In Vivo Perturbation of Membrane-Associated Calcium by Freeze-Thaw Stress in Onion Bulb Cells

SIMULATION OF THIS PERTURBATION IN EXTRACELLULAR KCl AND ALLEVIATION BY CALCIUM

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ABSTRACT

Incipient freeze-thaw stress in onion bulb scale tissue is known to cause enhanced efflux of K+, along with small but significant loss of cellular Ca2+. During the post-thaw period, irreversibly injured cells undergo a cytological aberration, namely, protoplasmic swelling. This cellular symptom is thought to be caused by replacement of Ca2+ from membrane by extracellular K+ and subsequent perturbation of K+ transport properties of plasma membrane. In the present study, onion (Allium cepa L. cv Sweet Sandwich) bulbs were slowly frozen to either −8.5°C or −11.5°C and thawed over ice. Inner epidermal peels from bulb scales were treated with fluorescein diacetate for assessing viability. In these cells, membrane-associated calcium was determined using chlorotetracycline fluorescence microscopy combined with image analysis. Increased freezing stress and tissue infiltration (visual water-soaking) were paralleled by increased ion leakage. Freezing injury (−11.5°C; irreversible) caused a specific and substantial loss of membrane-associated Ca2+ compared to control. Loss of membrane-associated Ca2+ caused by moderate stress (−8°C; reversible) was much less relative to −11.5°C treatment. Ion efflux and Ca2+-chlorotetracycline fluorescence showed a negative relationship. Extracellular KCl treatment simulated freeze-thaw stress by causing a similar loss of membrane-associated calcium. This loss was dramatically reduced by presence of extracellular CaCl2. Our results suggest that the loss of membrane-associated Ca2+, in part, plays a role in initiation and progression of freezing injury.

Freezing injury is known to result in increased efflux of ions and organic solutes and in water soaking of plant tissue (16, 17). The major cation that leaks out of cells is known to be K+ (16). Due to this leakage, cells after thawing experience a high extracellular K+ concentration. The first microscopic symptom observed in irreversibly injured onion bulb scale cells was protoplasmic swelling (2, 19). In parallel with K+ efflux, a small but significant Ca2+ efflux was also noted, which increased with progress of injury (16). From these observations, Palta and Li (19) hypothesized that secondary injury occurs to onion bulb cells during postthaw as a consequence of removal of Ca2+ from the membrane by extracellular K+. Calcium is an important factor in the maintenance of membrane integrity and has been found to regulate ion transport processes (6, 8–10). Arora and Palta (2) suggested that removal of Ca2+ from the membrane by extracellular K+ results in perturbation of structural integrity of plasma membrane and induces cytological aberration similar to that caused by freeze-thaw stress, namely, protoplasmic swelling. In these studies, it was also demonstrated that elevated concentrations of Ca2+ in extracellular solution protected cell membrane against the adverse effect of K+ and prevented the cytological alteration from occurring. However, direct evidence supporting perturbation of membrane or cellular calcium following freeze-thaw stress in plants is lacking.

Several studies have used CTC,2 a Ca2+ binding antibiotic, as a fluorescent probe for membrane-associated calcium (14, 21, 28, 32). Recently, using this probe, membrane-associated Ca2+ status has been investigated in response to salt (5, 13) and chilling (33) stress. In the present study, this method was adopted to microscopically observe and quantitatively estimate the perturbation in membrane-associated Ca2+ of onion epidermal cells in response to freeze-thaw stress and extracellular KCl solution. Our results provide direct evidence for our hypothesis that one aspect of freezing injury (which can be simulated by extracellular KCl) in onion epidermal cells, is the loss of membrane-associated calcium. By using FDA, a fluorescent probe for cell viability (11, 31), simultaneously with CTC, we show that a selective loss of membrane-associated Ca2+ from otherwise viable cells occurs during early stages of freeze-thaw injury.

MATERIALS AND METHODS

Onion (Allium cepa L. cv Sweet Sandwich) bulb scale tissue was used for this study. These bulbs were grown at the University of Wisconsin-Madison Experimental Farm located at Arlington, WI.

Freeze-Thaw Protocol. Medium-sized onion bulbs were frozen by cooling at the rate of 1.5°C/h, to either −8.5°C ± 0.5°C or −11.5°C ± 0.5°C, and then were thawed slowly over ice (2, 15). To prevent supercooling, bulbs were nucleated at −1°C. A continuous record of the temperature inside the bulb was obtained using a copper-constantan thermocouple inserted into the middle of the bulb. Freezing and thawing process of bulbs resembled the freeze-thaw curves reported earlier (15). All the measurements on stressed tissue were completed within 24 h after thawing.

Cell Viability. Sections of inner epidermis of onion bulb scales, which can be peeled off as a single cell layer, were prepared from the third scale (counting from outermost) of freeze-thaw stressed bulbs (20). Viability and physiological condition of epidermal cells were assessed with a variety of criteria, e.g. microscopic

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2 Abbreviations: CTC, chlorotetracycline; FDA, fluorescein diacetate.
observations, cytoplasmic streaming, ability to plasmolyze, and ability to exhibit flochrome reaction to FDA (11, 18). Stock solution of FDA was prepared in acetone (50 mg/10 ml). The FDA staining solution was then prepared by diluting 50 μl of stock solution in 5 ml of 2.5 mM Tris-HCl buffer (pH 6.0) (12). Epidermal peels were exposed to the staining solution for 5 min, then rinsed with buffer, and the cells were plasmolyzed in 0.8 M mannitol solution.

These cells were then microscopically examined and photographed using a Standard 16 microscope, equipped with epifluorescence optics and ×20 objective lens and mounted with MC63A photomicrographic camera (Carl Zeiss, Inc., Thornwood, NY). For comparison, unfrozen control cells were examined and photographed.

**Efflux of Ions from Bulk Scale Tissue.** The third scale (counting from outermost) of thawed onion bulbs was visually examined for water soaking and later used for microscopic observations of Ca2+-CTC fluorescence. This was then used to measure ion efflux using the conductivity method (2, 16). Two pieces (1 x 1 cm2) of the third scale were vacuum infiltrated in 20 ml of distilled-deionized H2O and were shaken for 1 h. Conductivity of effusate was measured using a conductivity meter (YSI model 31), and it was again recorded for the same tissue at room temperature after it was heat-killed.

**Membrane-Associated Ca2+-CTC Fluorescence in Freeze-Thaw Stress Cells.** Inner epidermal sections from the third scale of freeze-thaw stressed onion bulbs were made as described above and were exposed to CTC staining solution for 30 min, at which time the CTC fluorescence had stabilized. Staining solution consisted of 0.2 mM CaCl2, 20 μM CTC (Sigma), and 10 mM Tris-HCl buffer (pH 6.8). Sections were then rinsed with the same buffer, and the cells were plasmolyzed in 0.8 M mannitol solution with an intent to observe membrane-associated Ca2+-CTC fluorescence without interference from neighboring cells.

These cells were then observed under a Universal microscope, equipped with epifluorescence optics and an ×40 objective lens and mounted with MC 63 photomicrographic camera (Carl Zeiss, Inc.). No detrimental effect on cell cytolysis (cytophysiology) was observed due to exposure to 20 μM CTC for 30 min under bright field. A voltage-stabilized 50 W DC HBO mercury lamp (Carl Zeiss, Inc.) was used as a light source. Excitation and emission wavelengths for Ca2+-CTC were isolated with 400 nm and 510 nm interference filters (Carl Zeiss, Inc.). Cells were photographed on Kodak Tri-X Pan 35 mm film (400 ASA). Illumination with blue excitation light and duration of photographic exposure were kept uniform for all pictures. For comparison, these observations were made and pictures were taken under identical conditions for unfrozen control cells.

**Cell Viability and Membrane-Associated Ca2+-CTC Fluorescence as Affected by Extracellular KCl and Its Response to CaCl2.** Inner epidermal sections were made from unfrozen onion bulb scale as described above. To study the effect of extracellular KCl on cell viability, onion epidermal cells were bathed in 50 mM KCl and in solutions containing 50 mM KCl and various concentrations of CaCl2. At various times after transfer, epidermal cells were examined under low microscope to assess cell viability. Ability to plasmolyse and microscopic observations on protoplasmic coagulation were used as criteria of cell viability.

We have shown (2) that injurious effect of freeze-thaw stress on these cells (occurrence of protoplasmic swelling) can be simulated by bathing them in extracellular 50 mM KCl and can be prevented by presence of extracellular 20 mM CaCl2. To examine the effect of this treatment on cellular/membrane calcium, some of the epidermal sections were bathed in 50 mM KCl containing 0.2 mM CaCl2 for 30 min, whereas others were bathed in a solution containing 50 mM KCl and 20 mM CaCl2 for same duration. Following these treatments, the sections were stained with CTC staining solution, plasmolyzed, and photographed as described for freeze-thaw stressed samples. For comparison, the same observations were made for control cells bathed in 0.2 mM CaCl2.

**Quantitative Image Analysis of Ca2+-CTC Fluorescence Intensity.** A prototype two-dimensional photodiode array camera (TN 6100 detector head, Tracor Northern, Middleton, WI) equipped with Reticon RA 256 x 256A two-dimensional array of photodiodes was used for quantitative image analysis of Ca2+-CTC fluorescence intensity. Thirty-five mm negatives of photomicrographs obtained from epifluorescence microscopy were used to obtain pictures by the help of this camera. Software on high-performance Hewlett-Packard Unix Workstation (HP 320) was used to acquire and store these pictures. Operating temperature of the camera, exposure time, and gain and distance of object to photographic lens were kept constant for all the pictures from different treatments. The camera responded to light in linear fashion. Using Collage, a software program, individual fluorescent areas (protoplasmic surface) from different cells in these pictures were selected, and the brightness due to fluorescence in these areas was converted to optical density (milli absorbance) units. Fog area on the negative was used as blank. Knowing the number of pixels (center to center distance between adjacent detectors on detector chip in camera) for a given fluorescent area (protoplasmic surface) and the area/pixel, these measurements of optical density were converted to absorbance units/mm2 of fluorescent area (protoplasmic surface) (Fig. 1).

The freeze-thaw experiment was repeated five times. Similar results were obtained each time. Results from one experiment are presented here.

**RESULTS**

**Cell Viability.** Both unfrozen control and freeze-injured (−11.5°C ± 0.5°C) onion epidermal cells exhibited ability to plasmolyze and bright fluorescence when treated with FDA (Fig. 1, a and b). Cytoplasmic streaming was clearly evident in both control and freeze-injured cells. Cells from the bulbs stressed to −8.5°C also exhibited these characteristics similar to bulbs stressed to −11.5°C and unfrozen control (picture not shown). However, it is important to note that epidermal cells of onion bulb which was stressed to −11.5°C died (assessed by cytoplasmic streaming and ability to plasmolyze) in 4 to 7 d when kept at 5°C. These cells were, thus, irreversibly damaged. On the other hand, those stressed to −8.5°C eventually recovered almost completely (visual water-soaking of the tissue disappeared). These cells were, therefore, reversibly damaged (15, 17; see also Table I, discussed below).

**Efflux of Ions from Scale Tissue.** About 15 and 65% of bulkscale tissue showed visual water soaking when onion bulbs were subjected to freezing stress of −8.5 and −11.5°C, respectively (Table I). Leakage of ions after 1 h of shaking of tissue pieces was expressed as percent of total ions that leaked out in distilled-deionized H2O after they were heat-killed. Ion leakage increased from about 32 to 52% as the freezing temperature was lowered from −8.5 to −11.5°C (Table I). In comparison, about 15% of total ions leaked out from the unfrozen bulb scale tissue. Visual water soaking almost disappeared from the scale tissue of bulbs frozen to −8.5°C, when stored at 5 ± 0.5°C for 3 d, whereas it did not for the bulbs frozen to −11.5°C. The extent of water-soaking very closely follows the electrolyte leakage from these scale tissues.

**Effect of Freeze-Thaw Stress on Membrane-Associated Ca2+-CTC Fluorescence.** Inner epidermal cells of unfrozen control onion bulb scale tissue showed very bright fluorescence due to chelate complex of CTC and membrane-associated Ca2+ (Fig. 1c). Cytoplasmic streaming and plasmolyzing were clearly evident in these cells (Fig. 1d). Freeze-injured cells (frozen to −11.5 ±
FIG. 1. Photomicrographs of adaxial epidermal cells of onion bulb (both unfrozen control and freeze-thaw stressed) treated with FDA and CTC staining solutions. a, Control cells showing fluorescence from FDA staining; b, freeze-thaw stressed (−11.5°C) cells showing fluorescence from FDA staining; c, control cells exhibiting Ca²⁺-CTC fluorescence; d, bright field picture of (c); e, freeze-thaw stressed (−8.5°C) cells showing Ca²⁺-CTC fluorescence; f, bright field picture of (e); g, freeze-thaw stressed (−11.5°C) cells showing Ca²⁺-CTC fluorescence; h, bright field picture of (g). p, Protoplasmic surface (plasma membrane); cw, cell wall. Magnification in (a) and (b) was ×180 and in (c) to (h) was ×500.
Table I. Ion Leakage and Visual Observations on Water Soaking of Freeze-Thaw Stressed and Control Onion Bulb Scale Tissue

<table>
<thead>
<tr>
<th>Treatment Temperature</th>
<th>Extent of Visual Water Soaking</th>
<th>Ion Leakage as % of Total Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>+5 (control)</td>
<td>0*</td>
<td>15.3 ± 1.2*</td>
</tr>
<tr>
<td>−8.5</td>
<td>15</td>
<td>32.5 ± 2.4</td>
</tr>
<tr>
<td>−11.5</td>
<td>65</td>
<td>52.0 ± 7.0</td>
</tr>
</tbody>
</table>

* Values for frozen scale tissue are within ±5%.  b Means ± sd of four separate measurements.

Table II. Quantitative Estimates of Membrane-Associated Ca2+-CTC Fluorescence Intensity in Onion Scale Epidermal Cells following a Freeze-Thaw Stress

<table>
<thead>
<tr>
<th>Treatment Temperature</th>
<th>No. of Measurements</th>
<th>Ca2+-CTC Fluorescence Intensity absorbance/mm² fluorescent area (protoplasmic surface)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+5 (control)</td>
<td>13</td>
<td>35.5 ± 10.8b</td>
</tr>
<tr>
<td>−8.5</td>
<td>5</td>
<td>20.2 ± 3.4</td>
</tr>
<tr>
<td>−11.5</td>
<td>9</td>
<td>6.0 ± 2.5</td>
</tr>
</tbody>
</table>

* Calculated from original data obtained as milli absorbance/pixel (pixel area = 0.0083 mm²).  b Mean ± sd.

Table III. Cell Viability as Affected by Extracellular 50 mM KCl in the Presence or Absence of Various Concentrations of CaCl₂

<table>
<thead>
<tr>
<th>Bathing Medium</th>
<th>Percentage of Cells Appearing Dead at Various Durations (h) of Exposure to Different Solutions*</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM KCl</td>
<td>6 h 12 h 24 h 34 h</td>
</tr>
<tr>
<td>50 mM KCl + 0.2 mM CaCl₂</td>
<td>10* 60 90 100</td>
</tr>
<tr>
<td>50 mM KCl + 2.0 mM CaCl₂</td>
<td>10 50 80 100</td>
</tr>
<tr>
<td>50 mM KCl + 10.0 mM CaCl₂</td>
<td>0 5 5 5</td>
</tr>
<tr>
<td>50 mM KCl + 20.0 mM CaCl₂</td>
<td>0 0 0 0</td>
</tr>
</tbody>
</table>

* A cell was counted dead if it failed to plasmolyse in hypertonic mannitol solution and at the same time exhibited granulation of protoplasm.  b Average of three separate observations.

0.5°C depicted very dim fluorescence as compared to control (Fig. 1g); however, they exhibited cytoplasmic streaming and ability to plasmolyze when bathed in 0.8 mM mannitol solution (Fig. 1h) and were able to perform fluorochromic reaction when treated with FDA (Fig. 1b). For the epidermal cells from onion bulbs frozen to −8.5°C, Ca2+-CTC fluorescence appeared intermediate between −11.5°C and control treatment (Fig. 1e). Again, these cells exhibited cytoplasmic streaming and ability to plasmolyze (Fig. 1f). These cells also performed fluorochromic reaction with FDA (picture not shown).

Qualitative estimate of Ca2+-CTC fluorescence intensity showed that freeze-injured cells (−11.5°C) had only 15% membrane-associated Ca2+-CTC fluorescence, compared to control (Table II). However, this fluorescence was about 60% of control for the cells frozen to −8.5°C (Table II).

Effect of Extracellular KCl on Cell Viability and on Ca2+-CTC Fluorescence and Its Response to CaCl₂. There was progressive damage to the onion epidermal cells exposed to 50 mM KCl (Table III). All cells died after exposure to 50 mM KCl for 34 h. However, all cells were alive after 34 h, when bathing medium contained 20 mM CaCl₂ in addition to 50 mM KCl (Table III).

Addition of CaCl₂, up to 2.0 mM, to 50 mM KCl solution did not reverse the injurious effect of KCl on cells. However, at 10 mM CaCl₂, almost all cells survived up to 34 h.

Onion epidermal cells that were bathed in 50 mM KCl for 30 min showed very dim Ca2+-CTC fluorescence, as compared to control (Fig. 2a; cf. Fig. 1c with Fig. 2a). Visually, the intensity of fluorescence in these cells was close to that in freeze-injured (−11.5°C) cells (cf. Fig. 1g with Fig. 2a). This was further substantiated by the quantitative analysis for fluorescence intensity of these cells, which revealed that the intensity was only 15% compared to control (untreated) cells (Table IV). It is important to note that in spite of the loss of 85% of membrane-associated Ca2+, these cells were able to plasmolyze (Fig. 2b) and exhibited fluorochromic reaction to FDA (picture not shown). In the presence of 20 mM CaCl₂ and 50 mM KCl the cells exhibited relatively high Ca2+-CTC fluorescence (Fig. 2c). These cells also exhibited cytoplasmic streaming and ability to plasmolyze (Fig. 2d). The Ca2+-CTC fluorescence intensity in these cells was about 70% compared to the control (Table IV). Thus, there was a loss of only 30% of membrane-associated Ca2+ in the presence of 20 mM CaCl₂ compared to an 85% loss when 0.2 mM CaCl₂ was present.

DISCUSSION

The perturbation by Ca2+ in the thermotropic behavior and permeability properties of cell membrane has been shown to be also produced by Mg2+ (24, 29). However the concentration of Mg2+ required to mimic the response of Ca2+ has been found to be much higher (23). In accordance with this, we have found that the concentration of MgCl₂ required to prevent protoplasmic swelling (a cellular symptom caused by freezing injury and simulated by extracellular KCl treatment) in onion bulb cells was over 2 times higher than the concentration of CaCl₂ (data not shown). The uniqueness of calcium as a second messenger in mediating many metabolic responses and in membrane stability in plant cells is well established (6, 9, 10, 23). Furthermore, our earlier studies have suggested a role of calcium in freezing injury (1, 2). Therefore, in the present study, we examined the role of calcium rather than other divalent cations.

Freeze-Thaw Stress and Membrane-Associated Ca2+. The results of the present study suggest for the first time, that a loss of membrane-associated calcium occurs during the early stages of freeze-thaw stress injury. This loss of membrane calcium appears to be specific. We used CTC, a Ca2+ binding fluorescence probe to investigate membrane-associated Ca2+. Several reports have indicated that by using optical filters selective for Ca2+-CTC, changes in the fluorescence signal may be considered indicative of changes in membrane-associated Ca2+ (5, 14, 21, 33). Support for such a consideration comes from earlier work of Caswell and Hutchison (4) whereby they showed that Ca2+-CTC fluorescence intensity was a function of polarity of the medium. They also showed that the fluorescence intensity of Ca2+-CTC complex increased by sixfold in the presence of detergents or biological membranes or less polar solvents, when compared to in aqueous medium. Hallet et al. (7) found that in the presence of red blood cell membrane the Ca2+-CTC complex underwent sevenfold increase in peak emission whereas Mg2+-CTC complex increased only by 20%, as compared to fluorescence in aqueous medium. This observation strengthens the argument that the signal we get in our system is primarily from Ca2+-CTC in membrane.

Reduction of Ca2+-CTC fluorescence by freeze-thaw stress (Fig. 1g) thus could be interpreted to mean that membrane-associated Ca2+ was lost. Frequent observations that Ca2+ plays an important role in membrane-associated processes, such as maintenance of membrane integrity and reduction in ion leakage (6, 8, 9, 24, 30), suggest possibilities for alleviation of the negative effects of low temperature stress by Ca2+. Our results show that loss of...
membrane-associated Ca\textsuperscript{2+} from freeze-thaw stressed cells is accompanied by leakage of ions and water soaking of tissue (Table I). It is important to note that in freeze-stressed onion cells efflux of ions and Ca\textsuperscript{2+}-CTC fluorescence (membrane calcium) are negatively related (Fig. 3), suggesting that one of the possible reasons for freeze-thaw stress induced membrane leakiness is the loss of membrane associated Ca\textsuperscript{2+}. This is supported by the findings of Pomeroy and Andrews (22) whereby they showed that viability of winter wheat cells was significantly enhanced by the presence of Ca\textsuperscript{2+} in cell suspensions exposed to ice encasement stress, resulting in lower ion efflux following injury. In these studies, it was also suggested that a similar protective effect against freezing stress is not elicited by either Mg\textsuperscript{2+} or La\textsuperscript{3+}, providing further evidence of a specific effect of Ca\textsuperscript{2+} on membrane functions (22). Interestingly, in spite of the loss of 85% of membrane-associated Ca\textsuperscript{2+} as compared to control (Table II; −11.5°C treatment), the freeze-thaw injured cells exhibited flow-
rochromatic reaction, hence were viable, right after thawing. However, the cells from bulbs freeze-stressed to −8.5°C (which lost about 32% of total ions and about 40% of membrane-associated Ca2+ as compared to control) eventually recovered completely, whereas those stressed to −11.5°C (which lost about 52% of total ions and about 85% of membrane-associated Ca2+ as compared to control) ultimately died. These results suggest that there is a critical amount of Ca2+ which cell membrane must retain to enable cells to ultimately survive freeze-thaw stress.

Simulation of Freeze-Thaw Induced loss of Membrane-Associated Ca2+ by Bathing Cells in Extracellular KCl and Its alleviation by Extracellular CaCl2. In our earlier work (2) we have shown that the cellular symptoms of freeze-thaw injury could be simulated by bathing onion scale epidermal cells in 50 mM KCl and could be prevented by adding 20 mM CaCl2 to the extracellular KCl. Results of the present study support and extend this finding. It is apparent from our results that KCl treatment not only caused a loss of membrane-associated calcium (Fig. 2a), but also the extent of the Ca2+ loss was similar (both visual and quantitative estimates) to that caused by freeze-thaw stress (cf. Fig. 2a with Fig. 1g; cf. Table II with Table IV). It is important to note that 50 mM KCl was injurious to the onion cells (Table III). Furthermore, this injurious effect could be prevented only by a specific amount of CaCl2.

We have previously hypothesized (2, 19) that K+-induced replacement/loss of membrane Ca2+ might be responsible for membrane leakiness to passive influx of K+ during post thaw period thus resulting in the initiation of one of the cellular symptoms of freeze-thaw stress, namely protoplasmic swelling. Results presented here support this hypothesis. We have also reported that this cellular aberration is an indication of irreversible injury (2, 19). Hence, removal of K+ from extracellular water during post thaw should help in enhancing the viability of bulb scale cells. Support for this consideration comes from earlier experiments whereby it was shown that the onion bulb cells remained viable for a longer time if freeze-thaw stressed (irreversible injury) tissue was washed with distilled deionized H2O, spring H2O, or 10 mM CaCl2 (1, 17). In a comparative study, freeze-thaw stressed tissue, when washed in 20 mM CaCl2, showed better recovery of turgor, as compared to control washed in distilled-deionized H2O (1). Caswell and Hutchison (4), as early as 1971, reported a decrease in fluorescence of Mg2+-CTC when 100 mM NaCl was added to human erythrocyte membrane or rabbit skeletal muscle microsomal preparation. Only recently, both Na+ and K+ have been shown to displace Ca2+ from the plasma membrane of intact cotton root hairs and corn root protoplasts (5, 12). Lynch et al. (13) have shown that reduction of Ca2+-CTC fluorescence due to cations (Na+, in particular) is neither due to displacement of CTC from membrane nor can it be attributed to its interference with Ca2+-CTC binding. Thus, reduction in Ca2+-CTC fluorescence due to extracellular KCl in the present study reflects displacement of Ca2+ from the membrane. In our experiments, EGTA treatments reduced Ca2+-CTC fluorescence to about the same extent as did KCl (data not shown). It has been shown that EGTA does not cross the membrane (3). Thus, it is possible that K+-displacement of membrane-associated Ca2+ may be occurring primarily at the external surface of plasma membrane.

Our results indicate that when 20 mM CaCl2 was added to extracellular 50 mM KCl, it was able to maintain higher levels of Ca2+-CTC fluorescence. Similar protective effect of Ca2+ against Na+ stress has also been reported (5). These results are also consistent with our earlier observations (2) where it was shown that addition of 20 mM CaCl2 to extracellular KCl prevented protoplasmic swelling.

Viability and Membrane-Associated Calcium. Although there was a large reduction in membrane-associated Ca2+ in freeze-injured cells, yet these cells showed a fluorochromatic reaction to FDA (a lipophilic fluorescence probe for cell viability). FDA, when hydrolyzed by cell esterases, gives off a polar product ‘fluorescein,’ which is retained by the cell membrane, gets accumulated, and fluoresces when hit by the excitation wavelength. This test has been used as an effective method for assessing viability (27). Hence, our results show that although, after experiencing freeze-thaw stress (irreversible, −11.5°C), cells have lost a significant amount of membrane calcium, they still are ‘viable’ immediately after thawing. Furthermore, injurious effects (protoplasmic swelling and cell death) of 50 mM KCl were either mitigated or prevented by the addition of CaCl2. These results can be interpreted as a selective loss of membrane calcium, which probably results in specific alteration in membrane properties. These alterations, our results suggest, play in part an important role in ultimate cell survival.

The fate of calcium that is lost from membrane following freeze-thaw stress is not clear. It could probably be lost into the cytoplasm or to the extracellular environment. Earlier observations on increased Ca2+ efflux following freeze-thaw injury (16) suggest that some of the cellular Ca2+ is lost to the extracellular water. In addition, some of the free calcium can probably be sequestered in certain compartments of the cell. A high Ca2+-CTC fluorescence signal from swollen protoplasm (irreversibly damaged cells) was observed (picture not shown), which indicates that some of the Ca2+ released from the cell membrane is sequestered in the hydrophobic part of cytoplasm. It is widely assumed that cytoplasmic calcium levels are very low in plant cells, suggesting a tight regulation of cytosolic calcium concentration (9, 25). It is also believed that changes in cytosolic calcium levels could lead to changes in cellular processes that eventually result in a physiological response (25, 26). Alteration in the function of plasma membrane-bound ATPases has long been proposed as a site of incipient freezing injury (17, 19). The present study suggests that this functional alteration in ATPases could be brought about by changes in cellular/membrane calcium. Recent reports (25) on the changes in activity of membrane-bound ATPases via either Ca2+ or Ca2+-calmodulin dependent protein kinases lend support to this hypothesis. Further studies are underway to understand the role of membrane-associated calcium in molecular mechanism of freezing injury.

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