Freezing presents a combination of interrelated physiological challenges to tissues that undergo freeze/thaw cycles, complicating the determination of specific cause(s) of freezing damage to cells. One of the greatest challenges to avoiding freezing damage is thought to involve the exposure to a large increase in osmolarity that occurs as both solutes and tissues are excluded into residual, unfrozen channels (Zachariassen, 1985; Hincha and Schmitt, 1992; Storey and Storey, 1996). The osmolarity of the solution in these unfrozen channels can be calculated from the equation

$$m_{\text{ext}} = \frac{D}{1.86},$$

where \(m_{\text{ext}}\) is the solution osmolarity and \(D\) is the number of degrees below zero (Mazur, 1984). Thus, the magnitude of the osmotic shock is extremely large even at relatively mild freezing temperatures; at \(-4^\circ C\), for instance, the solution osmolarity will be 2.1 osmol l\(^{-1}\). Also, the magnitude of this osmotic shock is determined solely by the freezing temperature of that measured at \(20^\circ C\), no RVI was observed at \(0^\circ C\); cross-sectional area was 39\% below the control value immediately after the hyperosmotic exposure and 36\% below the control value 30 min after hyperosmotic exposure. Dibutyryl cyclic AMP potentiated the RVI observed at \(20^\circ C\), but failed to elicit an RVI at \(0^\circ C\). A substantial RVI was also absent when the saline contained trehalose rather than glucose, regardless of whether the tubules were held at \(20^\circ C\) or \(0^\circ C\). The cross-sectional area of cells in saline containing glucose remained at approximately 30\% below the control value during an entire 30 min period of actual freezing to \(-4^\circ C\), suggesting that an acute volume regulatory response was in fact inhibited during mild freezing. The inhibition of an acute RVI during mild freezing may serve to avoid the energetic expenditure associated with volume regulation at a time when the normal defence of cell volume appears to be unnecessary.

**Key words:** New Zealand alpine weta, *Hemideina maori*, Malpighian tubule, freezing tolerance, cell volume regulation.

**Summary**

*Cells in freeze-tolerant tissues must survive substantial shrinkage during exposure to the hyperosmolarity that results as solutes are excluded from extracellular ice. We investigated the possibility that this hyperosmotic shock elicits an acute regulatory volume increase (RVI) by monitoring the response of epithelial cell volume in the Malpighian tubules of the New Zealand alpine weta (*Hemideina maori*) during exposure to low temperature/hyperosmolarity (mimicking freezing conditions) or during an actual freeze/thaw cycle. The cross-sectional area of cells in isolated Malpighian tubules was measured using differential interference contrast microscopy. At \(20^\circ C\), cells held in saline containing 400 mmol l\(^{-1}\) glucose exhibit an RVI in response to hyperosmotic shock. Cross-sectional area decreased by 30\% immediately after a change from iso-osmotic (0.7 osmol l\(^{-1}\)) to hyper-osmotic saline (2.1 osmol l\(^{-1}\), equal to the osmotic shock encountered during freezing to \(-4^\circ C\)) and then returned to 21\% below the control value 30 min after the exposure. Although substantial cellular function of Malpighian tubules was retained at low temperature (the rate of fluid secretion by isolated tubules at \(4^\circ C\) was 72\% of that measured at \(20^\circ C\)), no RVI was observed at \(0^\circ C\); cross-sectional area was 39\% below the control value immediately after the hyperosmotic exposure and 36\% below the control value 30 min after hyperosmotic exposure. Dibutyryl cyclic AMP potentiated the RVI observed at \(20^\circ C\), but failed to elicit an RVI at \(0^\circ C\). A substantial RVI was also absent when the saline contained trehalose rather than glucose, regardless of whether the tubules were held at \(20^\circ C\) or \(0^\circ C\). The cross-sectional area of cells in saline containing glucose remained at approximately 30\% below the control value during an entire 30 min period of actual freezing to \(-4^\circ C\), suggesting that an acute volume regulatory response was in fact inhibited during mild freezing. The inhibition of an acute RVI during mild freezing may serve to avoid the energetic expenditure associated with volume regulation at a time when the normal defence of cell volume appears to be unnecessary.*

**Key words:** New Zealand alpine weta, *Hemideina maori*, Malpighian tubule, freezing tolerance, cell volume regulation.
(Costanzo et al. 1993; Storey, 1997). In much the same manner that an acute regulatory volume increase (RVI) would prevent cell damage during hyperosmotic exposure (such as dehydration), an acute RVI could also prevent excessive shrinkage during freezing and thus prevent volume-related cell damage. However, Holden and Storey (1996) recently suggested that volume regulation in response to the osmotic shock associated with freezing might serve little function, provided that the shrinkage did not reach a critical minimal value and, thus, that it might be more adaptive to inhibit volume regulation during mild freezing. To date, there are no studies of which we are aware that have specifically investigated whether an acute RVI is invoked by the increase in osmolarity resulting from freezing.

In the present study, we have used a transport epithelium, that of the insect Malpighian tubule, as a model system for investigating the degree to which acute volume regulation occurs during an actual freezing event. Several recent studies demonstrate that cells of the Malpighian tubule of at least one insect (Rhodnius prolixus) do indeed possess the capacity for either a regulatory volume decrease (RVD) or an RVI (O’Donnell and Mandelzys, 1988; Arenstein et al. 1995). The Malpighian tubules of the New Zealand alpine weta (Hemideina maori), an orthopteran insect which naturally undergoes freeze/thaw cycles (Ramlov et al. 1992), survive in vitro freezing to below -5°C so long as trehalose (a glucose dimer and the naturally occurring cryoprotectant) or glucose is present in the medium (Neufeld and Leader, 1995). Since cells of the Malpighian tubules of H. maori are normally exposed to the volumetric challenges associated with freezing, we used this tissue to investigate whether volume regulation occurs at the low temperature associated with freezing. It was suggested that volume regulation in response to the osmotic drop away the dorsal portion of the abdominal cuticle and then severed the Malpighian tubules at the point where they connect to the gut. Many Malpighian tubules were easily obtained from a single animal in this manner. Dissected Malpighian tubules were then transferred to a Petri dish containing cold saline and subsequently stored at 4°C until use. In all experiments, we equilibrated tubules in saline at 4°C for several hours before any experiments were performed.

Materials and methods

Animal collection and maintenance

The New Zealand alpine weta Hemideina maori Hutton is a nocturnal insect found above 1200 m on the mountain ranges of the central South Island of New Zealand. After obtaining permission from the New Zealand Department of Conservation, we collected animals from beneath slabs of schist rock on the Rock and Pillar Range of Central Otago.

Membrane potential and intracellular cations

We also measured the effect of temperature on the basolateral membrane potential and the intracellular activities of Na+ and K+. Microelectrodes for the measurement of basolateral membrane potentials were pulled to a resistance of 25 MΩ from borosilicate glass tubing (Clark Electromedical Instruments) and backfilled with saturated KCl. Separate ion-selective microelectrodes were constructed from silanised microelectrodes (exposed to the vapour of dichlorodimethyl silane for 30 s and baked at 104°C for 1 h) backfilled with either K+-selective resin (Coming Medical, no. 477317) or Na+-selective resin (Fluka). A chorided silver wire was then inserted directly into the resin. We calibrated the ion-selective microelectrodes with a series of pure solutions of the
appropriate ion at 20 °C. Ion activities in tubules held at 0 °C were calculated using a Nernstian slope adjusted for the lower temperature.

We impaled cells from isolated tubules held by the ends to a glass coverslip using small droplets of Vaseline. The impalement of cells was judged successful if the electrode potential abruptly changed to a new, stable value after it had been advanced through the basolateral surface of the tubule. The calculation of intracellular ion activities was based on 3–4 impalements in separate cells with both the reference electrode and the ion-selective electrode. The sample size therefore represents the number of tubules from which mean intracellular ion activities were calculated. Kanno and Loewenstein (1966) showed that the epithelial cells are electrically connected, and we found a mean difference of 4.0 mV between the maximum and minimum basolateral membrane potentials from a single tubule, indicating that impalements from separate cells using single-barreled electrodes provided a sufficiently accurate measure of intracellular ion activities.

Measurement of cell size

All volumetric measurements of Malpighian tubules were made using differential interference contrast (DIC) microscopy, a proven technique in assessing volume changes of cells of renal tubules (Kirk et al. 1987). We took cross-sectional images of tubules using an inverted microscope (Nikon Diaphot-TMD) equipped with a long-working-distance condenser and a Leitz 25× objective (numerical aperture 0.75). Images were collected with a Cohu television camera equipped with an image intensifier, summed into an image board (Matrox), and then stored on a computer for later analysis using Image-Pro II (Media Cybernetics, Inc.). The cross-sectional area of cells was calculated by adjusting the plane of focus to the level at which the tubule was widest (with the basolateral surfaces in sharp focus) and tracing the luminal and total tubule areas on the image. Dividing this area by the length of tubule gave the mean luminal and tubule diameters for that tubule length, from which cross-sectional areas could then be calculated (methods are described in detail by Kirk et al. 1987).

Since the basolateral surface of the cell itself could not always be distinguished from the layer of connective and muscular tissue that surrounds the tubule, the basolateral point of measurement was taken as the outer edge of the total tubule. This layer of connective and muscular tissue is very thin relative to the cell thickness (see Fig. 4 in Neufeld and Leader, 1998b) and thus had a negligible effect on the calculated cross-sectional area of cells. Malpighian tubules have a microvillar border on the apical surface, which also complicates the accurate calculation of cell volume. Since the microvillar tips were more consistently observed in images than was the base of the microvilli, we made luminal measurements from the tips of the microvilli. For estimating changes in cell length, we focused on the bottom surface of the tubule and measured the distance between the defined points on the cell surface (usually the border between several cells).

For all measurements, tubules were secured to a coverslip by gently pressing the ends into small drops of Vaseline on a no. 0 coverslip. A small nick was made at one end of the tubule to allow for the movement of fluid between the bathing solution and the luminal space (O’Donnell and Mandelzys, 1998). A positive luminal potential could still be measured several millimetres distant from the site of the nick (data not shown), suggesting that the nick in the tubule had not substantially disrupted the normal activity of the tubule epithelium. Tubules were allowed to equilibrate in the chamber for 20 min before the start of any experiments. In experiments testing the effects of 10^{-5} mol l^{-1} 5-hydroxytryptamine (5-HT) or 10^{-4} mol l^{-1} dbcAMP, the secretagogue was present in the saline during this equilibration period. The ability of tubules to regulate volume over the long term was investigated in tubules held for 6 h at 4 °C and then warmed for 30 min at 20 °C prior to making measurements. In experiments that did not involve the use of dbcAMP, saline was superfused into the holding chamber at a rate of approximately 0.5 ml min^{-1}. Because of the expense of using large quantities of dbcAMP, we allowed a superfusion of 0.5 ml min^{-1} for the first 10 min after the change of solution and we then stopped saline superfusion until the next change of solution. A vacuum line maintained the normal saline volume in the holding chamber at approximately 0.5 ml. During a change of solutions, we briefly advanced the vacuum line to reduce the chamber volume and to provide for a more rapid change of solutions.

The effect of low temperature on cellular parameters was investigated by using a microscope stage cooled to 0 °C by a Peltier unit. A refrigeration bath circulated water through the stage, providing a heat sink for the Peltier unit. Iso-osmotic solutions superfused through the chamber first passed through an outer metal block, which held the coverslip, thus cooling the solution to the desired temperature before it entered the tubule chamber. Further cooling of the stage to -4 °C caused freezing of the saline in the chamber, allowing us to observe the volumetric response of tubule cells during an actual freezing event. While the image quality degraded significantly in the presence of ice crystals, cell boundaries were still visible in saline containing glucose. Cell boundaries were generally not visible in saline containing trehalose, however, because of a difference in crystal structure which made the ice more opaque.

Statistics

Comparisons of the long-term volumetric responses to various conditions were made using analysis of variance (ANOVA) followed by the Student–Newman–Keuls post-hoc test. All other statistical comparisons of data were made using the t-test. The statistical analysis of the volumetric response to hyperosmolarity was limited to the following three comparisons: (1) maximum shrinkage versus 30 min after hyperosmotic exposure, (2) maximum swelling upon re-exposure to iso-osmolarity versus control, and (3) maximum swelling upon re-exposure to iso-osmolarity versus 30 min after re-exposure to iso-osmolarity. Similarly, during the freezing trials, we limited statistical comparisons to (1) shrinkage at 15 min after the initiation of freezing (immediately
after ice covers the entire chamber) versus 45 min after the initiation of freezing, (2) maximum swelling upon thawing versus control, and (3) maximum swelling upon thawing versus 30 min after the initiation of thawing.

All data are presented as means ± S.E.M.

Results

Effects of cold on the function of Malpighian tubules

Given the range of temperatures encountered by *H. maori* (−5 to 28 °C) and the rapidity at which temperatures can change in its natural environment (Bliss and Mark, 1974), we hypothesized that tubules should be adapted to maintaining function at different incubation temperatures. While the rate of fluid secretion was reduced significantly by 28% in tubules held at 0 °C. Tubules at 0 °C did, however, maintain a significantly higher intracellular K⁺ activity and a significantly lower intracellular Na⁺ activity, relative to tubules held at 20 °C (Table 1). There was no effect of the different incubation temperatures on the cross-sectional area of cells (Table 1).

Long-term regulation of cell volume

The cells of most tissues demonstrate some degree of cell volume regulation, especially over longer-term exposures to altered osmolarity (Chamberlin and Strange, 1989). We therefore investigated the ability of tubule cells to regulate their volume after a 6 h exposure to various solutions. Tubules held in iso-osmotic saline had a cross-sectional area not significantly different (*P* > 0.05) from that of tubules held in hypo-osmotic saline (saline composition equivalent to iso-osmotic saline, but without the high concentration of glucose or trehalose) (Fig. 1), despite the fact that the much lower osmolalities of hypo-osmotic saline (300 versus 700 mosmol l⁻¹) should be reflected by a 133% increase in cell volume in the absence of cell volume regulation. Therefore, tubule cells were able to regulate cell volume over the long term.

Regulatory volume increase after hyperosmotic shock

In order to investigate the effects on cell volume of the abrupt increase in osmolarity caused by a freezing event, we exposed tubules to a threefold increase in saline osmolarity. Exposure to saline of 2.1 osmol l⁻¹ is equivalent to the osmotic concentration caused by freezing a solution to −4 °C (Mazur, 1984), a temperature commonly encountered by *H. maori* throughout the year (Bliss and Mark, 1974) and a freezing temperature that *H. maori* normally survives (Ramlov et al. 1992). When tubules held at 20 °C in saline containing 400 mmol l⁻¹ glucose were given an abrupt hyperosmotic shock, there was an immediate decrease in cross-sectional area of 30% (Figs 2, 3). This was rapidly countered by an RVI; the cross-sectional area was 24% below the control level at 10 min after the shock and 21% below the control level 30 min after the shock. The presence of an RVI response should be manifested by an overswelling when cells are returned to their original osmolarity due to the increased solute content in the cells. Such an overswelling was observed in tubules when they were returned to iso-osmotic saline (Figs 2, 3). Thus, the volumetric response of tubule cells was consistent with the presence of volume regulation at 20 °C.

We also measured the response of tubule length to a

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Table 1. Effect on cellular function of a 1 h exposure to low temperature

<table>
<thead>
<tr>
<th>Variable measured</th>
<th>Temperature (°C)</th>
<th>Value</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secretion rate (nl h⁻¹)</td>
<td>20</td>
<td>79.8±9.8*</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>57.2±8.2*</td>
<td>13</td>
</tr>
<tr>
<td>Basolateral membrane potential† (mV)</td>
<td>20</td>
<td>−61.3±1.8</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>−63.4±0.5</td>
<td>16</td>
</tr>
<tr>
<td>Intracellular K⁺ activity (mmol l⁻¹)</td>
<td>20</td>
<td>104±6*</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>137±6*</td>
<td>8</td>
</tr>
<tr>
<td>Intracellular Na⁺ activity (mmol l⁻¹)</td>
<td>20</td>
<td>37.9±5.4</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>18.8±1.6*</td>
<td>8</td>
</tr>
<tr>
<td>Cross-sectional area of cells (µm²)</td>
<td>20</td>
<td>6460±330</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>6360±290</td>
<td>32</td>
</tr>
</tbody>
</table>

All tests were performed in iso-osmotic saline containing 400 mmol l⁻¹ trehalose.

*Indicates that the value at cold temperature was significantly different (*P* < 0.05) from the value at warm temperature.

†Basolateral membrane potential is measured as intracellular potential relative to the bathing saline.

Values are means ± S.E.M.
hyperosmotic shock to verify that changes in cross-sectional area reflected changes in actual cell volume. The change in tubule length at 2 and 30 min after the hyperosmotic shock was only 0.1±1.6 % and 0.5±0.9 % (N=7 for each) of the control length, respectively. At 2 and 30 min after a return to iso-osmotic saline, tubule length increased by 3.9±2.0 % (N=4) and 3.9±2.2 % (N=3) relative to the control value, respectively. Any length-related changes in cell volume were therefore minimal and were not measured in further experiments.

Tubules held at 20 °C in iso-osmotic saline for the entire 1 h experimental period did not demonstrate a significant change (P>0.05) in tubule length or cellular cross-sectional area (−1.8±2.5 % and −0.5±2.2 %, respectively, of the control value after 60 min in iso-osmotic saline; N=6), indicating that there was no change in cell volume due solely to the time held at 20 °C.

The shrinkage of the lumen during hyperosmotic exposure was nearly osmometric: the cross-sectional area of the lumen was reduced by approximately 60 % for the full 30 min period of hyperosmotic exposure (Fig. 4) compared with an expected decrease of 66 %. There was a high degree of variability in the subsequent increase in cross-sectional area of the lumen upon a return to iso-osmotic saline.

We similarly measured the response of cross-sectional area to hyperosmotic shock at 0 °C, a condition that mimics that occurring during freezing of tubules (Fig. 2). There was no indication of an RVI at this low temperature: cross-sectional area remained at approximately 39 % for the full 30 min period after the hyperosmotic shock. As expected in the absence of volume regulation, there was no overswelling of cells upon return to iso-osmotic saline.

**Modulation of volume regulation by secretagogues**

Given the ability of second messengers to potentiate both cell volume regulation (Hebert, 1986) and freezing survival (Holden and Storey, 1996), we tested the effect on cell volume regulation of two proven secretagogues (5-HT and dbcAMP) of Malpighian tubules. Cell volume regulation in tubules at 20 °C in saline containing 400 mmol l⁻¹ glucose; 0.7 osmol l⁻¹) to a 30 min exposure to hyperosmolarity (2.1 osmol l⁻¹) at 20 °C followed by a return to control saline.
sectional area decreased to a minimum of 30.3±1.8 % (N=8 for all conditions) below the control value after the hyperosmotic shock, returned to 22.1±3.5 % below after a 30 min exposure to hyperosmolarity, increased to 24.5±6.9 % upon return to iso-osmotic saline and then returned to 14.4±6.1 % at 30 min after the return (cf. Fig. 2). Cells demonstrate a greater ability to regulate volume, however, in the presence of dbcAMP, the compound with the more potent secretory effect in Malpighian tubules of *H. maori* (Neufeld and Leader, 1998a). In the presence of 10⁻⁴ mol l⁻¹ dbcAMP, shrinkage was only 25 % below the control value immediately after the hyperosmotic shock and returned to 10 % below the control value at 30 min after the shock (Fig. 5; Table 2). A regulatory volume decrease was evident upon return to iso-osmotic saline, as the cross-sectional area at 30 min after a return to iso-osmotic saline was significantly smaller than the maximal swelling that occurred shortly after a return (Table 2).

In tubules held at 0 °C in the presence of dbcAMP (saline containing 400 mmol l⁻¹ glucose), the cross-sectional area after a 30 min exposure to hyperosmotic saline was not significantly different from that reached immediately after the shock (Fig. 5), although this shrinkage was smaller than that occurring in the absence of dbcAMP. While cells did overswell significantly upon return to iso-osmotic saline, the magnitude was smaller than that occurring at 20 °C in the presence of dbcAMP (Table 2). Thus, low temperature appeared to inhibit volume regulation of tubules held in saline containing 10⁻⁴ mol l⁻¹ dbcAMP.

**Effect of trehalose on volume regulation**

Volume regulation was largely abolished by using saline containing 400 mmol l⁻¹ trehalose (rather than glucose), despite the presence of 10⁻⁴ mol l⁻¹ dbcAMP (Fig. 6). At both 20 °C and 0 °C, the cross-sectional area after a 30 min exposure to hyperosmotic saline was not significantly different from that
attained immediately after the osmotic shock (Table 2). At 20 °C, cells briefly overswelled by 19 % upon return to iso-osmotic saline and then returned to a value 7 % above the control value. At 0 °C, the mean swelling upon return to iso-osmotic saline did not exceed 5 % above the control value.

Cell volume during a freezing cycle

We observed the response of tubule cell cross-sectional area during a 30 min freezing cycle to −4 °C (producing a threefold concentration of the unfrozen saline to which the tubule cells are exposed). In our chamber, freezing of saline occurred first around the edge and then proceeded over approximately 10 min to the centre of the chamber where the tubules were mounted. Exposure of tubules to hyperosmolarity was therefore a more gradual process than in the previous experiments, as reflected by the slower shrinkage of cells (Fig. 7). In saline containing 400 mmol l⁻¹ glucose and dbcAMP, cells shrank by approximately 35 % and remained at this value for the 30 min period (Fig. 7; Table 2). Upon thawing, cells swelled to approximately 10 % above the control value.

Accurate measurements of cross-sectional area could not be made in frozen saline containing trehalose, since the ice was too opaque to distinguish cell boundaries. Measurements before and after freezing, however, suggested that volume regulation was also absent in the presence of trehalose and

<table>
<thead>
<tr>
<th>Saline containing (at 400 mmol l⁻¹)</th>
<th>dbcAMP present?</th>
<th>% Change in cell volume at 5 min</th>
<th>% Change in cell volume at 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Yes</td>
<td>−29.2±2.8</td>
<td>−33.8±2.3</td>
</tr>
<tr>
<td>Trehalose</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± S.E.M.; values of N are given in Figs 2, 5–7. Positive values indicate swelling; negative values indicate shrinkage.

% Change in cell volume (at 30 min after hyperosmotic exposure) significantly different from maximum shrinkage after exposure to hyperosmotic saline (P<0.05).
% Change in cell volume (at maximum swelling after iso-osmotic return) significantly different from control (P<0.05).
% Change in cell volume (at 30 min after iso-osmotic return) significantly different from maximum swelling after return to iso-osmotic saline (P<0.05).
% Change in cell volume (at 15 min after thawing) significantly different from control (P<0.05).

Values of N are given in Figs 2, 5–7.

Table 2. Statistical comparisons of the volumetric responses of tubule cells

<table>
<thead>
<tr>
<th>Response of tubules exposed to hyperosmotic saline</th>
<th>Hyperosmotic</th>
<th>Iso-osmotic return</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline containing (at 400 mmol l⁻¹)</td>
<td>Temperature (°C)</td>
<td>dbcAMP present?</td>
</tr>
<tr>
<td>Glucose 20 No</td>
<td>−30.0±1.6</td>
<td>−20.7±2.8</td>
</tr>
<tr>
<td>Glucose 0 No</td>
<td>−39.0±2.2</td>
<td>−36.1±2.5</td>
</tr>
<tr>
<td>Glucose 20 Yes</td>
<td>−24.5±2.9</td>
<td>−9.9±2.1</td>
</tr>
<tr>
<td>Glucose 0 Yes</td>
<td>−27.3±2.6</td>
<td>−24.0±3.1</td>
</tr>
<tr>
<td>Trehalose 20 Yes</td>
<td>−27.8±2.2</td>
<td>−24.2±2.0</td>
</tr>
<tr>
<td>Trehalose 0 Yes</td>
<td>−31.1±9.1</td>
<td>−30.6±2.9</td>
</tr>
</tbody>
</table>

Fig. 7. Effect of a freeze/thaw cycle on the volume regulatory decrease of cells from Malpighian tubules held in saline containing 400 mmol l⁻¹ glucose or trehalose in the presence of 10⁻⁴ mol l⁻¹ dbcAMP. Tubules were held frozen for approximately 30 min after complete freezing in the holding chamber and then followed for 30 min after the initiation of thawing. Data could not be collected for tubules frozen in trehalose because of the opacity of the ice. See Table 2 for statistics. Sample sizes were 6–9 for tubules in saline containing glucose and nine for tubules in saline containing trehalose. Values are means ± S.E.M.
dbcAMP (Fig. 7). Cross-sectional area immediately after thawing, before mixing of saline abolished local osmotic gradients, indicated that cells had shrunk by at least 35% during freezing, a value similar to that observed during low-temperature exposure to hyperosmotic saline. Once mixing of saline had been complete, the cross-sectional area was only approximately 5% above the control value (Table 2).

In the presence of either glucose or trehalose, the volumetric behaviour during freezing to −4 °C (Fig. 7) was therefore virtually identical to that observed during hyperosmotic exposure at low temperature in the absence of freezing (Figs 5, 6). Any volume regulation was minimal in both instances.

**Discussion**

Our demonstration that low temperature inhibits an acute RVI in epithelial cells of Malpighian tubules suggests that cell volume regulation is not a necessary adaptation for the survival of mild freezing conditions. Substantial volume regulation in tubule cells appeared to be absent at 0 °C (and during freezing to −4 °C) despite the fact that (1) cellular function was substantially maintained at low temperature (Table 1), and (2) tubule cells demonstrated an obvious RVI at 20 °C (Figs 2, 3, 5). We briefly discuss these two observations before returning to the issue of freeze-related changes in cell volume.

On the basis of differing temperature coefficients for the active and passive transport components of a cell’s pump–leak system, which maintains a constant intracellular concentration of ions, a change in temperature is generally accepted to present a significant challenge to cellular functions. Alterations in either active transport or passive permeabilities to maintain a balance of ion influxes versus effluxes are therefore one basis for cellular tolerance of temperature alterations (Kamm et al., 1979; Hochachka, 1986; Willis, 1987). Tissues of non-hibernating mammals often show marked alterations in intracellular ion concentrations (Reisin and Gulati, 1972; Kamm et al., 1979; Sudo and Morel, 1984) when exposed to low temperature. In contrast, tissues from animals that naturally experience large temperature fluctuations (ectotherms and hibernating mammals) more consistently maintain a high intracellular K+ and low intracellular Na+ concentration during long-term exposure to low temperature (Gulati and Reisin, 1972; Kamm et al., 1979; Willis, 1987; Morgunov and Hirsch, 1991). In the Malpighian tubules of *H. maori*, cold temperature actually caused a decrease in intracellular Na+ concentration and an increase in intracellular K+ concentration. Coupled with the observations that membrane potential and cell cross-sectional area were unchanged at 0 °C, and that there was significant fluid secretion at 4 °C (Table 1), we conclude that the Malpighian tubules of *H. maori* maintained substantial function at low temperature, as expected for an animal that naturally encounters temperature fluctuations.

While there is a large database on the characteristics of fluid and ion secretion by insect Malpighian tubules (reviewed by Nicholson, 1993), only recently has the ability of this transporting epithelium to regulate cell volume been studied. The Malpighian tubule of the blood-sucking insects *Rhodnius prolixus* and *R. neglectus* is now known to demonstrate an RVD or an RVI in response to hypo- or hyperosmolality, respectively (O’Donnell and Mandelzys, 1988; Arenstein et al., 1995). Cell volume regulation in many transport epithelia could function primarily to maintain a constant cell volume during the large changes in fluid and ion fluxes rather than simply as an adaptation to altered osmolarity (Spring and Ericson, 1982). This suggestion is particularly relevant for Malpighian tubules, which can sustain extraordinarily high rates of fluid and ion transport (Maddrell, 1991). Thus, the presence of cell volume regulation in Malpighian tubules of *H. maori* (present study, at 20 °C) and *Rhodnius prolixus* and *R. neglectus* (O’Donnell and Mandelzys, 1988; Arenstein et al., 1995) confirms the expectation that these cells are capable of rapidly altering transmembrane solute fluxes in order to maintain a constant volume.

Two observations are noteworthy with regard to volume regulation under non-freezing conditions in the present study. First, the substantial secretion at low temperature (4 °C) implies that the challenges to cell volume normally associated with transporting epithelia are also present, yet the ability to regulate cell volume was inhibited. In other studies, different volume regulatory pathways are invoked depending on the type of volume challenge (Strange, 1994), raising the possibility that volume regulation in response to transport-related volume challenges may still operate at low temperature in Malpighian tubules. Second, although trehalose is normally present at high levels in the haemolymph of *H. maori* and acts as the naturally occurring cryoprotectant (Neufeld and Leader, 1998b), short-term volume regulation was significantly inhibited by the presence of trehalose (Fig. 6). At present, we have no evidence as to either the mechanism of inhibition or why short-term volume regulation might normally be inhibited even at high temperatures.

Intracellular ion concentrations, cell volume, membrane potential and fluid secretion rate are all physiological variables dependent on the control of solute influx versus efflux and all were well-maintained at low temperature in *H. maori* (Table 1). Since the regulation of cell volume is also primarily an issue of solute influx versus efflux, we expected that the cell volume regulation observed at 20 °C would also be operative at low temperatures. Instead, we found that volume regulation was largely inhibited by exposure to 0 °C. Volume regulation at low temperature was not elicited by the presence of dbcAMP, which not only enhanced volume regulation at 20 °C (cf. Figs 2, 5) but has also recently been shown to facilitate freezing survival in tissues of the wood frog *Rana sylvatica* (Holden and Storey, 1996). Observations of cell volume during an actual freezing cycle (Fig. 7) confirmed the results obtained from hyperosmotic exposure at low temperature (designed to mimic freezing). Since Malpighian tubules of *H. maori* show excellent survival of freezing to at least −5 °C (Neufeld and Leader, 1998b), we conclude that an acute RVI is not necessary for cell survival under these mild freezing conditions.
However, short-term volume regulation in *H. maori* could become operative at a lower freezing temperature (and therefore higher osmolarity), when a critical minimum cell volume is passed, causing cell damage (Meryman, 1971), or over a longer period than that used in the present study. Likewise, tissues from other organs may show a different volumetric response to freezing.

While studies have investigated the kinetics of the initial volume decrease occurring at the time of freezing (Korber, 1988), less attention has been given to the implications of any subsequent solute redistributions on cell volume. Griffiths et al. (1979) noted that cell volume increased briefly above the control value when cells were thawed following freezing to −15°C, a result suggesting that some solute loading had occurred during freezing. This post-freeze swelling was, however, correlated with increased cell damage, suggesting that it resulted from non-specific membrane damage that increased solute permeability. Other studies have also shown redistributions of ions linked to general membrane damage (Daw et al. 1973; Griffiths, 1978; Hincha and Schmitt, 1992) rather than via the specific transport mechanisms normally associated with cell volume regulation. More recently, carrier-mediated redistribution of glucose has been clearly demonstrated in wood frogs during freezing (King et al. 1993). Exposure of frog hepatocytes to mild freezing (−4°C) both initiates glucose accumulation and reduces cell shrinkage when the freezing temperature is subsequently (after 24 h) lowered to −20°C (Storey et al. 1992). Thus, glucose accumulation in tissues is suggested to function in the regulation of cell volume (Storey and Storey, 1996). The observations that cells of Malpighian tubules are permeable to glucose (O’Donnell et al. 1984) and trehalose (Knowles, 1975) indicates that the redistribution of these solutes could play a role in the volumetric response to freezing. Our present study is, however, the only one of which we are aware that has monitored the time course of changes in cell size immediately following the initial volume decrease to investigate whether an acute RVI actually occurs in response to the hyperosmotic exposure of freezing.

Given that cells survived freezing to −4°C despite the significant inhibition of volume regulation, we conclude that long-term cellular integrity in Malpighian tubules was not compromised by the increase in solution osmolarity of 300%. This represents a theoretical shrinkage to one-third of the original volume. Our measurements of shrinkage only account for approximately 50% of the expected shrinkage; however, optical measurements do not take into account the compartments that are not responsive to osmotic pressure (either dry mass or ‘bound water’). For instance, osmotically inactive water has been estimated to constitute approximately 25% of cell space in a number of animals (Zachariassen, 1985; Storey and Storey, 1988). A number of cell types have shown a remarkable resiliency in the face of large volumetric changes due to osmotic shifts (e.g. Clegg and Gordon, 1985). It is not, in fact, necessary that cellular function be maintained during the shrinkage associated with actual freezing, since system-level functions are largely inoperative in the frozen state. Rather, it is necessary that cellular function be restored once thawing (and reswelling) occurs. Our results therefore support the suggestion of Holden and Storey (1996) in wood frogs that volume regulation during freezing itself may be unnecessary, so long as osmotic shrinkage does not reduce cell volume below a point that would be detrimental to cellular function upon subsequent thawing. Volume regulation under conditions in which the full maintenance of cellular function is not needed anyway (as during freezing) would serve only to incur a large metabolic expense to little advantage at a time when the cell’s ability to maintain ATP is compromised.

In summary, we have demonstrated that substantial short-term regulation of cell volume in Malpighian tubules is not necessary for survival of the osmotic stress associated with a freeze/thaw cycle to −4°C. An RVI was not detected at low temperature despite the fact that cells of the Malpighian tubules (1) maintained cellular function at low temperatures and (2) were capable of obvious volume regulation at higher temperatures. We suggest that the lack of an acute RVI at low temperature reflects the fact that full maintenance of cell function is unnecessary during freezing and, therefore, that cell volume need not be defended unless cell shrinkage would be great enough to cause permanent damage, such as an irreversible disruption of the plasma membrane structure.

References


