Furthermore, to determine if the RCH response depends on whether the body fluids freeze, the RCH treatment was conducted in both the supercooled and frozen states. In the RCH supercooled treatment fluid in the undisturbed microcentrifuge tubes remained unfrozen for 1 h at  $-5^{\circ}\text{C}$ , while in the RCH frozen treatment an ice crystal was added after 10 min at  $-5^{\circ}\text{C}$

to initiate freezing. After the in vitro treatments, cell viability was assessed as described above.

**Assessing cell viability with functional viability assay.** To complement the dye exclusion assay for viability described above, we also assessed cell viability in select experiments with a functional viability assay. We dissected entire guts of *B. antarctica* larvae, including Malpighian tubules, and subjected them to control, frozen, and RCH supercooled treatments as described above. The treatments were carried out both with calcium present and after pretreatment with BAPTA (see below). To measure functional viability, we used the CellTiter 96 Non-radioactive Cell Proliferation Assay (Promega, Madison, WI), a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based assay. In this assay, the mitochondria of living cells reduce yellow MTT to a purple formazan product, so that the number of living cells is proportional to the optical density of the purple solution (40). After treatment, tissue samples were transferred to 96-well plates, 4 samples per well, and cell viability was assessed according to the manufacturer's protocol. During incubation with the MTT dye, tissues were transferred to  $18^{\circ}\text{C}$  to increase their metabolic activity. Optical density was measured at 570 nm with a Dynex MRX Microplate Reader, with a correction wavelength of 630 nm. Cell viability is expressed as a percentage relative to the mean optical density of the  $\text{Ca}^{2+}$ -present control treatment.

**Manipulation of calcium availability.** To assess the role of calcium in RCH, two approaches were used to manipulate calcium availability. These experiments were conducted only on midgut and Malpighian tubules, because fat body did not lend itself to in vitro preparation (see RESULTS).

First, the in vitro experiments described above were repeated except that Coast's solution prepared without calcium was substituted. This prevented external sources of calcium from entering the cell. In the second set of experiments, tissues were loaded with the cell-permeant calcium chelator BAPTA-AM (Molecular Probes), which is cleaved intracellularly to the active form of BAPTA and binds free cytosolic calcium. BAPTA-AM was added in a stock solution prepared with DMSO. Isolated midgut and Malpighian tubules were incubated for 1 h in calcium-free Coast's solution containing  $100\ \mu\text{M}$  BAPTA-AM (6). The tissues were then rinsed in calcium-free Coast's solution to remove excess BAPTA-AM before being subjected to the same in vitro temperature treatments as above.

**Inhibition of calmodulin.** To assess the role of calmodulin during RCH, the above in vitro experiments were conducted in the presence of *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), a calmodulin antagonist. Since these experiments were conducted on a different group of larvae collected a year later (2007), the above in vitro RCH experiments and BAPTA experiments were repeated to confirm that the tissues behaved similarly and to provide a means for comparison with the W-7 treatments. A stock solution of W-7 (Sigma-Aldrich, St. Louis, MO) was prepared in ethanol, and the W-7 stock solution was diluted with Coast's solution to a final working concentration of  $50\ \mu\text{M}$ . Midgut and Malpighian tubules were dissected in Coast's solution and placed in 1.6-ml microcentrifuge tubes containing  $50\ \mu\text{l}$  of the dilute W-7 solution. The tissues were then subjected to the same in vitro temperature treatments as above.

**Statistical analysis.** Mean values for organismal and cellular survival were compared with Student's *t*-test or analysis of variance and Bonferroni-Dunn tests (Statview, SAS Institute, Cary, NC). Since the data are presented as percentages, the survival data were arcsin square-root transformed to stabilize the variances before analysis (44). All data are presented as means  $\pm$  SE. Statistical significance was set at  $P < 0.05$ .

## RESULTS

**Effect of RCH on organismal survival.** Nearly all larvae were freeze-tolerant at  $-10^{\circ}\text{C}$ , as 95% of the larvae survived

freezing for 24 h (Fig. 1). Larval survival decreased significantly to 58% after a 24-h freeze at  $-15^{\circ}\text{C}$ , and only 5% of the larvae survived freezing at  $-20^{\circ}\text{C}$ . However, as demonstrated previously (31), 1 h of RCH at  $-5^{\circ}\text{C}$  significantly increased the freeze tolerance of larvae frozen at both  $-15^{\circ}\text{C}$  and  $-20^{\circ}\text{C}$  (Fig. 1). After RCH, 88% and 70% of the larvae survived freezing at  $-15^{\circ}\text{C}$  and  $-20^{\circ}\text{C}$ , respectively.

**Effect of in vivo RCH on cellular survival.** The increase in organismal survival after RCH was reflected in the body tissues, as shown by Lee et al. (31). Overall, RCH significantly increased the cell survival of fat body and midgut frozen at  $-20^{\circ}\text{C}$  (Table 1). In fat body tissue, RCH at  $-5^{\circ}\text{C}$  for 1 h significantly increased cell viability by  $\sim 50\%$  relative to larvae frozen to  $-20^{\circ}\text{C}$  with no prior treatment. A similar pattern was observed in the midgut, as RCH significantly increased cell viability by  $\sim 30\%$  over larvae frozen to  $-20^{\circ}\text{C}$  with no prior treatment (Table 1). In the Malpighian tubules, RCH increased cell viability by nearly 20%; however, this increase was not statistically significant ( $P > 0.05$ ). The lack of statistical significance in Malpighian tubules is likely due to the intrinsically high level of freeze tolerance in Malpighian tubules without RCH and the relatively high susceptibility of Malpighian tubules to mechanical damage during dissection, which may have caused greater variability in survival values.

**Effect of in vitro RCH on cellular survival.** In isolated midgut and Malpighian tubules, RCH in vitro had an effect on cell survival similar to that observed in vivo. RCH at  $-5^{\circ}\text{C}$  for 1 h caused a significant increase in cell survival in isolated midgut and Malpighian tubules compared with those tissues frozen with no prior treatment (Table 1; Fig. 2). In the midgut, freezing at  $-20^{\circ}\text{C}$  for 24 h significantly reduced cell viability by  $\sim 55\%$  relative to the controls held at  $4^{\circ}\text{C}$  (Table 1; Fig. 2). However, RCH in the frozen state at  $-5^{\circ}\text{C}$  for 1 h significantly increased cell survival  $>25\%$  relative to tissues frozen with no prior treatment. Likewise, in the Malpighian tubules 24 h of freezing at  $-20^{\circ}\text{C}$  reduced cell viability by  $\sim 55\%$  relative to control tissues held at  $4^{\circ}\text{C}$ , while RCH in the frozen state significantly increased cell survival by  $\sim 50\%$  (Table 1; Fig. 2). These results indicate that isolated tissues retain the capacity to undergo RCH in the absence of input from the CNS and associated endocrine glands.

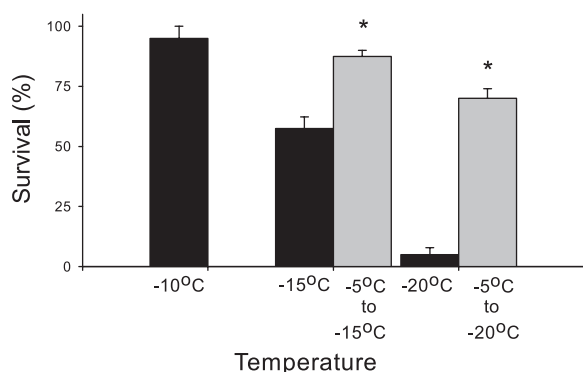


Fig. 1. Effect of rapid cold-hardening on the freeze tolerance of *Belgica antarctica* larvae. Values are means  $\pm$  SE based on 6 replicates of 10 individuals. \*Significant (Student's *t*-test,  $P < 0.05$ ) difference in survival between larvae that were directly exposed for 24 h to the test temperature (black bars) and those that were held at  $-5^{\circ}\text{C}$  for 1 h before exposure (gray bars).

Table 1. Effect of RCH in vivo and in vitro on cell viability of fat body, midgut, and Malpighian tubules

Tissue Type	Control	Frozen	RCH (Frozen)	RCH (Supercooled)
<i>In vivo</i>				
Fat body	98.5 $\pm$ 0.6 <sup>a</sup>	23.3 $\pm$ 6.5 <sup>b</sup>	77.7 $\pm$ 1.6 <sup>c</sup>	-
Midgut	97.3 $\pm$ 0.6 <sup>a</sup>	49.0 $\pm$ 5.5 <sup>b</sup>	77.7 $\pm$ 1.6 <sup>c</sup>	-
Malpighian tubules	91.2 $\pm$ 7.4 <sup>a</sup>	67.5 $\pm$ 7.5 <sup>a</sup>	86.7 $\pm$ 4.1 <sup>a</sup>	-
<i>In vitro</i>				
Fat body	79.4 $\pm$ 4.6 <sup>a</sup>	6.1 $\pm$ 1.7 <sup>b</sup>	5.6 $\pm$ 1.2 <sup>b</sup>	1.3 $\pm$ 0.8 <sup>b</sup>
Midgut	93.1 $\pm$ 1.2 <sup>a</sup>	38.3 $\pm$ 3.6 <sup>b</sup>	56.0 $\pm$ 3.6 <sup>c</sup>	53.7 $\pm$ 2.7 <sup>c</sup>
Malpighian tubules	81.6 $\pm$ 5.4 <sup>a</sup>	27.9 $\pm$ 8.0 <sup>b</sup>	74.5 $\pm$ 5.2 <sup>a</sup>	65.2 $\pm$ 7.4 <sup>a</sup>

Values (in %) are mean  $\pm$  SE cell viability for  $n = 4$  individuals. For midgut, cell survival was based on the mean of 3 counts of 100 cells, while for the Malpighian tubules all cells were counted. The treatment groups are control ( $4^{\circ}\text{C}$ ), frozen ( $-20^{\circ}\text{C}/24$  h), and rapid cold-hardening (RCH;  $-5^{\circ}\text{C}/1$  h to  $-20^{\circ}\text{C}/24$  h). For the RCH frozen treatment in vitro, an ice crystal was added after 10 min. to induce freezing. In the in vivo treatments larvae were subjected to the temperature treatment before dissection, while in the in vitro treatments larvae were dissected and isolated tissues were subjected to the temperature treatments. Different superscript letters indicate significant (ANOVA, Bonferroni-Dunn test,  $P < 0.05$ ) differences between treatments within a tissue type.

Fat body was the tissue most susceptible to freezing in vitro, as only  $\sim 5\%$  of the cells survived freezing at  $-20^{\circ}\text{C}$  (Table 1; Fig. 2). Also, freezing in vitro caused considerable structural damage to fat body tissue, because the cells became disassociated and a number of cells lysed (Fig. 2). Unlike the midgut and Malpighian tubules, in vitro RCH had no effect on the freeze tolerance of fat body at these temperatures (Table 1; Fig. 2). Since isolated fat body tissue was highly susceptible to freezing under all of the experimental conditions, we did not use fat body for the calcium experiments (see below).

**Comparison of RCH in frozen and supercooled states.** Interestingly, in the midgut and Malpighian tubules similar results were observed when the tissues were supercooled rather than frozen at  $-5^{\circ}\text{C}$  during RCH. In the midgut, RCH in the supercooled state significantly enhanced cell viability by  $\sim 55\%$  over tissues frozen at  $-20^{\circ}\text{C}$  with no prior treatment, which is the same increase in survival as that in tissues frozen during RCH (Table 1; Fig. 2). Likewise, in the Malpighian tubules RCH in the supercooled state significantly increased cell survival by  $\sim 40\%$  over tissues frozen at  $-20^{\circ}\text{C}$  with no prior treatment, while RCH in the frozen state increased cell survival by  $\sim 50\%$  (Table 1; Fig. 2). These results suggest that RCH is induced because of the change in temperature and is not dependent on freezing. Therefore, even though whole larvae froze at  $-5^{\circ}\text{C}$  during RCH, isolated midgut and Malpighian tubules demonstrated the capacity for RCH in both the frozen and supercooled states.

**Role of calcium in RCH.** To assess the role of calcium in RCH, we manipulated calcium availability by removing it from the incubating solution and by loading tissues with the calcium chelator BAPTA-AM. In the midgut, neither removing calcium from the Coast's solution nor adding BAPTA-AM to the tissues had a significant effect on the survival of control tissues or tissues frozen directly at  $-20^{\circ}\text{C}$  with no prior treatment (Fig. 3A). On the other hand, depriving the tissues of calcium reduced cell survival in both RCH treatments. In the RCH supercooled treatment, removing calcium from the Coast's solution caused a 20% reduction in cell survival, although the

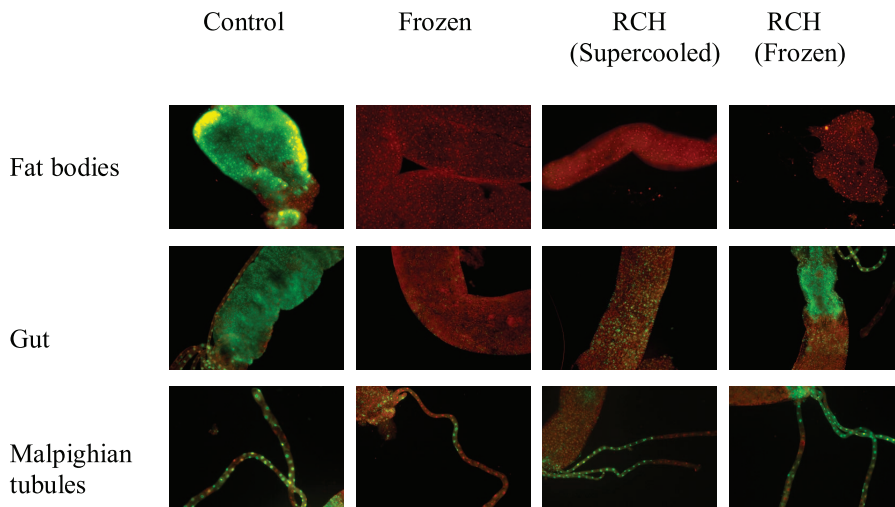


Fig. 2. Effect of rapid cold-hardening (RCH) *in vitro* on the cell viability of fat body, midgut, and Malpighian tubules. The control group was held at 4°C for 48 h, the frozen group was directly exposed to -20°C for 24 h, while the RCH groups were exposed to -5°C for 1 h followed by -20°C for 24 h. For the RCH frozen treatment, an ice crystal was added after 10 min at -5°C to initiate freezing. Live cells fluoresce green, while dead cells fluoresce red.

relationship was not significant ( $P > 0.05$ ). However, adding BAPTA-AM to the tissues, which binds free cytosolic calcium from both extracellular and intracellular sources, caused a significant 30% reduction in survival. Similarly, in the RCH frozen treatment, removing calcium from the Coast's solution

and adding BAPTA-AM significantly decreased survival by 28% and 29%, respectively.

The response of the Malpighian tubules to calcium manipulation was similar to that of the midgut. Once again, removing calcium from the Coast's solution or adding BAPTA-AM had no effect on the control and frozen treatments (Fig. 3B). In both RCH treatments (supercooled and frozen), removing calcium from the Coast's solution caused a reduction in cell survival, although this reduction was not significant ( $P > 0.05$ ). However, adding BAPTA-AM to the tissues caused a drastic decrease in cell survival in both RCH treatments. Compared with tissues that had access to calcium, the addition of BAPTA-AM caused a 44% reduction in cell survival in the RCH supercooled treatment and a 47% reduction in survival in the RCH frozen treatment. These results suggest that intracellular calcium stores are more important in Malpighian tubules than in the midgut.

*Validation of dye exclusion assay as accurate measure of cell viability.* Whereas the above results were obtained with a dye exclusion assay that assesses membrane integrity, we also used a functional, MTT-based assay to demonstrate a role for RCH and calcium in preserving cell function. With calcium present, the cell viability of tissues directly exposed to -20°C was only 35.6% that of control tissues (Fig. 4). However, after 1 h of RCH at -5°C in the supercooled state, cell viability was significantly higher at 77.9% of that of the control tissues (ANOVA, Bonferroni-Dunn,  $P < 0.05$ ). Thus, in addition to preserving membrane integrity, RCH also preserves cell function during low-temperature stress. Also, as before, pretreatment with BAPTA inhibited the RCH response (Fig. 4). Treatment with BAPTA did not significantly alter cell viability in the control or frozen treatments (Student's *t*-test,  $P > 0.05$ ), but viability of RCH-treated tissues was significantly lower by nearly 50% (Student's *t*-test,  $P < 0.05$ ). Thus nearly identical results were obtained with both a dye exclusion assay that measures membrane integrity and an MTT-based assay that measures functional viability.

*Role of calmodulin in RCH.* To assess the role of calmodulin in RCH, we inhibited calmodulin activity with the calmodulin antagonist W-7. In the midgut, inhibiting calmodulin had no effect on cell viability of control tissues and tissues frozen

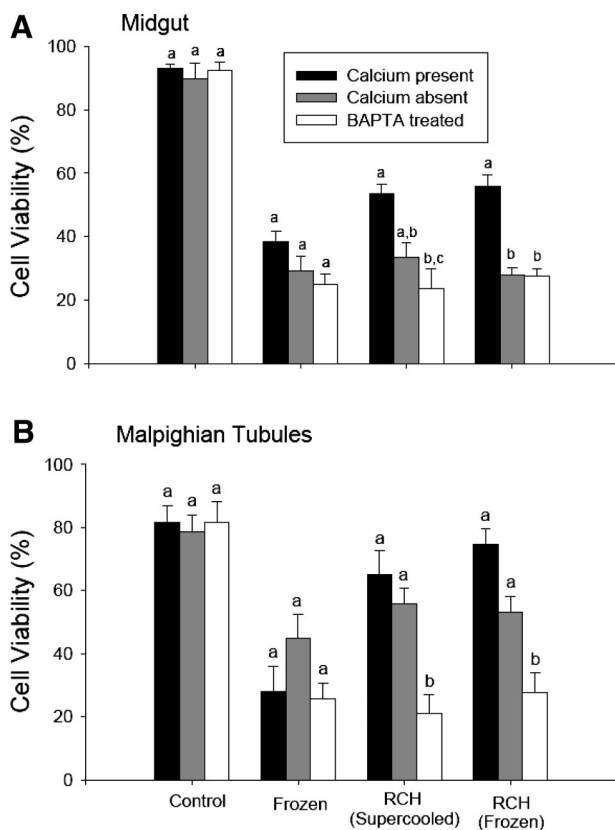


Fig. 3. Effect of calcium availability on the cell viability of midgut (A) and Malpighian tubules (B) after control (4°C), frozen (-20°C/24 h), RCH supercooled (-5°C/1 h to -20°C/24 h), and RCH frozen (-5°C/1 h + ice crystal to -20°C/24 h) treatments. Different letters indicate significant (ANOVA, Bonferroni-Dunn test,  $P < 0.05$ ) differences within a treatment group. Values are means + SE;  $n = 4$  individuals. For midgut cell survival was based on the mean of 3 counts of 100 cells in each of the 4 individuals, while for the Malpighian tubules all cells were counted in each of the 4 individuals.

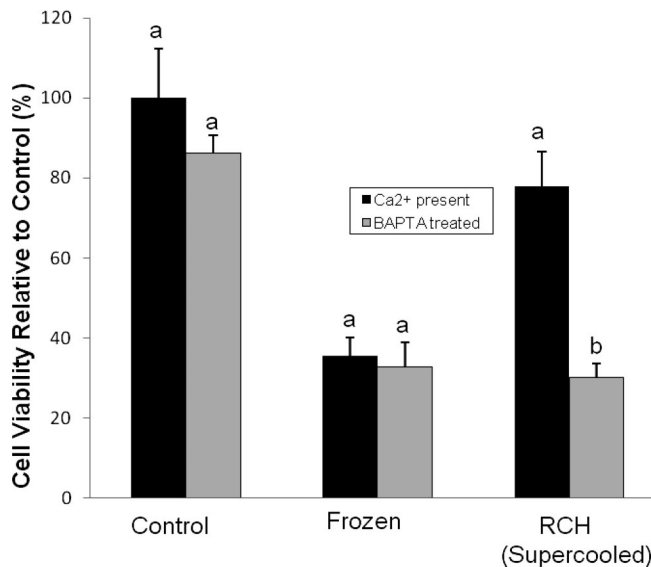


Fig. 4. Effect of RCH and calcium availability on cell viability of isolated digestive tracts measured with a functional, MTT-based viability assay. Treatments are control (4°C), frozen (−20°C/24 h), and RCH supercooled (−5°C/1 h to −20°C/24 h). In these treatments, fluid in the microcentrifuge tubes remained supercooled at −5°C and was frozen at −20°C. Different letters indicate a significant (Student's *t*-test,  $P < 0.05$ ) difference within a treatment group. Values are means  $\pm$  SE;  $n = 4$  groups of 4 tissue samples. Cell viability is expressed relative to the mean optical density of the control tissues incubated with calcium present.

directly at −20°C with no prior treatment (Fig. 5A). However, antagonizing calmodulin action inhibited RCH in both the frozen and supercooled states. The addition of W-7 to Coast's solution caused cell survival of midgut to decrease significantly by 29% in the RCH supercooled treatment and by 33% in the RCH frozen treatment (Fig. 5A). In the Malpighian tubules, the addition of W-7 had no effect on cell viability of control tissues (Fig. 5B). Meanwhile, inhibiting calmodulin caused a significant reduction in survival in frozen, RCH supercooled, and RCH frozen treatments. For tissues frozen directly at −20°C, the addition of W-7 caused a significant 26% decrease in survival (Fig. 5B). Also, similar to the midgut, antagonizing calmodulin resulted in a significant 54% decrease in survival in the RCH supercooled treatment and a 51% decrease in survival in the RCH frozen treatment (Fig. 5B). Overall, inhibiting calmodulin inhibited RCH to the same degree as chelating intracellular calcium with BAPTA-AM.

## DISCUSSION

*RCH enhances organismal and cellular freeze tolerance.* As established previously (31), RCH at −5°C for 1 h significantly enhanced the freeze tolerance of cold-acclimated larvae, resulting in a significant increase in survival at both −15°C and −20°C (Fig. 1). Also, consistent with Lee et al. (31), the level of protection afforded by RCH at the organismal level was matched by increases in cold tolerance at the cellular level. In fat body, midgut, and Malpighian tubules, RCH at −5°C for 1 h significantly enhanced cell survival compared with larvae directly exposed to −20°C (Table 1).

Only 5% of the larvae survived direct exposure to −20°C, which is a relatively modest level of freeze tolerance compared with some freeze-tolerant invertebrates. However, this limited

freeze tolerance is sufficient to allow survival within *B. antarctica*'s relatively stable microclimate conditions on islands, which are thermally buffered by the surrounding ocean throughout the year (2). On the other hand, larvae of the temperate goldenrod gall fly, *Eurosta solidaginis*, which live in more exposed microclimates, survive to adulthood after freezing at −25°C (32), and fully freeze-tolerant larvae can survive freezing to −80°C, although they are unable to complete development (3). Similarly, larvae of another Dipteran, *Heleomyza borealis*, survive temperatures as low as −60°C and require a low-temperature stimulus to complete development (61). Perhaps the most freeze-tolerant invertebrates are nematodes and tardigrades, which can survive freezing in liquid nitrogen (7, 23, 41, 56, 59). At the other extreme, two species of sub-Antarctic beetles, *Hydromedion sparsatum* and *Perimylops antarcticus*, can only survive temperatures a few degrees below their freezing points, which is sufficient in their thermally buffered microhabitats (5, 63).

The capacity to quickly increase cold tolerance has been documented in a number of Antarctic arthropods (54, 55, 62). However, in these other studies, insects were acclimated at temperatures near 0°C. In contrast, in our study −5°C was effective in enhancing cold tolerance, the lowest temperature documented to induce RCH in any insect. Also, *B. antarctica*

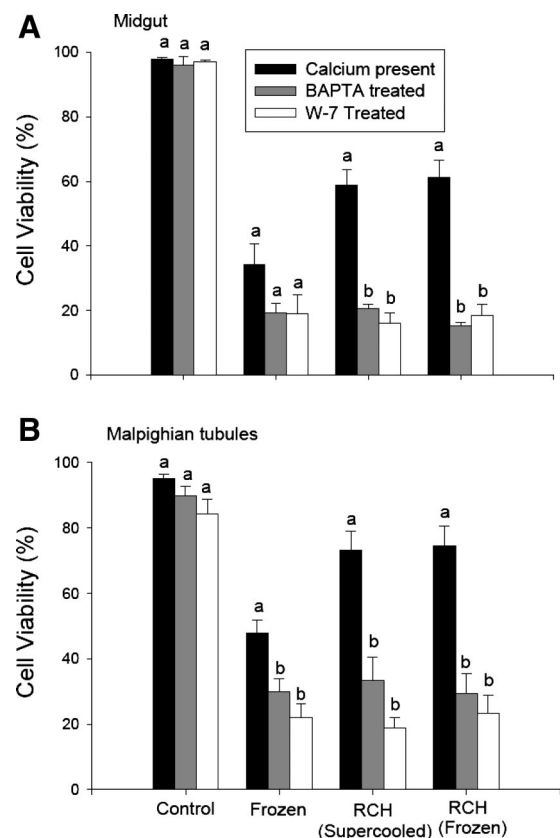


Fig. 5. Effect of calmodulin inhibition on the cell viability of midgut (A) and Malpighian tubules (B) after control (4°C), frozen (−20°C/24 h), RCH supercooled (−5°C/1 h to −20°C/24 h), and RCH frozen (−5°C/1 h + ice crystal to −20°C/24 h) treatments. Different letters indicate significant (ANOVA, Bonferroni-Dunn test,  $P < 0.05$ ) differences within a treatment group. Values are means  $\pm$  SE;  $n = 5$  individuals. For midgut cell survival was based on the mean of 3 counts of 100 cells in each of the 4 individuals, while for Malpighian tubules all cells were counted in each of the 4 individuals.

is the first known freeze-tolerant insect with RCH capacity. RCH may be particularly important during Antarctic summers, when microclimate temperatures can range anywhere from  $>30^{\circ}\text{C}$  to below freezing (11). Thus it appears that RCH allows larvae to fine-tune their response to changes in environmental temperature.

*In vitro RCH improves cellular viability.* A notable finding of this study is that cellular cold-sensing and RCH occurred in isolated tissues of *B. antarctica*. Isolated midgut and Malpighian tubules exposed to  $-5^{\circ}\text{C}$  for 1 h before freezing at  $-20^{\circ}\text{C}$  exhibited significantly higher survival than tissues directly exposed to  $-20^{\circ}\text{C}$  (Table 1; Fig. 2). Thus midgut and Malpighian tubules retain the capacity for RCH in the absence of nervous or hormonal input. This enhancement of cell survival by RCH was detected with both a dye exclusion assay that assesses membrane integrity (Fig. 2) and an MTT-based assay that measures cell function (Fig. 4). A similar study by Yi and Lee (67) demonstrated that cold-sensing and the RCH response of the freeze-susceptible flesh fly *Sarcophaga crassipalpis* occur in isolated tissues, and our study establishes that the RCH response of a freeze-tolerant insect operates in a similar manner.

Several other studies have also shown that cellular level changes conferring stress tolerance occur in the absence of neuroendocrine stimulation. For example, the chironomid *Polypedilum vanderplanki* can undergo cryptobiosis even when the brain is removed (60). Also, fat body cells that are chilled to  $5^{\circ}\text{C}$  in vitro secrete measurable amounts of the cryoprotectant glycerol (69). Nonetheless, cryoprotectant synthesis does not appear to be the mechanism of RCH in *B. antarctica*, since there is no change in hemolymph osmolality or supercooling point during RCH (31). However, this inference is based only on the colligative benefits of cryoprotectants, so it is possible that cryoprotectants are upregulated without a detectable change in osmolality. Changes in membrane composition are likely involved in RCH, because recent studies found that both membrane lipid composition and membrane fluidity change during RCH (30, 36, 45). However, RCH does not appear to require the synthesis of new proteins, because RCH can occur even when protein synthesis is inhibited (37). Overall, this study provides further evidence that cold-sensing occurs in isolated tissues without neuroendocrine modulation, which may contribute to the speed of the aptly named RCH response.

An important result of the in vitro experiments is that RCH occurred in both the supercooled and frozen states at  $-5^{\circ}\text{C}$ , and thus was not dependent on ice formation. In the midgut and Malpighian tubules, there were no significant differences in cell survival between the RCH supercooled and RCH frozen groups (Table 1). When RCH was carried out in vivo, the larvae most likely froze at  $-5^{\circ}\text{C}$  because of their high susceptibility to inoculative freezing (31). However, the in vitro experiments confirmed that freezing was not a prerequisite for RCH to occur in *B. antarctica*. This result is not surprising, because even in frozen tissues the cytoplasm is expected to remain supercooled at these test temperatures. During freezing, ice formation begins outside the cells, and because only water molecules join the ice lattice, solutes in the extracellular fluid become freeze-concentrated, thereby creating an osmotic gradient that draws water out of the cytoplasm. As a result, the cytoplasm becomes increasingly concentrated and remains

supercooled even when the extracellular fluids are frozen. Since freezing is not a prerequisite for RCH in *B. antarctica*, the response we observed in *B. antarctica* is fundamentally the same as RCH in other insects, except with a lower induction temperature. Ecologically, the ability to undergo RCH in both the supercooled and frozen states may enhance the ability of larvae to track changes in temperature, as would occur during natural diurnal thermal cycles and abrupt changes in ambient temperature.

*Calcium is required for RCH protection.* In many stress responses, calcium is involved as a second messenger (34). To assess the role of calcium in RCH, we incubated tissues in calcium-free Coast's solution to remove external calcium sources, or we loaded them with BAPTA-AM before the temperature treatments to prevent signaling by both extracellular and intracellular calcium. In both the midgut and Malpighian tubules, removing calcium had no effect on control and frozen treatments but inhibited the RCH response, as indicated by both the dye exclusion and functional viability assays (Figs. 3 and 4). In the midgut, both protocols for removing calcium had a negative effect on cell survival in both the RCH supercooled and RCH frozen groups. In the Malpighian tubule, removal of calcium likewise had a negative impact on cell survival. However, in this case removal of external calcium caused only a slight ( $\sim 10\text{--}20\%$ ) reduction in cell survival, while the chelation of intracellular calcium had a more dramatic ( $\sim 45\%$ ) effect.

To further elucidate the role of calcium in RCH, we examined the role of calmodulin in RCH. Calmodulin is a ubiquitous  $\sim 17\text{-kDa}$  protein that is located both in the cytoplasm and in association with biomembranes and subcellular organelles (26). On binding with calcium, calmodulin undergoes a conformational change that allows it to interact with other signaling proteins (20). Calmodulin mediates cell function by regulating the activity of certain protein kinases and phosphatases (58) and certain transcription factors (21). In *B. antarctica*, inhibition of calmodulin by the calmodulin antagonist W-7 repressed the RCH response. In both the midgut and Malpighian tubules, treatment with W-7 resulted in a significant reduction in survival in both RCH treatments (Fig. 5). This result suggests that calmodulin is an integral part of the calcium-signaling pathway mediating RCH.

Overall, the above results indicate that calcium flux is involved in cellular cold-sensing and signal transduction during RCH. Our data are the first to indicate a role for calcium and calmodulin in cold-hardening in insects. It is well established in plants that calcium flux is responsible for triggering cold acclimation events (39), and our results suggest a similar mechanism in insects. In plants, low temperature decreases membrane fluidity, which in turn destabilizes the actin cytoskeleton and causes an increase in cytosolic calcium within seconds of cold exposure (43). This increase in calcium is due to both influx across the cell membrane and release from internal stores (27). A similar role for the actin cytoskeleton occurs in animal cells, as mechanical disruptions to the actin cytoskeleton result in calcium release from intracellular compartments in human gingival fibroblasts (64). In insects, exposure to low temperature induces a change in both the composition and fluidity of cell membranes (30, 36, 45), which could facilitate calcium flux from either extracellular or intracellular

compartments through interactions with the cytoskeleton and calcium channels.

In plants, calcium flux triggers a number of signaling pathways involved in cold acclimation. In particular, calcium increases cold tolerance in plants by mediating the expression of cold-specific genes and activating certain protein kinases. In freeze-tolerant alfalfa, cold-induced calcium flux upregulates the transcription of two cold acclimation-specific (cas) genes, *cas15* and *cas18* (38). While alfalfa requires several days of cold acclimation to become freeze-tolerant, the increase in cytosolic calcium is transient and occurs at the very onset of cold exposure. In *Arabidopsis*, increased expression of calcium-regulated low-temperature genes was detected within 30 min of cold exposure, a timescale very similar to that of RCH in insects (18). While protein synthesis is not required for RCH to occur in insects (37), a number of genes are upregulated during brief exposure to cold (35, 48), and it is possible that the expression of these genes may be triggered by calcium.

Since specific protein synthesis is not required (37), a more likely action of calcium and calmodulin in RCH is the activation of protein kinases. In the freeze-intolerant flesh fly, *S. crassipalpis*, RCH at 0°C activates p38 MAP kinase (15), which is known to be activated by calcium/calmodulin-dependent signaling pathways (13). Also, in several freeze-tolerant animals, the activities of various kinases and phosphatases change in response to temperature. For example, in the freeze-tolerant wood frog, *Rana sylvatica*, the AMP-activated protein kinase is activated during freezing (49), while in *E. solidaginis* the activities of protein kinase A and several phosphatases change over the course of the winter (46). Likewise, in plants several classes of protein kinases are involved in cold acclimation. Calcium-dependent protein kinases (CDPKs) are particularly important in cold tolerance, because they regulate the activity of several membrane transport proteins. For example, CDPKs regulate the transport of intracellular ion stores by phosphorylating the slow vacuolar ion channel (4). In addition, the H<sup>+</sup>-ATPase, which in plants provides the electrochemical gradient for active transport and consumes roughly 25% of the cell's ATP, is also inhibited by CDPKs (33). Another action of protein kinases is regulation of metabolism; for example, NAD kinase stimulates the accumulation of the amino acid proline during cold shock in plants (51).

Since RCH inhibits apoptosis (68), it is likely that there is a relationship between calcium and the apoptotic pathway during RCH. Intracellular calcium overload induces apoptosis (42), but modest levels of calcium flux can inhibit apoptosis by activating a member of the Bcl-2 family via a Ca<sup>2+</sup>/calmodulin-dependent protein kinase kinase that phosphorylates protein kinase B (65). Finally, aquaporins, which are important in maintaining water balance during freezing in *E. solidaginis* (47), are activated by a calcium-regulated kinase in plants (17). Additionally, in the kidney, calcium and calmodulin are required for the translocation of aquaporins from intracellular vesicles to the plasma membrane (9).

### Perspectives

Calcium signaling is used by diverse taxa to initiate cellular level responses to environmental stresses, including heat stress (16), osmotic and drought stress (1, 12, 70), and oxidative stress (14). Low temperature induces several concurrent phys-

iological stresses (22, 57), and quite possibly RCH in *B. antarctica* has its evolutionary origins in a generalized calcium-mediated stress response. The fact that RCH blocks cold-induced apoptosis (68) provides further evidence of a link between RCH and calcium signaling. Freeze tolerance and RCH are frequently viewed as distinct adaptations to low temperature (53), but it is evident that RCH is nearly ubiquitous, thus suggesting that RCH is a basal adaptation retained in freeze-tolerant species, as is the case for *B. antarctica*. There are some situations in which it is impossible to observe RCH, e.g., in freeze-tolerant insects that are already maximally freeze-tolerant through seasonal acclimation and in freeze-susceptible insects in a developmental stage (such as diapause) that can survive down to the supercooling point without displaying cold shock. But the fact that RCH can be exhibited in most insects suggests that it is a basal trait that is highly conserved and may indeed provide an evolutionary link to other adaptive stress responses. The putative role we demonstrate here for calcium certainly suggests a common link between multiple forms of stress tolerance.

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