Perturbation of Membrane Calcium as a Molecular Mechanism of Freezing Injury[1]

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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 Ca^{2+} (Palta *et al.*, 1977a; Palta *et al.*, 1977b). Based on these results Palta and Li (1978) proposed a hypothesis for possible sequence of events leading to cell death or recovery depending upon the initial injury (Palta and Li, 1978). According to this hypothesis, loss of cellular calcium was thought to play an important role in freezing injury. Recently we have presented evidence that loss of membrane/cellular Ca^{2+} occurs in the early stages of freezing injury in onion scale epidermal cells (Arora and Palta, 1988).

In the present study we document experimental results which provide evidence for the loss of cellular calcium in the early stages of freezing injury. We give a possible sequence of events explaining the role of calcium in freezing injury or recovery.

I. *In Vivo* Perturbation and Detection of Membrane-Associated Ca^{2+} following Freeze-Thaw Stress

Onion (*Allium cepa* L cv. Sweet Sandwich) bulbs were slowly frozen by cooling at the rate of 1.5°C/h to -11.5°C ± 0.5°C (irreversible damage) and then thawed slowly over ice (Arora and Palta, 1988). Inner epidermal cell layer from these bulb scales was used to investigate the perturbation and detect membrane-associated Ca^{2+} using CTC, (chlorotetracycline) a Ca^{2+} binding fluorescent probe. A bright fluorescence due to chelate complex of Ca^{2+}-CTC was observed in control unfrozen cells (Fig. 1a). Freeze injured cells, however, exhibited a significantly reduced fluorescence (Fig. 1c). In reversibly damaged cells (able to repair and

[1]Supported by the USDA Competitive Grant (Agreement No. 85-CRCR-1-1673) and by the College of Agricultural and Life Sciences, University of

recover completely) the fluorescence intensity was in-between those of control and reversibly injured cells (Arora and Palta, 1988). Both control and freeze-injured cells exhibited fluorochromatic reaction with FDA (fluorescein diacetate), right after thawing (Fig. 1b & d). Our results indicate that freeze-thaw stress causes a selective perturbation of cell membranes. One of these perturbation, our results show, is the loss of membrane associated calcium in onion cells. This loss was more severe with increase in freezing stress. When this loss of Ca^{2+} reaches beyond a threshold the cells experience an irreversibly injury (Arora and Palta, 1988).

II. Enhanced K^+ Leakage in Freeze-Injured Onion Scale Tissue and Its Mitigation by Extracellular Ca^{2+}

Onion bulb was frozen to -11.0°C ± 0.5°C and was slowly thawed over ice. Two pieces (1cm x 1cm) of onion scale tissue were shaken in 20 ml distilled-deionized H_2O or 20 mM $CaCl_2$ over a period of 5 h. K^+ was measured in the effusate by atomic absorption spectrophotometer and K^+ leakage rate was calculated. Freeze-thaw injured scale tissue showed an enhanced K^+ leakage rate to more than 3 times of control (Table I). Both in control and freeze-injured tissue, presence of extracellular $CaCl_2$ reduced the leakage by 50 percent (Table I). There is ample evidence in the literature supporting a protective role of Ca^{2+} in the membrane (Hepler and Wayne, 1985). Reduced ion leakage in the presence of extracellular Ca^{2+} has been suggested to be due to blockage of aqueous channels in the membrane through which hydrated ions can leak out (Poovaiah and Leopold, 1976), or formation of Ca^{2+} bridges between polar head groups of membrane lipids (Geerard and Humphreys, 1967). It is thus possible that extracellular Ca^{2+} reduces K^+ leakage from control cells via one of these mechanisms. However, in freeze-injured scale tissue, enhanced K^+ leakage (more than three times that of control) was noticed. This, has been explained, in part due to the inactivation of active transport system in the membrane (Palta *et al.*, 1977a; Palta *et al.*, 1977b; Palta and Li 1978). A sharp reduction in the K^+ leakage from freeze-injured cells in the presence of extracellular Ca^{2+} could be due to its direct effect on stabilizing of membrane lipid or indirect effect on activation of H^+-ATPase.

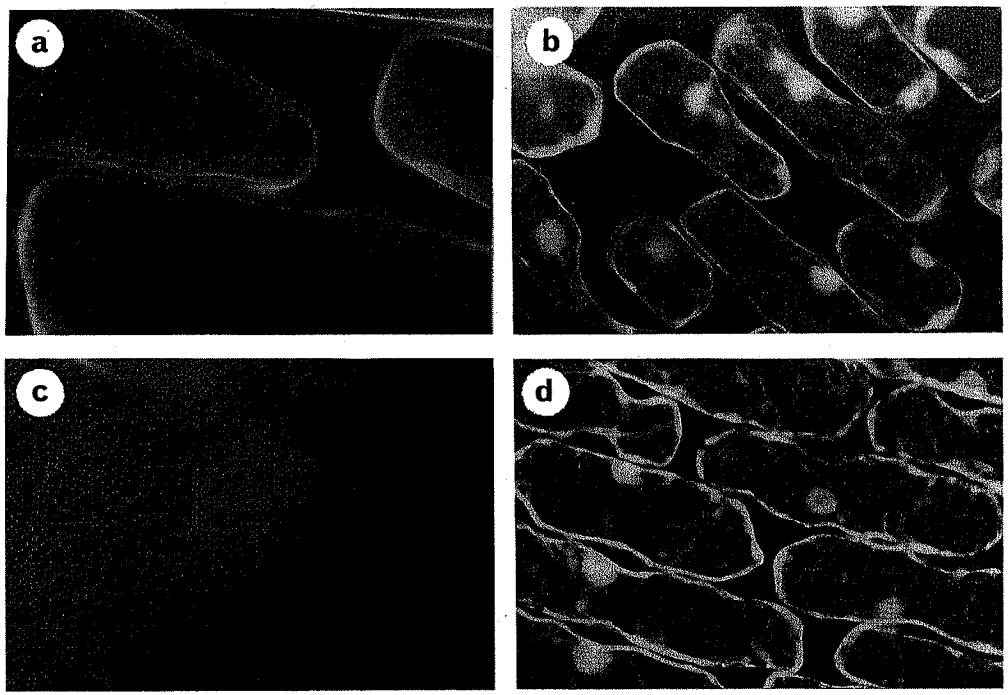


Figure 1. Photomicrographs of adaxial epidermal cells of onion bulb (both unfrozen control and freeze-thaw stressed), treated with CTC and FDA solutions (CTC solution contained 0.2 mM $CaCl_2$, 20 M CTC and 10 mM Tris-HCL buffer, pH 6.8. Cells were subjected to this solution for 30 minutes, quickly rinsed, and then plasmolysed in 0.8M mannitol. FDA staining solution was prepared by diluting 50 1 of stock (50 mg/10 ml in acetone) in 5 ml of 2.5 mM Tris-HCl buffer pH 6.0. Cells were bathed in this solution for 5 minutes, quickly rinsed and then plasmolysed in 0.8M mannitol). a) control cells exhibiting Ca^{2+}-CTC fluorescence; b) control cells showing fluorescence from FDA staining; c) free-thaw stressed (-11.5°C) cells showing Ca^{2+}-CTC fluorescence; d) freeze-thaw stressed cells showing fluorescence from FDA staining, right after thawing. Magnification for a and c was X 500 and b and d was X 235.

III. Pretreatment with Extracellular Ca^{2+} Enhances Tolerance to Freeze-Thaw Stress

Weighed pieces of onion scale tissue were pretreated with either $CaCl_2$ or EGTA for 2 h and then subjected to varying degrees of freeze-stress. After thawing, tissue was examined for cell viability using a TTC (Triphenyl tetrazolium chloride) reduction test (Palta *et al.*, 1981). Our results show that 20 mM $CaCl_2$ pretreatment increased the tolerance of onion tissue

Table I. Effect of Extracellular $CaCl_2$ on K^+ Efflux Rate in Control and Freeze-Injured Onion Scale Tissue.

Treatment	K^+Efflux/h(Calculated as % of total cellular K^+) in the presence of	
	Distilled-deionized H_2O	20 mM $CaCl_2$
Control (unfrozen)	14.8 ± 1.6[a]	8.1 ± 0.3
(-11.0°C) Frozen	47.1 ± 5.0	24.6 ± 2.3

[a] Mean of three replications ± S.E.

to freeze-thaw stress (of -3°C) by two-fold (Table II). Pretreated tissue with EGTA, however, (which is known to remove Ca^{2+} from outer surface of plasma membrane and also from cell wall) suffered greater injury by a freeze-thaw stress (of-3°C) compared to control (Table II). Decreased injury by pretreatment of $CaCl_2$ was also supported by visual observations on water soaking (data not presented). This protective effect of $CaCl_2$ in freezing injury is in accordance with findings of Pomeroy and Andrews (Pomeroy and Andrews, 1985). These authors showed that Ca^{2+} treatment resulted in less ion efflux and enhanced survival after ice encasement of winter wheat cell suspension. The effect of Ca^{2+} on cell survival is thought to be specific since it is not elicited by either Mg^{2+} or La^{3+} (Pomeroy and Andrews, 1985). We have also found that freeze-thaw injured onion scale tissue was able to recover turgor when washed in 20 mM $CaCl_2$ (Arora and Palta, 1986a).

IV. Protoplasmic Swelling: A Symptom of Freezing Injury

Red onion bulbs (*Allium cepa* L. cv. Big Red) were frozen to -11.5°C ± 0.5°C (irreversible injury) and thawed over ice (Arora and Palta, 1986). Immediately after thawing transverse sections of anthocyanin containing outer epidermal cells were observed under microscope. Freeze-thaw injured cells, exhibited a distinct swollen protoplasm which could be easily observed upon plasmolysis (Fig. 2a). This cellular aberration was not evident in unfrozen control cells (Fig. 2b). Similar cellular alteration can be simulated by bathing these cells in 50 mM KCl for an hour (Arora

Table II. Effect of $CaCl_2$ and a Two Hour EGTA Pretreatment on Cell Viability of Onion Scale Tissue After Freeze-Thaw Stress. Cell Viability was assessed by TTC reduction method (Palta *et al.*, 1981).

Treatment	TTC reduction (%) when pretreated with		
	0.2mM $CaCl_2$	20 mM $CaCl_2$	1 mM EGTA
Control (unfrozen)	100	100	100
-3°C	37.6 ± 4.2[a]	79.5 ± 8.3	28.6 ± 2.7
-5°C	11.6 ± 1.3	24.2 ± 3.1	13.5 ± 1.6
-7°C	8.0 ± 0.9	6.3 ± 0.5	10.0 ± 0.8

[a] Mean of 6 replications ± S.E.

and Palta, 1986b). However, such symptoms are not evident in the cells which were bathed in a solution of 50 mM KCl that contained 20 mM $CaCl_2$ (Arora and Palta, 1986b). Protoplasmic swelling was first reported to occur in cells plasmolysed in a hypertonic solution of an alkali salt (Hofler 1940; Stadelmann, 1966). The cells exhibit normal plasmolysis at first but in a short time the protoplasmic swelling follows. This increase in protoplasmic volume (50 to 100 times of original) was described by Hofler (1940) as "Cap Plasmolysis". In this condition, the cell eventually dies. In these studies an inhibition of cap plasmolysis was found in the presence of Ca^{2+}. We hypothesize that during the post-thaw period, a high extracellular K^+ accumulates in the extracellular solution as a result of K^+ leakage caused by a secondary injury of these cells by removing membrane-associated Ca^{2+}. A detailed account of these results supporting this hypothesis is presented elsewhere (Arora and Palta, 1988).

A Possible Role of Cytosolic and Membrane Calcium in Freezing Injury

The level of cytosolic Ca^{2+} is kept low (10^{-6}M to 10^{-8}M)in both plants and animal cells alike. In this case, the change in cytosolic Ca^{2+} concentrations is considered to be the primary event in triggering the cellular response. It is believed that primary signals in plants (hormones, light and gravity) alter cytosolic Ca^{2+} levels. Evidence is

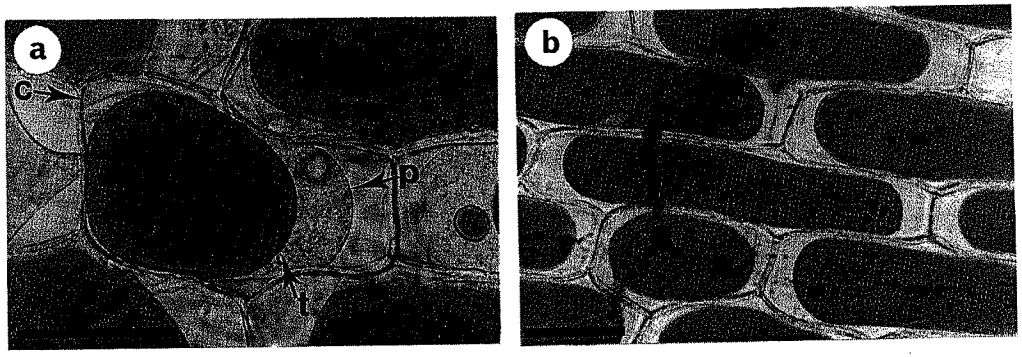


Figure 2. Photomicrograph of abaxial onion epidermal cell showing protoplasmic swelling, a symptom of irreversible freezing injury: a) freeze-injured cells plasmolysed in 0.8 M mannitol, showing swollen protoplasm (note separation of plasma membrane and tonoplast with protoplasm in between); b) control cells (unfrozen) plasmolysed in 0.8M mannitol. C: cell wall; p: plasma membrane; t: tonoplast. Bar in a and b equals 50 m (Source: Arora and Palta, 1986).

accumulating for a role of phosphoinositides in altering cytosolic Ca^{2+} levels (Poovaiah and Reddy, 1986). Extracellular signals stimulate the hydrolysis of phosphatidyl inositol 4,5 bisphosphate (PIP_2), an inositol phospholipid in inner leaflets of plasma membrane resulting in production of diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP_3), both of which have been shown to play roles in signal transduction (Poovaiah and Reddy 1987). IP_3 has been shown by several investigators to mobilize intracellular Ca^{2+}. This free cytosolic Ca^{2+}, then, can activate Ca^{2+} and/or Ca^{2+}-calmodulin-dependent protein kinase, that results into phosphorylation of cellular proteins and physiological response (Fig. 3).

In plants, several enzymes such as Ca^{2+}-ATPase (Dieter and Marme, 1981), H^+-ATPase (Zocchi *et al.*, 1983), phospholipase (Youpa *et al.*, 1986) and some others have been shown to be regulated by cytosolic concentration of Ca^{2+}. Based on such supportive evidence and our results we propose a possible sequence of events that may lead to the recovery or irreversible injury after a freeze-thaw stress (Fig. 4). It has been shown by Palta *et al.* (1977a; 1977b; 1981) that after a reversible freeze-thaw injury, water soaking disappears from the extracellular spaces of onion scale tissue. This will only follow the re-entry of effluxed ions (K^+) into the cell.

It is therefore conceivable that after a moderate freeze-thaw injury (Ca^{2+} loss below a threshold) membrane bound H^+-ATPase may get activated (via some mechanism) and thus lead to recovery (Fig. 4). On the other hand, a severe injury (Ca^{2+} loss more than threshold) may cause inhibition of H^+-ATPase and/or stimulation of phospholipase either directly by weakening the membrane structure or by the cascade of reactions described in Fig. 3. These alterations could result in irreversible injury (Fig. 4). Further experiments are needed to systematically document these events in freezing injury.

Figure 3. A Scheme for Ca^{2+} as a Second Messenger (for details see Pomeroy and Andrews, 1985)

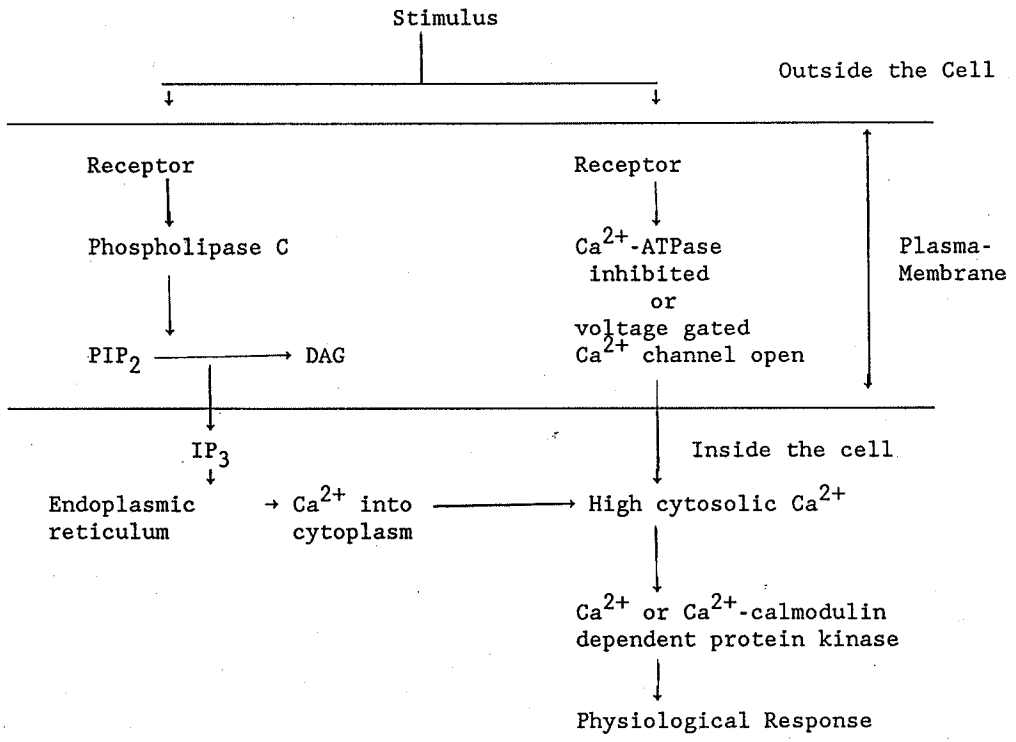
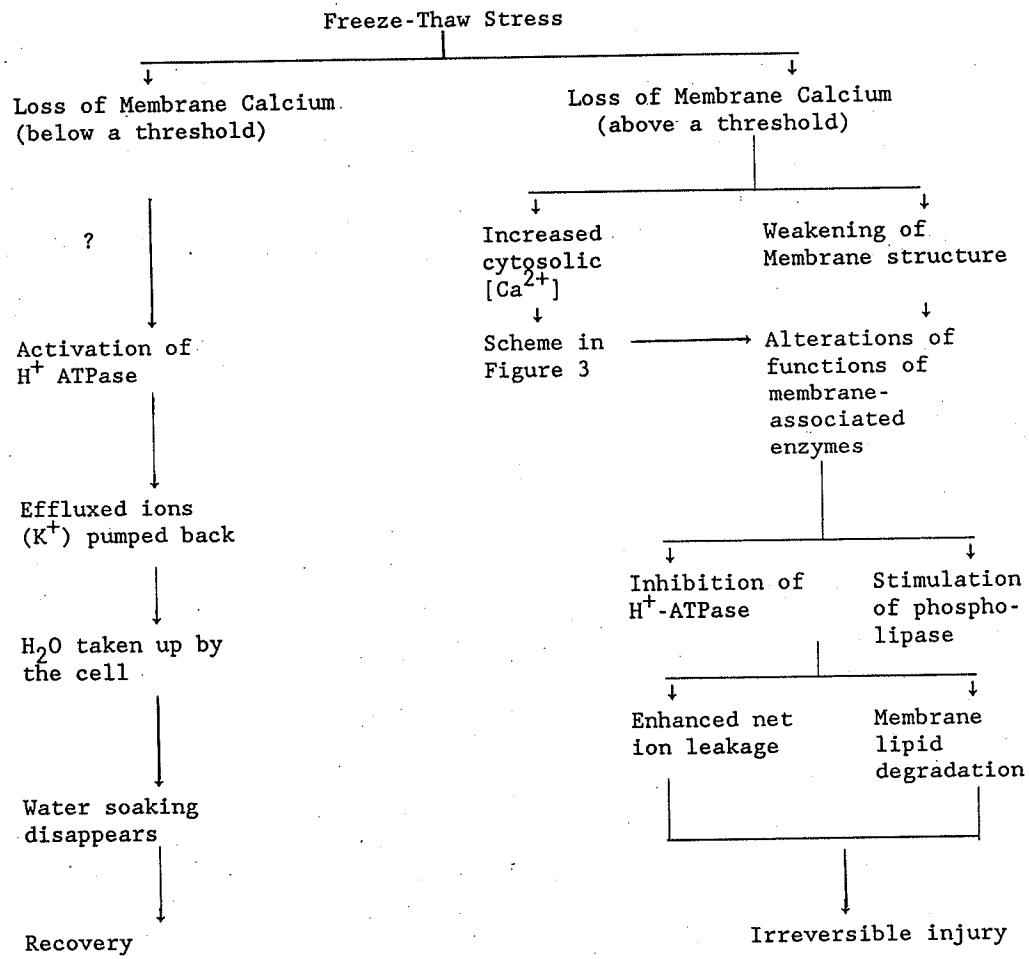


Figure 4. Possible Sequence of Events Involving Membrane Calcium Leading to Cell Recovery or Irreversible Injury After Freeze-Thaw Stress.



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