Communication

Protoplasmic Swelling as a Symptom of Freezing Injury in Onion Bulb Cells

ITS SIMULATION IN EXTRACELLULAR KCI AND PREVENTION BY CALCIUM

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ABSTRACT

Freezing injury, in onion bulb tissue, is known to cause enhanced K⁺ efflux accompanied by a small but significant loss of Ca²⁺ following incipient freezing injury and swelling of protoplasm during the postthaw secondary injury. The protoplasmic swelling of the cell is thought to be caused by the passive influx of extracellular K⁺ into the cell followed by water uptake. Using outer epidermal layer of unfrozen onion bulb scales (Allium cepa L. cv Big Red), we were able to stimulate the irreversible freezing injury symptoms, by bathing epidermal cells in 50 millimolar KCI. These symptoms were prevented by adding 20 millimolar CaCl₂ to the extracellular KCI solution. Our results provide evidence that loss of cellular Ca²⁺ plays an important role in the initiation and the progression of freezing injury.

In nature, herbaceous plants are usually exposed to slow freezing which produces extracellular ice and subsequent cellular dehydration (6, 7, 16). After a slow thaw this freezing stress is known to result in an increased efflux of K⁺ and water soaking of tissue (14, 15). This increased K⁺ efflux was shown to be from the freeze injured, yet alive, onion bulb cells (14). Using these cells it was demonstrated that incipient freezing injury leads to specific alterations in the transport properties of the cell membranes, rather than membrane rupture (14, 15, 18). Depending upon the degree of freezing stress, the injury to onion bulb cells is either reversible or irreversible (15, 18). All the cells were viable (determined by vital staining, presence of protoplasmic streaming and ability to plasmolyze), immediately after thaw, when the onion bulb was frozen to -11°C (15). In these cells injury progressed with time during the postthaw period and the first microscopic alteration observed was protoplasmic swelling (18). In parallel with this symptom, a small but significant Ca²⁺ efflux along with increased K⁺ efflux was noted (14). Furthermore, cell viability was significantly enhanced by washing the irreversibly freeze injured cells in CaCl₂ solution (15). From these studies it was proposed that Ca²⁺ may play a role in the prevention of secondary injury following a freeze-thaw cycle (15, 17). However, the exact nature of this role is not yet understood.

In the present study, we demonstrate that the cellular symptom of secondary freezing injury could be produced by placing the onion bulb epidermal cells in hypotonic KCl solution. Our results show that these symptoms were prevented when calcium was added to extracellular KCl solution. The anthocyanin containing onion epidermal cells proved to be an ideal experimental material for this investigation.

MATERIALS AND METHODS

This study was conducted using bulb scale tissue of red onions (Allium cepa L. cv Big Red). The outer epidermal layer of these bulb scales contain anthocyanin in the vacuole thus making it possible to discern vacuole from cytoplasm.

K⁺ Efflux in the Presence and Absence of Extracellular Calcium. The third healthy scale (counting inwards from the outer-most fleshy scale) from the middle portion of the bulb was selected and cut into approximately 1 cm² pieces. The inner epidermal layer was peeled off to ensure better contact of the tissue with the bathing solution. Two weighed tissue pieces were transferred to 50 ml of various concentrations of CaCl₂ and NaCl solutions, contained in 250 ml flasks. The concentrations utilized were 25, 50, and 100 mM and distilled-deionized H₂O was used as control. The tissue pieces were then vacuum infiltrated to ensure better cell contact with the bathing solution and shaken for 1 h. The solution was then decanted and analyzed for K⁺ content using an atomic absorption spectrophotometer (Perkin-Elmer model 2380). The K⁺ efflux was expressed on a fresh weight basis.

Freeze-Thaw Studies with Onion Bulb Tissue. The protocol employed for freezing and thawing onion bulb was similar to that of Palta et al. (13). Onion bulb weighing about 245 g was frozen at the rate of about 1.5°C/h, to -11 ± 0.5°C, held at this temperature for 3 h and then thawed slowly over ice. This temperature was selected for present study because it causes irreversible damage to bulb cells (14, 15). Lethal damage to these cells occurs at about -20°C as observed immediately after thawing (13). A continuous record of the temperature was obtained using a copper-constantan thermocouple inserted into the middle of the bulb.

After thawing, the third and fourth scale tissue were visually examined to determine the extent of water soaking (13) and then used for microscopic examination and efflux studies. All of these studies were completed within 24 h after thawing. During this period the tissue was stored at 5 ± 0.5°C. From the third scale, transverse sections of the anthocyanin containing outer epidermal layer were made with a razor blade. These were then examined under a Bausch and Lomb light microscope equipped with a 35 mm camera. Ion efflux was measured by a conductivity

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method (14, 15). For this purpose two pieces (2 × 2 cm) of the scale tissue were vacuum infiltrated in 20 ml of deionized H2O and shaken for 1 h. The conductivity of the effusate was measured using a conductivity meter (YSI model 32). The conductivity was again recorded for the same tissue after it was heat-killed. For comparison, all these studies were conducted on unfrozen bulb scale tissue. All the conductivity measurements and microscopic observations were made at room temperature.

Simulation of Freezing Injury with Extracellular KCI and Its Response to Calcium. Transverse sections of the outer epidermal layer were made from the third healthy scale tissue in a manner already described. Some sections were bathed in 50 mM KCI solution while others were transferred to solutions that contained 50 mM KCl and concentrations of CaCl2 up to 20 mM. At various times after transfer, intact epidermal cells were examined under a light microscope for protoplasmic swelling and photographs were taken. Similar observations were made on sections transferred to either tap or distilled-deionized H2O which served as controls.

RESULTS

Effect of Extracellular CaCl2 and NaCl on K+ Efflux. In the unfrozen bulb tissue, the presence of extracellular CaCl2 up to 50 mM, resulted in a sharp decrease in the K+ efflux, when compared to distilled H2O control (Table I). As the concentration of extracellular CaCl2 was increased from 50 to 100 mM, the reduction in K+ efflux was relatively small. There was comparatively little effect of equivalent concentration of extracellular NaCl on K+ efflux (Table I). Maximum reduction in K+ efflux resulting from extracellular NaCl was 21.4% and it occurred at 50 mM. Thus, CaCl2 was about three times more effective than an equivalent concentration of NaCl in preventing K+ efflux.

Freeze-Thaw Studies. About 60% of the bulb scale tissue showed visual water soaking after going through a freeze-thaw cycle (Table II). Conductivity of the effusate, collected by shaking the tissue in distilled-deionized H2O, for the frozen and subsequently thawed scale tissue was 2.5 to 3 times greater than the control (Table II). Since freeze-injured bulb scale tissue did not recover when kept at 5°C for several days (tissue remained partially water soaked and flaccid), we conclude that the injury was irreversible. However, all these cells were able to plasmolyze and exhibited cytoplasmic streaming, when observed after thawing.

In outer epidermal control cells, bathed in tap water, no separation of plasma-membrane from tonoplast was visible either before or after plasmolysis of these cells (Fig. 1, a and b). Cytoplasmic streaming and an intact nucleus were clearly evident in such cells. However, in freeze-injured cells bathed in tap water, the tonoplast, outlining the pigmented vacuole (Fig. 1c, i), retracted from cell wall, revealing a distinct protoplasmic swelling (Fig. 1c, sp). In these cells the plasma-membrane (which presumably was still next to cell wall) was not discernible. The plasma-membrane in these cells became conspicuous when they were plasmolyzed in hypertonic mannitol solution (Fig. 1d, p.). In between the two cell membranes the swollen protoplasm was clearly evident (Fig. 1d). In the swollen protoplasm, a distinct nucleus and in some cells, anthocyanin containing vacuole could be seen (Fig. 1d). Protoplasmic swelling is a transient event that takes place during the progression of freezing injury to the stressed cells. At any given time only a certain proportion of the cells exhibit this cytological aberration. These cytological alterations were absent in unfrozen control cells (Fig. 1, a and b).

Stimulation of Freezing Injury with Extracellular K* and Its Prevention by Ca**. A distinct protoplasmic swelling, similar to the freeze injured cells, was noted within 1 h when cells were bathed in 50 mM KCl solution (Fig. 1e). Once again, this cytological alteration was found to be a transient event, exhibited by a proportion of cells (in field), at a given time. The time required for the appearance of this symptom was concentration-dependent. At lower concentrations of bathing solution, this swelling took a longer time to appear whereas at relatively higher but still hypotonic concentrations, when compared with the concentration of cell sap, it was apparent in less than 30 min. Total cell sap concentration was found to be about 350 mOs/mal. On plasmolysis, once again a clear separation of the two cell membranes, similar to that in freeze injured cells, was noted (Fig. 1f). These observations were repeated in equimolar extracellular KCl solution which also contained various concentrations of CaCl2 (Table III). The appearance of protoplasmic swelling in 50 mM KCl treatment was prevented only at relatively higher concentrations of CaCl2 (Table III). At 10 and 20 mM CaCl2, added to 50 mM KCl, no swelling was noted even after 3 h of exposure (Fig. 1g, Table III). No separation of two membranes was evident when these cells were plasmolyzed in 0.8 m mannitol (Fig. 1h). Lower concentrations of CaCl2 (0.1, 0.2 mM) added to KCl, did not prevent the swelling (Table III). However, the time required for the microscopic symptoms to appear was slightly longer when relatively higher but still low concentrations of CaCl2 (0.5 and 1.0 mM) were present in bathing medium (Table III). Thus, the time required for producing protoplasmic swelling, at a given concentration of KCl, was dependent on the concentration of extracellular CaCl2 in the bathing medium (Table III).

It is important to note that distilled-deionized H2O alone was not able to produce the protoplasmic swelling, indicating that extracellular KCl is required to induce these symptoms (Table III). In the presence of 20 mM CaCl2 in the bathing medium, the cells appeared as in both tap and distilled-deionized water con-

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**Table I. Effect of Extracellular CaCl2 and NaCl on K+ Efflux from Onion Bulb Scale Tissue**

<table>
<thead>
<tr>
<th>Extracellular Concentration of CaCl2 or NaCl (mM)</th>
<th>Relative Concentration of K* in the Extracellular Solution after 1 h of Shaking in the Presence of NaCl</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl2</td>
<td>CaCl2</td>
<td>100</td>
</tr>
<tr>
<td>NaCl</td>
<td>NaCl</td>
<td>100</td>
</tr>
<tr>
<td>0†</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>25</td>
<td>57.8 ± 6.1†</td>
<td>98.2 ± 2.1</td>
</tr>
<tr>
<td>50</td>
<td>37.5 ± 11.7†</td>
<td>78.5 ± 4.5</td>
</tr>
<tr>
<td>100</td>
<td>32.2 ± 2.3†</td>
<td>79.9 ± 4.5</td>
</tr>
</tbody>
</table>

* Distilled deionized H2O used as control (in distilled-deionized H2O about 20% of total K* leaked out). † Means of three replications ± SD.

**Table II. Ion Leakage (in Distilled Deionized H2O) and Visual Observation on Water Soaking of Freeze-Thaw Injured and Control Onion Tissue**

<table>
<thead>
<tr>
<th>Treatment Temperature</th>
<th>Bulb Tissue Used</th>
<th>Ion Leakage</th>
<th>Extent of Water Soaking (Visual Observation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>Bulb Tissue Used</td>
<td>Ion Leakage</td>
<td>% of killed tissue</td>
</tr>
<tr>
<td>-11</td>
<td>3rd Scale</td>
<td>49.2 ± 3.0†</td>
<td>60</td>
</tr>
<tr>
<td>+5</td>
<td>3rd Scale</td>
<td>20.1 ± 2.42</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4th Scale</td>
<td>15.2 ± 0.93</td>
<td>0</td>
</tr>
</tbody>
</table>

* Means ± st of three different measurements. † All the figures are within ± 5%.
CALCIUM AND FREEZING INJURY

FIG. 1. Photomicrographs of abaxial onion epidermal cells showing the protoplasmic swelling which is a symptom of freezing injury, simulation of the symptom in the presence of KCl and its prevention by Ca\(^{2+}\), a, control cells in tap water; b, plasmolysis of (a) in 0.8 M mannitol solution; c, freeze-injured cells just after thawing showing swollen protoplasm; d, plasmolysis of (c) in 0.8 M mannitol; e, simulation of protoplasmic swelling by bathing control cells in 50 mM KCl for 1 h (compare c with 3); f, plasmolysis of (e) in 0.8 M mannitol (compare d with f); g, control cells bathed in 50 mM KCl containing 20 mM CaCl\(_2\) showing absence of protoplasmic swelling; h, control cells bathed in 50 mM KCl containing 20 mM CaCl\(_2\) showing absence of protoplasmic swelling. Bar in a and c equals 50 \(\mu\)m.

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Table III. Time Course for the Appearance or Absence of Protoplasmic Swelling in Onion Bulb Epidermal Cells, when Subjected to Various Concentrations of Extracellular CaCl2 in the Presence of KCl

<table>
<thead>
<tr>
<th>Bathing Medium</th>
<th>Time of Exposure, at which Protoplasmic Swelling was First Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM KCl</td>
<td>min</td>
</tr>
<tr>
<td>+ 0.1 mM CaCl2</td>
<td>30</td>
</tr>
<tr>
<td>+ 0.2 mM CaCl2</td>
<td>30</td>
</tr>
<tr>
<td>+ 0.5 mM CaCl2</td>
<td>45</td>
</tr>
<tr>
<td>+ 1.0 mM CaCl2</td>
<td>45</td>
</tr>
<tr>
<td>+ 1.0 mM CaCl2</td>
<td>45</td>
</tr>
<tr>
<td>+ 20.0 mM CaCl2</td>
<td>Not seen in 3 h</td>
</tr>
</tbody>
</table>

*In a separate study, cells bathed in this treatment did not show protoplasmic swelling even after 24 h.*

The occurrence of protoplasmic swelling in the presence of extracellular KCl suggests for alterations in the properties of plasma-membrane, which are not manifested in the tonoplast. Thus, our results show that the two cell membranes respond differently, *in vivo*, to extracellular KCl.

The exact mechanism by which extracellular K*+* induces protoplasmic swelling is unknown. Palta and Li (18) explained this swelling in terms of the removal of membrane Ca*++* by extracellular K*+*, during the postthaw period. Recent studies (2) on the displacement of Ca*++* by Na*+* from the extracellular space of root cells and the prevention of protoplasmic swelling in onion cells by Ca*++* in 50 mM KCl solution, (Table III; Fig. 1, g and h), support this view. Loss of Ca*++* from plasma membrane was envisaged to result in passive influx of extracellular K*+*, followed by water uptake thus leading to protoplasmic swelling (18). Water movement into the cytoplasm can be explained in terms of water potential gradient that is created following K*+* influx into the cytoplasm. Under the present experimental conditions, at any given time, the water potential of the vacuole, the cytoplasm and cell wall microcapillaries, is identical. Due to relatively high extracellular K*+*, plasma membrane becomes leaky resulting in K*+* influx. This causes a decrease in the water potential of cytoplasm thus water moves in the cytoplasm to equilibrate the water potential. The end result will be the increase in the volume of protoplasm and retraction of tonoplast thus showing the protoplasmic swelling (Fig. 1, c and e).

Alteration in the K*+* transport properties of cells is known to result from incipient freezing injury (14, 15, 18). A small but significant loss of Ca*++* concomitant with K*+* leakage from freeze injured cells has also been observed (14, 15). This loss of Ca*++* during the early stages of freezing injury may cause membrane alteration that results in enhanced K*++* efflux and subsequent chain of events that lead to irreversible injury. The exact mechanism by which Ca*++* influences the K*+* transport (both influx and efflux) has yet to be elucidated. The possible suggested mechanisms could be membrane packing in the presence of Ca*++* (19) or occurrence of Ca*++* bridges between the polar head groups of membrane lipids (1, 3, 11). Another possible explanation could be an indirect effect of Ca*++* on the membrane ATPases involved in K*+* transport, resulting from the calcium-induced alterations of the membrane lipid environment (4).

**DISCUSSION**

The present study demonstrates that extracellular calcium is able to prevent the protoplasmic swelling, a symptom of irreversible freezing injury, in onion bulb scale cells. Our results show that the K*+* transport (efflux/influx) of these cells is markedly reduced by the extracellular Ca*++* both before and after the cell experiences freeze-thaw stress. Calcium was over three times more effective than equivalent concentration of Na*+* in reducing K*+* efflux from the bulb scale tissue (Table I). This is consistent with the general protective effect of Ca*++* on many cellular processes during stress (5, 8, 10, 12) and postulated role of Ca*++* on the maintenance of membrane integrity (9).

One of the consequences of incipient freezing injury in bulb scale cells is the alteration of K*+* transport properties of the cell membranes (18). Depending upon the degree of injury these alterations are either reversible or irreversible (18). One of the first microscopic symptoms in irreversibly injured onion cells has been shown to be protoplasmic swelling (18). Leakage of K*+* from the cells and its accumulation in the extracellular space is associated with freezing injury (14, 15). Furthermore, removal of this K*+* from extracellular solution was shown to halt the progress of injury during the postthaw period (15). From these observations it was proposed that protoplasmic swelling results from the perturbation of the cell membrane by high extracellular K*+* and passive influx of K*+* followed by water uptake. The present study supports this proposal since a protoplasmic swelling could be induced in un frozen cells when bathed in 50 mM extracellular KCl solution (Fig. 1c). Clearly, extracellular KCl was required to produce protoplasmic swelling and these symptoms were prevented in the presence of CaCl2 in the bathing medium (Fig. 1, g and h; Table III). These results suggest that the progress of injury due to freezing stress can be retarded by providing extracellular Ca*++*. This suggestion is supported by earlier studies by Palta et al. (15). It is important to note that this protective role of Ca*++* was not evident at relatively lower concentrations of CaCl2 (Table III).

**LITERATURE CITED**

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