Parallel Effects of Freezing and Osmotic Stress on the ATPase Activity and Protein Composition of the Plasma Membrane of Winter Rye Seedlings

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ABSTRACT

The objective of this study was to determine the influence of freezing versus hypertonic stress on the ATPase activity and polypeptide profile of the plasma membrane of nonacclimated winter rye leaves (Secale cereale L. cv Puma). Exposure of leaves to hypertonic sorbitol solutions resulted in a similar extent of injury as did freezing to subzero temperatures that resulted in equivalent osmotic stresses. When isolated with a two-phase partition system of aqueous polymers, the plasma membrane fractions of control, frozen, or hypertoinically stressed leaves were of similar purity as judged by the distribution of marker enzyme activities. When assayed in the presence of Triton X-100 (0.05% w/w), ATPase activity was decreased only slightly in plasma membrane fractions isolated from either frozen or hypertoinically stressed leaves. In contrast, the specific ATPase activity of the plasma membrane fractions assayed in the absence of Triton X-100 increased following freezing or hypertonic stress. As a result, the Triton X-100 stimulation of the ATPase activity decreased significantly from sixfold in control leaves to threefold in lethally stressed leaves and reflects an increase in the permeability of the plasma membrane vesicles. The increased permeability was also manifested as a decrease in H+ transport following exposure to freezing or hypertonic stress. Both freezing and hypertonic exposure at subzero temperatures altered the polypeptide profile of the plasma membrane, but with the exception of one polypeptide, there was no difference between the two treatments.

Although there is a general consensus that the plasma membrane is a primary site of freezing injury (17, 23), most studies have been directed to factors responsible for lysis or disruption of the plasma membrane (23). In contrast, there have been relatively few studies of alterations in the biochemical composition or enzymatic characteristics of the plasma membrane that occur as a result of a freezing/thaw excursion. Such studies have only been possible since the development of techniques for the isolation of the plasma membrane in relatively high purity following a freeze/thaw excursion (27). For example, compositional changes in the plasma membrane of Jerusalem artichoke tubers that were frozen to lethal temperatures include (a) a decrease in sterols and phosphatidylethanolamine and (b) changes in the plasma membrane polypeptide profile, including a shift from relatively high to low molecular mass polypeptides.

Freeze/thaw-induced alterations in the functional characteristics of the plasma membrane (e.g., ATPase activity) have also been reported. However, the relation between the inactivation of plasma membrane ATPase and freezing injury is controversial. On the basis of microscopic observations of onion cells following slow freezing, Palta and Li (20) suggested that plasma membrane ATPase, presumably associated with the active transport system, was inactivated as a result of freezing. Subsequent studies of Jian et al. (12), which involved cytochemical determinations of ATPase activity in wheat cells, suggested that ATPase activity was first lowered and then completely inactivated during exposure to lethal temperatures. However, these studies are equivocal because of the association of acid phosphatases with the plasma membrane (3) and the possibility that phosphatases other than H+-ATPase can be detected cytochemically (14). Nevertheless, Helleberg et al. (8) and Iswari and Palta (11) reported that the plasma membrane ATPase (of pine needles and potato leaves, respectively) is inactivated following a lethal freeze/thaw cycle, and they proposed that inactivation of plasma membrane ATPase is a primary cause of freezing injury. In contrast, Uemura and Yoshida (27) reported only a slight decrease (less than 8% of the activity in the unfrozen controls) in the ATPase activity of plasma membrane fractions isolated from Jerusalem artichoke tubers frozen in vitro to -10°C, which resulted in less than 20% survival of the cells. They also observed a 6% decrease in the ATPase activity of samples that were frozen to -5°C, which resulted in 95% survival of the cells.

Collectively, these studies suggest that freezing may result in compositional and/or functional changes in the plasma membrane; however, the specific cause(s) of the membrane alterations has not been investigated. During a freeze/thaw cycle, cells are subjected to several perturbations including low temperatures per se, high concentrations of extracellular solutes, and cell dehydration. Although there is a general consensus that cell dehydration during slow cooling is a primary cause of freezing injury, the effect of osmotic dehydration versus freezing on compositional or functional alterations in the plasma membrane has not been reported.

The purpose of this study was to determine the influence...
of osmotic dehydration versus freezing on the survival of winter rye leaves in relation to the K⁺-stimulated ATPase activity, H⁺-transport, and the polypeptide composition of the plasma membrane. The experimental approach included exposure of rye leaves to either freezing or hypertonic solutions in vivo, with the hypertonic exposures at either 0°C or at the freezing point of the particular solution. With this approach, the influence of osmotic stresses and the effect of temperature could be determined in the absence of ice formation.

MATERIALS AND METHODS

Plant Materials

Seeds of winter rye (Secale cereale L. cv Puma) were germinated in moist vermiculite and grown in a controlled environment at 20°C d (16 h) and 15°C night (8 h) for 10 to 14 d. After germination, the seedlings were irrigated with Hoagland solution.

Survival of Leaves after a Freeze/Thaw Cycle or Hypertonic Exposure

Survival of leaves was evaluated by the electrolyte leakage test. Rye leaves (1 g fresh weight) were cut into small pieces and placed into test tubes at 0°C. In freezing experiments, the tissues were suspended in 5 mL of isotonic (0.53 osm)³ sorbitol solution and cooled to −2.5°C for 1 h. Thereafter the tissues were cooled in steps of 2.5°C at 1-h intervals. After holding at the desired temperature for 1 h, the tissues were thawed at 4°C and then incubated at room temperature for 2 h with 10 mL of 0.53 osm sorbitol solution before measuring the solution conductivity. In hypertonic exposure experiments, the tissues were suspended in 10 mL of a hypertonic sorbitol solution at 0°C or a subzero temperature equal to the freezing point of the particular hypertonic sorbitol solution. Therefore, hypertonic exposure would result in the same extent of cell dehydration as exposure in the frozen state to the particular temperature. After holding for 1 h, the solution was removed and 10 mL of distilled water was added to the tissues to dilute the remaining sorbitol. This procedure was repeated at least three times. After incubating the washed tissues with 10 mL of distilled water at room temperature for 2 h, the conductivity of each solution was measured separately. The conductivity of the solution containing sorbitol was recalculated based on the weight of water in the solution. The sum of conductivity values in each solution (i.e., hypertonic sorbitol solution, distilled water used for washing, and postincubating distilled water) was used for the evaluation of survival following hypertonic exposure. Samples that were boiled at 100°C for 15 min and those that were kept at 0°C were taken as 100% and 0% relative conductivity, respectively. The conductivity of the tissues boiled in distilled water or in a sorbitol solution was the same after the calculation described above.

³ Abbreviations: osm, osmolality; SHAM, salicylhydroxamic acid; PVP-25, polyvinylpyrrolidone (mol wt 24,500); BHT, tert-butylhydroxytoluene.

Isolation of Plasma Membranes

Plasma membranes were isolated from control, frozen-thawed, and hypertonically exposed tissues as follows. The standard (isotonic) homogenizing medium consisted of 0.5 M sorbitol, 50 mM Mops/KOH (pH 7.6), 5 mM EDTA/KOH, 5 mM EGTA/KOH, 2.5 mM K₂₃O₇, 4 mM SHAM, 1 mM PMSF, 1 mM KF, 3% (w/v) PVP-25, 0.5% (w/v) defatted BSA and 10 μg/mL BHT. The osmolality of the homogenizing medium was adjusted by the addition of sorbitol. Rye leaves (30 g fresh weight) were frozen in 30 mL of an isotonic homogenizing medium to the desired temperature for 2 h as described above and then 70 mL of hypertonic homogenizing medium, precooled at the temperature to which the tissues were frozen, was added to the frozen tissues. The freezing point of the final solution was equal to the temperature at which the tissue was frozen (1.24, 2.69, or 5.38 osm in tissues frozen to −2.3, −5, and −10°C, respectively). After incubation for 2 h, all of the ice was melted at the subzero temperatures.

In the hypertonic exposure experiments, rye leaves (30 g fresh weight) were incubated in 100 mL of a hypertonic homogenizing medium for 1 h at 0°C or at subzero temperatures equal to the freezing point of the homogenizing medium used. After these treatments, the tissues were homogenized with a Polytron PT20 for 60 s. When the tissues were homogenized in either a 2.69 or 5.38 osm homogenizing medium, 200 mL of a hypertonic homogenizing medium was added immediately after homogenization to adjust the final osmolality of the medium to 1.24 osm. This resulted in sedimentation of membrane materials as usual. The standard procedure (25) was applied for unstressed (control) tissues. The microsomal fraction (10,000–80,000 g) was used for plasma membrane isolation using an aqueous two-polymer phase partition system described elsewhere (25), with a slight modification in that PMSF (0.2 mM) was included in the two-phase system. The purity of the isolated plasma membrane was assessed by evaluation of the distribution of various marker enzyme activities as previously described (25). The isolated plasma membranes were finally suspended in a solution of 0.25 M sucrose–10 mM Tris/Mes (pH 7.3) containing 0.2 mM PMSF and immediately frozen in liquid nitrogen and then kept frozen at −80°C until use.

ATPase Assay

Mg²⁺-ATPase activity associated with the isolated plasma membrane was measured by the method of Hodges et al. (9) with a slight modification. The standard mixture consisted of 3 mM ATP, 3 mM MgSO₄, 25 mM Tris/Mes (pH 6.5), 50 mM KCl, 0.25 mM sucrose, and 50 μL of enzyme preparation (10–20 μg protein) in a final volume of 0.25 mL. The reaction was carried out at 30°C and stopped by the addition of 2.5 mL of 1.3% (w/v) SDS–0.25% (w/v) Na₂MoO₄ made up in 0.5 mM H₂SO₄ at 0°C. The released Pi was determined by the method of Fiske-SubbaRow (6) with K₂HPO₄ as a standard. The Triton X-100 stimulation of ATPase activity was maximal at a concentration of 0.05% (w/v) (data not shown), and this concentration was used to determine the stimulation of ATPase activity in subsequent experiments. Triton X-100, at a concentration of 0.05% (w/v), did not have any effect on
the color development in the Pi assay. Membrane protein was quantified by the method of Bradford (2) with a slight modification by using methanol in place of ethanol. BSA was used as a standard.

H⁺-Transport Assay

The transmembrane H⁺-transport activity of plasma membrane vesicles was measured by quenching of acridine orange fluorescence according to the method of Bennett et al. (1) with a slight modification. An aliquot of isolated plasma membrane suspension (equivalent to 200 µg protein) was preincubated at 30°C in a reaction mixture containing 0.25 M sucrose, 25 mM Tris/Mes (pH 6.5), and 5 µM acridine orange. After equilibration of temperature and fluorescence intensity (approximately 10-min incubation), ATP/MgSO₄ solution was added to a final concentration of 2.5 mM. The relative fluorescence intensity was monitored at 30°C with a Perkin-Elmer spectrofluorometer model MPF-44B with a thermostated sample holder. Excitation and emission wavelengths were 472 and 531 nm, respectively. The relative H⁺-transport activity was calculated from the initial rate of fluorescence quenching and that of plasma membrane vesicles from control tissues was taken as 100% activity.

SDS-PAGE

One-dimensional slab SDS-PAGE of solubilized plasma membrane proteins was performed as described previously (26) with a slight modification. The proteins were solubilized in 5% (w/v) SDS, 100 mM DTT, 62.5 mM Tris/HCl (pH 6.8), 0.25 M sucrose, and 0.2 mM PMSF with heating at 100°C for 60 s and separated with a linear gradient separation gel (7–12.5%). After electrophoresis, gels were stained with Comassie brilliant blue G250 (5). The stained gels were scanned with an LKB gel scanner equipped with a computerized data processing apparatus.

RESULTS AND DISCUSSION

Survival of the Leaves following Exposure to Stresses

In leaves frozen in an isotonic sorbitol solution (0.53 osm), 50% electrolyte leakage (i.e. the LT₅₀) occurred following freezing to −6.5°C (Fig. 1). This was only slightly lower than the LT₅₀ determined for leaves frozen in the absence of any suspending medium (i.e. −5.5°C; data not shown). We considered leaves frozen in an isotonic sorbitol solution to be the most appropriate treatment for comparison with the treatments involving hypertonic solutions in the subsequent experiments.

During the freezing of a solution, solutes are largely excluded from the ice phase and become concentrated in an unfrozen portion of the mixture. The fraction of the solution that remains unfrozen is a function of the subfreezing temperature and the initial osmolality of the solution. The osmolality of the unfrozen solution varies only as a function of the subfreezing temperature, i.e. osmolality = (273 – T)/1.86, where T is a subfreezing temperature. For example, at −6.5°C, the osmolality of the unfrozen solution is 3.49 osm.

Thus, the osmolality of the unfrozen solution at any subzero temperature can be determined and osmotic manipulation at any temperature, either at or above the freezing point of the solution, can be used to affect the same extent of cellular dehydration as freezing.

Using this approach, 50% electrolyte leakage occurred following a 1-h exposure in a 2.43 osm sorbitol solution at 0°C or a 3.41 osm solution at −6.35°C (Fig. 1). These results show that the increase in electrolyte leakage in both treatments, i.e. freezing in an isotonic sorbitol solution and hypertonic exposure at subzero temperatures, occurs over a similar range of osmolalities. Also, the results demonstrate that hypertonic exposure at 0°C has a more deleterious effect than hypertonic exposure at subzero temperatures. Previously, Gordon-Kamm (7) also observed that dehydration-induced injury in nonacclimated and acclimated rye protoplasts was much greater at 0°C than at subzero temperatures. Further, the incidence of lamellar-to-hexagonal β phase transitions in the plasma membrane of nonacclimated protoplasts in hypertonic solutions, which is thought to be a primary cause of freezing injury of nonacclimated protoplasts frozen to temperatures below −5°C, was also greater if the hypertonic stress occurred at 0°C rather than at subzero temperatures. Low temperature also has a protective effect against injury of thylakoid membranes (18, 24). Thus, the contribution of osmotic stress to freezing injury must be determined by comparing leaves frozen in the presence of sorbitol with leaves exposed to hypertonic solution at subzero temperatures. Further, the influence of temperature on the osmotic stress can be deduced

![Figure 1. Electrolyte leakage from rye leaves following freezing or hypertonic exposure.](image-url)
from studies of hypertonic exposure at 0°C relative to those at subzero temperatures.

**Plasma Membrane Isolation from Frozen/Hypertonically Stressed Leaves**

To determine the influence of freezing or hypertonic stress on the ATPase activity and protein composition of the plasma membrane, purified plasma membrane fractions were isolated from leaves exposed to the various treatments by using a two-phase partition system. The purity of the isolated plasma membrane fractions was determined by assay of the distribution of various marker enzyme activities associated with cellular membranes (Tables I and II). ATPase activity was determined in the presence of 0.05% (w/v) Triton X-100 (see "ATPase Activity" for the rationale). The inhibition of ATPase activity by vanadate, an inhibitor of plasma membrane ATPase, was quite constant (about 50%). In contrast, NO3敏感 ATPase activity, an ATPase associated with the tonoplast, was low in all treatments, i.e. no more than 5% of the ATPase activity was NO3-sensitive in the isolated plasma membrane preparations. The specific activities of marker enzymes such as Triton X-100 stimulated UDPase (Golgi body), Cyt c oxidase (mitochondrion), and NADH Cyt c reductase (antimycin A-insensitive, ER) increased only slightly as a result of freezing or hypertonic exposure of leaves. Chl was not detected in the plasma membrane fractions. These results indicated that the plasma membrane fractions obtained from control, hypertonically stressed, and frozen leaves were free of contamination by other cellular membranes. However, acid phosphatase activity was greater following exposure to lower freezing temperatures or higher osmolarities of sorbitol solutions. Although the activity of acid phosphatase is thought to originate from various kinds of phosphatases in the cell, the increase in acid phosphatase activity was not a result of contamination by vacuolar vesicles in the plasma membrane fractions because of the low levels of NO3-sensitive ATPase activity. There is the possibility that acid phosphatase is associated with the plasma membrane in plant cells (3). Nevertheless, these results show that the purity of the isolated plasma membranes was high in all treatments—even after freezing to −10°C or equilibration in a 5.38 osm sorbitol solution. Uemura and Yoshida (27) also demonstrated the high purity of plasma membrane fractions isolated from frozen Jerusalem artichoke tubers and orchard grass crown tissues using a two-phase system.

For leaves frozen to −5°C or −10°C, the recovery of plasma membrane (based on quantity of protein) decreased to 68 and 39% of the unfrozen control, respectively. On the other hand, the decrease in plasma membrane recovery from the leaves following hypertonic exposure to 2.69 osm or 5.38 osm sorbitol solution was not as great as that of the frozen tissues (i.e. 88 and 81%, respectively, of that recovered from leaves maintained in 0.53 osm sorbitol solution). Although the decrease in protein recovery in plasma membrane fractions was quite different in samples frozen to −5°C or −10°C in comparison to those subjected to a hypertonic exposure in either a 2.69 or 5.38 osm sorbitol solution, the degree of injury was the same (35–40% following freezing to −5°C or exposure in 2.69 osm and 65–70% following freezing to −10°C or exposure to 5.38 osm). Thus, the reduction in protein recovery following freezing does not appear to be a consequence of injury per se. Instead, it appears to be a result of less effective isolation of the plasma membrane from frozen tissues. This interpretation is consistent with the fact that the recovery of plasma membrane (based on protein content) decreased to 84% of the control following freezing to −2.3°C, which resulted in less than 5% electrolyte leakage, whereas there was only a 3% decrease in the protein recovery in the plasma membrane.

**Table I. Marker Enzyme Activities in Plasma Membrane Fractions Isolated from Rye Leaves Frozen in Isotonic Sorbitol and Thawed in Hypertonic Solutions**

Concentrations of Triton X-100, vanadate (as Na3VO4), NO3 (as KNO3), and antimycin A are 0.05% (w/v), 100 μM, 50 mM and 3 μM, respectively.

<table>
<thead>
<tr>
<th>Marker enzymes</th>
<th>Unfrozen Control</th>
<th>Frozen to −2.3°C Thawed in 1.24 osm</th>
<th>Frozen to −5°C Thawed in 2.69 osm</th>
<th>Frozen to −10°C Thawed in 5.38 osm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activitya</td>
<td>Total activityb</td>
<td>Specific activitya</td>
<td>Total activityb</td>
</tr>
<tr>
<td>ATPase (pH 6.5, Triton X-100)</td>
<td>64.25</td>
<td>80.31</td>
<td>60.93</td>
<td>64.04</td>
</tr>
<tr>
<td>+ vanadate</td>
<td>32.01</td>
<td>40.01</td>
<td>29.27</td>
<td>30.76</td>
</tr>
<tr>
<td>+ NO3</td>
<td>63.91</td>
<td>78.89</td>
<td>58.21</td>
<td>61.18</td>
</tr>
<tr>
<td>Triton-stimulated UDPase (pH 7.3)</td>
<td>3.29</td>
<td>4.11</td>
<td>3.57</td>
<td>4.41</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>5.11</td>
<td>6.39</td>
<td>5.88</td>
<td>6.18</td>
</tr>
<tr>
<td>Cyt c oxidase</td>
<td>0.021</td>
<td>0.026</td>
<td>0.022</td>
<td>0.023</td>
</tr>
<tr>
<td>NADH Cyt c reductase (+ antimycin A)</td>
<td>0.013</td>
<td>0.016</td>
<td>0.015</td>
<td>0.016</td>
</tr>
<tr>
<td>Protein, mg (% of unfrozen control)</td>
<td>1.250</td>
<td>1.051</td>
<td>0.851</td>
<td>0.492</td>
</tr>
</tbody>
</table>

a μmol substrate/mg protein/h.  b μmol substrate/30 g fresh weight/h.
fraction following exposure in a 1.24 osm solution, which resulted in a similar degree of injury (electrolyte release). Uemura and Yoshida (27) also observed a decrease (20%) in the recovery of plasma membrane from Jerusalem artichoke tubers following freezing to −5°C, even though survival remained unaffected. These results suggest that the decreased recovery of plasma membrane from frozen tissues is not a consequence of injury to the tissues.

**ATPase Activity**

There are conflicting reports of the effect of freezing on plasma membrane ATPase activity. Uemura and Yoshida (27) reported only a slight decrease in the ATPase activity of plasma membrane isolated from Jerusalem artichoke tubers frozen to lethal temperatures. In contrast, Hellergren et al. (8) and Iswari and Palta (11) reported that the plasma membrane ATPase activity (of pine needles and potato leaves, respectively) decreased following a lethal freeze/thaw cycle. One reason for the apparent conflicting results may be the difference of the method of determining ATPase activity, i.e. assay in the presence (8, 11) versus absence (27) of detergent.

Because the catalytic site of plasma membrane ATPase is thought to be located on the cytoplasmic surface of plasma membrane in situ (21), the orientation (right-side out versus inside-out) and whether or not the vesicles are sealed (i.e. permeable to the substrate, ATP-Mg) will influence the amount of ATPase activity determined. Therefore, the neutral detergent, Triton X-100, has been used to increase the permeability of plasma membrane vesicles obtained by the two-phase system and to determine the total intrinsic activity (10, 15, 29), with a caution on the direct effect of Triton X-100 on ATPase per se (22).

When assayed in the presence of Triton X-100 (0.05% w/v), the specific activity of plasma membrane ATPase decreased only slightly after freezing or hypertonic exposure of the leaves (Table III). In the plasma membrane fraction of leaves frozen in an isotonic sorbitol solution, the ATPase activity decreased to only 89% of the unfrozen control, with no further decrease after freezing to −10°C. Similar results were obtained if the leaves were exposed to hypertonic solutions at subzero temperatures. Following exposure to 1.24, 2.69, and 5.38 osm solutions, the ATPase activity was 88, 84, and 85% of the activity of the unstressed leaves, respectively. Assuming that the total intrinsic activity of the enzyme is assayed in the presence of Triton X-100, these results indicate that, under the conditions used, neither freezing nor hypertonic stress resulted in a substantial inactivation or change in plasma membrane ATPase activity.

These results are in contrast to those of Hellergren et al. (8) and Iswari and Palta (11) who concluded that freezing inactivated the plasma membrane ATPase. In the studies of both Hellergren et al. (8) and Iswari and Palta (11), the frozen samples were stored overnight at 0°C for thawing before isolation of the plasma membrane. In contrast, in our procedure the plasma membrane fractions were isolated from frozen tissues, which were not exposed to prolonged periods at 0°C. In the procedure of Hellergren et al. (8) and Iswari and Palta (11), there is the possibility that the inactivation of the plasma membrane ATPase occurred during the prolonged post-thaw period. Previously, Wang et al. (28) reported that the majority of the decrease in the plasma membrane ATPase activity occurred during prolonged periods of thawing. Hence, in the studies of Hellergren et al. (8) and Iswari and Palta (11), it is quite likely that the measured decrease in ATPase activity was a secondary pathological consequence that occurred during the overnight incubation rather than a primary consequence of the freeze/thaw cycle. This interpretation is consistent with several reports, including that of Hellergren et al.
Table III. Effect of Triton X-100 on ATPase Activity of Plasma Membrane Isolated from Leaves Subjected to Either a Freezing or Hypertonic Stress

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ATPase Activity</th>
<th>Triton Stimulation*</th>
<th>Latency of ATPaseb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−Triton</td>
<td>+Triton</td>
<td>(%)</td>
</tr>
<tr>
<td>Freezing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0°C</td>
<td>11.2 ± 0.2 (100)</td>
<td>63.3 ± 5.5 (100)</td>
<td>5.65 ± 0.62 (82)</td>
</tr>
<tr>
<td>−2.3°C</td>
<td>12.3 ± 0.9 (110)</td>
<td>56.0 ± 4.9 (88)</td>
<td>4.52 ± 0.43 (78)</td>
</tr>
<tr>
<td>−5°C</td>
<td>14.9 ± 1.2 (133)</td>
<td>57.4 ± 3.8 (81)</td>
<td>3.85 ± 0.32 (74)</td>
</tr>
<tr>
<td>−10°C</td>
<td>18.5 ± 0.9 (165)</td>
<td>54.0 ± 3.4 (85)</td>
<td>2.92 ± 0.34 (65)</td>
</tr>
<tr>
<td>Hypertonic exposure at subzero temperatures</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.53 osm</td>
<td>10.8 ± 0.5 (100)</td>
<td>63.5 ± 2.4 (100)</td>
<td>5.88 ± 0.52 (83)</td>
</tr>
<tr>
<td>1.24 osm</td>
<td>11.3 ± 1.1 (105)</td>
<td>55.9 ± 1.5 (88)</td>
<td>4.95 ± 0.38 (79)</td>
</tr>
<tr>
<td>2.69 osm</td>
<td>13.0 ± 0.8 (120)</td>
<td>53.3 ± 2.9 (84)</td>
<td>4.10 ± 0.40 (75)</td>
</tr>
<tr>
<td>5.38 osm</td>
<td>17.3 ± 1.3 (160)</td>
<td>54.0 ± 3.4 (85)</td>
<td>3.12 ± 0.30 (68)</td>
</tr>
<tr>
<td>Hypertonic exposure at 0°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.53 osm</td>
<td>9.5 ± 0.2 (100)</td>
<td>65.0 ± 3.3 (100)</td>
<td>6.84 ± 0.43 (85)</td>
</tr>
<tr>
<td>1.24 osm</td>
<td>10.4 ± 0.5 (109)</td>
<td>57.7 ± 3.6 (89)</td>
<td>5.55 ± 0.70 (82)</td>
</tr>
<tr>
<td>2.69 osm</td>
<td>11.9 ± 0.3 (125)</td>
<td>48.0 ± 1.3 (74)</td>
<td>4.03 ± 0.22 (75)</td>
</tr>
<tr>
<td>5.38 osm</td>
<td>18.4 ± 0.5 (194)</td>
<td>45.8 ± 4.1 (70)</td>
<td>2.49 ± 0.31 (59)</td>
</tr>
</tbody>
</table>

* Triton stimulation is expressed as the ratio of the activity assayed in the presence of Triton X-100 to the activity assayed in the absence of Triton X-100. b Latency of ATPase is estimated according to Larsson et al. (16), and the number in parentheses is the percentage of the unfrozen or isotonic control treatment.

al. (8), that indicate that in vitro freezing of plasma membrane preparations has little apparent effect on the ATPase activity.

Effects of Freezing/Hypertonic Stress on Plasma Membrane Vesicle Orientation and Semipermeability

The asymmetric localization of ATPase on the cytoplasmic side of the plasma membrane provides a means to determine if freezing or hypertonic stress alters the semipermeable characteristics or orientation of the plasma membrane vesicles that are isolated from the stressed leaves. Measurement of ATPase activity in both the presence and absence of Triton X-100 provides information on the proportion of vesicles that are right-side-out and sealed. A decrease in the Triton X-100 stimulation of the ATPase activity reflects a decrease in the proportion of right-side-out/sealed vesicles. Unfortunately one cannot use this approach to distinguish between right-side-out/leaky, inside-out/sealed, and inside-out/leaky vesicles in the remaining portion. Nevertheless, the approach provides a means to determine whether the native state of the membrane vesicles (right-side out and sealed) is perturbed by freezing or hypertonic stress.

Typically, plasma membrane vesicles that are partitioned into the upper phase of the polyethylene glycol-dextran two-phase system are apparently right-side-out and sealed. For example, based on the Triton X-100 stimulation of the ATPase activity, approximately 67% of the recovered vesicles are right-side out and sealed in the studies of Larsson et al. (15), whereas Yoshida et al. (29) reported a value of approximately 78%. In contrast, Canut et al. (4) reported only a two- to threefold Triton X-100 stimulation of ATPase activity in plasma membrane vesicles obtained by an aqueous two-phase system, and a strong stimulation (sevenfold) was only observed in plasma membrane fractions that were subfractionated by free-flow electrophoresis. It appears that the precise isolation procedure and the tissue may influence the proportion of right-side-out and sealed vesicles that will be isolated using the two-phase partitioning technique.

In the present study, there was nearly a sixfold stimulation of the ATPase activity in the plasma membrane fractions isolated from the unstressed leaves, i.e. the ATPase activity measured in the absence of Triton X-100, was approximately 17% of the activity measured in the presence of Triton X-100 (Table III). Thus, we estimate that approximately 83% of the plasma membrane vesicles isolated from the unstressed control tissues were right-side-out and sealed, with the remainder either right-side-out/leaky, inside-out/sealed, or inside-out/leaky.

The ATPase activity measured in the absence of Triton X-100 was greater in plasma membrane fractions isolated from leaves that were subjected to either a freezing or hypertonic stress (Table III). The magnitude of the increase was a function of the subzero temperature or osmolality of the hypertonic solution, with the proportional increase resulting from freezing being similar to that resulting from hypertonic exposures at subzero temperatures. However, if the leaves were exposed to hypertonic solutions at 0°C, the ATPase activity measured in the absence of Triton X-100 was even larger. These results are in contrast to the effect of freezing or hypertonic exposure on the activity of ATPase measured in the presence of Triton X-100 (i.e. only a slight proportional decrease). As a result, the Triton X-100 stimulation of the ATPase activity of plasma membrane was significantly decreased following freezing or hypertonic exposure of the leaves. Table III shows the Triton X-100 stimulation of ATPase activity as a ratio of the activity assayed in the presence of Triton X-100 (0.05% w/v) to the activity assayed in the absence of Triton X-100. Whereas
there was nearly a sixfold stimulation of the activity by Triton X-100 in the plasma membrane fractions isolated from un-stressed leaves (0°C or 0.53 osm), there was only a threefold stimulation in the fractions isolated from leaves frozen to −10°C or subjected to a 5.38 osm sorbitol solution at −10°C. Using these values, we estimate that the proportion of right-side out/sealed vesicles declined from 82% in the plasma membrane fractions isolated from the control tissues to 66% in those isolated from leaves frozen to −10°C and 68% in those isolated from leaves subjected to the 5.38 osm sorbitol solution (Table III). Thus, the proportion of the plasma membrane vesicles recovered in the upper fraction of the two-phase system that are not right-side out and sealed increased nearly twofold (from 18 to 34%). Whether these vesicles are right-side out/leaky, inside-out/sealed, or inside-out/leaky cannot be determined from these studies. One might speculate that they are right-side out/leaky given that the two-phase partition method separates vesicles on the basis of surface properties and that the surface properties of the cytoplasmic and protoplasmic surfaces are likely to be different. However, the results of Canut et al. (4) would tend to argue against this interpretation and other approaches are required to resolve this uncertainty.

H⁺-Transport Activity of the Plasma Membrane Vesicles

Assuming that ATP-dependent H⁺-transport is associated with the ATPase, which is located on the inner leaflet of the plasma membrane, Larsson et al. (16) suggested that H⁺-transport activity of plasma membrane vesicles would be detected only in inside-out and sealed vesicles. Thus, it is possible to estimate the proportion of inside-out/sealed vesicles in the plasma membrane fractions and to determine whether freezing or hypertonic stress alters this proportion. To examine this possibility, the transmembrane H⁺-transport activity of plasma membrane vesicles was measured by fluorescence spectroscopy using the fluorescence dye, acridine orange (1).

Figure 2 shows the relative H⁺-transport activity of the plasma membrane vesicles calculated from the initial quenching velocity of acridine orange fluorescence. Relative H⁺-transport activity was decreased in plasma membrane fractions isolated from leaves subjected to either a freezing or hypertonic stress. In leaves either frozen to −10°C or equilibrated in a 5.38 osm sorbitol solution, the H⁺-transport of the plasma membrane fraction decreased to less than 40% of that of unstressed tissues. It should be noted that the initial quenching velocity of the fluorescence in the controls (5% quenching/min) was relatively small compared with other reports (e.g. 10–20% quenching/min) (13, 19). However, given that the plasma membrane fractions from the control leaves contained an estimated 83% of right-side-out/sealed vesicles (on the basis of the Triton X-100 stimulation of ATPase activity), this low initial quenching velocity is not unexpected. Nevertheless, the results indicate that the proportion of sealed, inside-out vesicles in the plasma membrane samples decreased following freezing or hypertonic stress. Since the proportion of sealed, right-side-out plasma membrane vesicles also decreased under these same conditions (Table III), it appears that both freezing and hypertonic stress increase the permeability of the plasma membrane vesicles to both H⁺ as well as ATP. Further, because the decrease in the proportion of both sealed, right-side-out vesicles (Table III) and sealed, inside-out vesicles (Fig. 2) decreased as a function of the subzero temperature, whereas the decrease in the ATPase activity (measured in the presence of Triton X-100) decreased only slightly and to the same extent regardless of the freezing temperature, we submit that alterations in the semipermeable characteristics of the plasma membrane are of greater significance to freezing injury than the inactivation of ATPase.

SDS-PAGE

The effect of freezing or hypertonic exposure on the protein composition of the plasma membrane was determined using one-dimensional SDS-slab PAGE. Figure 3 shows the one-dimensional densitometric tracings of electrophoretograms of plasma membrane polypeptides stained with Coomassie brilliant blue. The polypeptides of plasma membranes from unstressed and stressed tissues that differed in staining intensity are indicated with letters and summarized in Table IV. Changes in several polypeptides (a decrease in bands A, B, F, and G and an increase in bands C and H) were common to all freezing and hypertonic treatments. When the plasma membrane polypeptides from leaves frozen in the presence of an isotonic sorbitol solution were compared with those exposed to hypertonic sorbitol solutions at subzero temperatures, there was no difference except for a decrease in band E of the plasma membrane from leaves frozen in the presence of isotonic sorbitol. If the hypertonic exposure occurred at 0°C, a decrease in band D also occurred. This difference may reflect the fact that injury in hypertonic solutions was greater at 0°C than that which occurred at subzero temperatures (Fig. 1).
Figure 3. Densitometric tracings of electrophoretograms of plasma membrane polypeptides from rye leaves. The plasma membrane was isolated from leaves either frozen in the presence of isotonic sorbitol (I), equilibrated in hypertonic solutions at either 0°C (II), or subzero temperatures (III). After electrophoresis, gels stained with Coomassie blue were scanned densitometrically. Polypeptides that changed in staining density following freezing and/or hypertonic exposure are labelled with letters. Mol wt determined with standard proteins are noted at the top of panel in kD.

Previously, Uemura and Yoshida (27) reported a decrease or elimination of several plasma membrane proteins of Jerusalem artichoke tubers following lethal freezing in vivo. These proteins were termed frost-susceptible proteins (FSPs). We also observed a decrease in several polypeptides of rye plasma membrane following freezing in the absence of sorbitol, which is the same freezing condition used by Uemura and Yoshida (27). However, except for one polypeptide (band A), the polypeptides that were altered in rye plasma membranes were not similar (on the basis of molecular mass on the SDS-PAGE gel, data not shown) to those that changed in Jerusalem artichoke tubers. Given that Uemura and Yoshida (27) also observed the FSPs of orchard grass plasma membranes were of a different molecular mass than the FSPs of Jerusalem artichoke, the specific FSPs are species dependent.

CONCLUSIONS

These studies demonstrate that exposure of rye leaves to a hypertonic stress at the freezing point of the solution results in the same extent of injury (as measured by electrolyte leakage) as does freezing the leaves in an isotonic solution. Because the two stresses result in the same alterations in the plasma membrane, including increased electrolyte leakage from the leaves, an increase in the proportion of plasma membrane vesicles that are permeable to ATP, a decrease in H+-transport activity of the vesicles, and changes in the plasma membrane polypeptide profiles in SDS-PAGE, we conclude that the large osmotic stress resulting from freezing is the principal cause of these manifestations of freezing injury. Further, under the conditions used in this study, we find little evidence to support the notion that inactivation of plasma membrane ATPase is a primary cause of freezing injury. Instead, injury is associated with an increase in the permeability (i.e. leakiness) of the plasma membrane. However, it should not be assumed that there is a global alteration in the semipermeable characteristics of the plasma membrane because, in spite of the fact that nearly 70% of the intracellular electrolytes were released from leaves that were frozen to −10°C (Fig. 1), more than 80% of the plasma membrane vesicles isolated from these tissues were apparently right-side-out and sealed (Table III). Thus, it appears that the release of a large amount of electrolytes from injured tissues occurs because of lesions in a relatively small proportion of the total surface area of the plasma membrane and is not associated with substantial changes in the polypeptide profile of the plasma membrane.

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Table IV. Summary of Compositional Changes in Plasma Membrane Polypeptides following Freezing or Hypertonic Exposure

<table>
<thead>
<tr>
<th>Band</th>
<th>Mol Wt</th>
<th>Freezing at 0°C</th>
<th>Hypertonic Exposure at subzero temperatures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kD</td>
<td>+ Sorbitol</td>
<td>At 0°C</td>
</tr>
<tr>
<td>A</td>
<td>121 ± 1</td>
<td>↑↓</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>86 ± 5</td>
<td>↑↓</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>63 ± 3</td>
<td>↑↓</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>49 ± 2</td>
<td>NC</td>
<td>↓</td>
</tr>
<tr>
<td>E</td>
<td>32 ± 1</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>26 ± 1</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>17 ± 1</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>15 ± 1</td>
<td>↑</td>
<td></td>
</tr>
</tbody>
</table>

* Decrease in staining intensity. † Increase in staining intensity. * No change in staining intensity.
LITERATURE CITED


