Plant Physiol. (1989) 90, 1271–1274
0032-0889/89/90/1271/04/$01.00/0

Received for publication March 13, 1989
and in revised form May 8, 1989

Communication

Salinity Stress Increases Cytoplasmic Ca Activity in Maize Root Protoplasts[1]

Jonathan Lynch[2], Vito S. Polito*, and André Läuchli

Department of Land, Air, and Water Resources (J.L., A.L.) *and Department of Pomology* (V.S.P.), *University of California, Davis, California 95616*

ABSTRACT

High concentrations of NaCl immediately elevated cytoplasmic Ca activity in maize (*Zea Mays* L. cv Pioneer 3377) root protoplasts, as measured with the fluorescent probe Indo-1. The effect of salinity was inhibited by Li pretreatment but restored by inositol, suggesting that phosphoinositides mediate the stress response.

We recently provided indirect evidence (14) that salinity stress disturbs intracellular Ca in maize (*Zea mays* L. cv Pioneer 3377) root protoplasts. We proposed that salinity initiated phosphoinositide-mediated release of Ca from intracellular pools, leading to elevated cytoplasmic Ca activity, which may have adaptive significance in salinity injury and salinity resistance. In this study our objective was to measure directly the effect of salinity on cytoplasmic Ca activity in maize root protoplasts using the fluorescent Ca probe, Indo-1.

MATERIALS AND METHODS

Protoplast Isolation

Protoplasts were isolated from the cortex of the primary roots of maize (*Zea mays* L. cv Pioneer 3377) seedlings as described previously (8, 14, 15).

Indo-1 Loading

Protoplasts were loaded with Indo-1 using the 'acid-loading' procedure of Bush and Jones (2). Protoplasts were transferred from the suspension medium to a loading medium of similar composition but buffered at pH 4.5 by 5 mM dimethylglutarate and supplemented with 20 mM galactose. Protoplasts were washed twice in loading medium, diluted to less than 10^5 mL^{-1}, and Indo-1 was added to a final concentration of 25 μM. After 2 h incubation, the protoplasts were washed in treatment solution without Indo-1 and were observed immediately.

Calcium Modulation

Ca-buffered suspension media were prepared in the range from 30 to 1000 nM Ca using Ca-EGTA buffers (17). Protoplasts were loaded with Indo-1 for 2 h, washed twice in Ca-buffered medium, and the nonfluorescent calcium ionophore, Br-A23187, was added to a final concentration of 10 μM. After 10 min in Br-A23187, the protoplasts were observed.

Treatment Application

Protoplasts were transferred from loading medium into suspension medium supplemented with NaCl immediately after the 2 h incubation in Indo-1. Li pretreatment was applied as LiCl to a final concentration of 10 mM 30 min prior to NaCl treatment. Inositol was applied to a final concentration of 20 mM 30 min after Li pretreatment and 30 min before NaCl treatment.

Microscope Fluorometry

A microscope fluorometer, as described (20), was used to quantify fluorescence intensity. Healthy protoplasts were identified using transmitted light optics on the basis of general cell morphology and the presence of vigorous streaming in the cytoplasmic strands. Excitation energy at 365 nm was isolated from a voltage-stabilized, 100 W D.C. mercury lamp with glass filters. Fluorescence emission filters at 405 and 480 nm were isolated with interference filters (Ditric Optical Co., Hudson, MA) having nominal bandwidths of 9.2 and 7.1 nm, respectively. Readings of fluorescence intensity for individual cells were taken at both wavelengths using a Zeiss Neofluar 63/1.25 objective and a Hamamatsu R-928 photomultiplier. A fast shutter in the excitation light path was used to limit exposure times to 25 ms, thereby minimizing fluorescence fading induced by prolonged exposure to the excitation source. Results were corrected for autofluorescence intensity values determined from nonloaded cells. Data are expressed as the ratio of the fluorescence signals at 405 and 480 nm which increases with increasing Ca activity (9).

RESULTS AND DISCUSSION

The loading technique produced a strong fluorescence signal that, in healthy cells, was clearly restricted to the cytoplasm without leakage of the probe into the vacuole (Fig. 1).

[1] Supported in part by a grant from the National Science Foundation (DCB-8514451) to V. S. P.

[2] Present address: Centro Internacional de Agricultura Tropical (CIAT), AA 6713, Cali, Colombia.

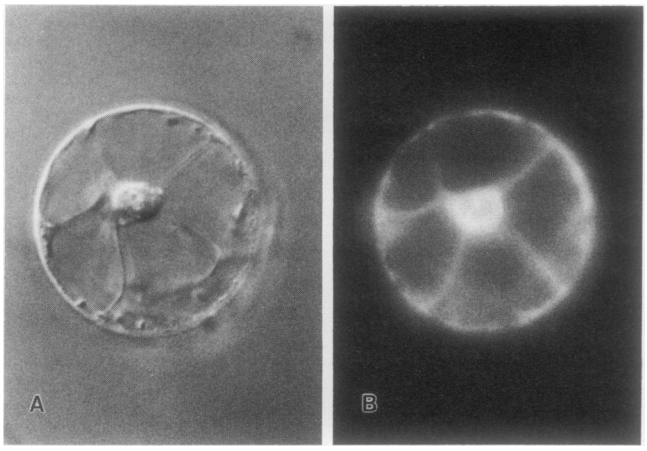

Figure 1. Maize root protoplast loaded with the fluorescent intracellular calcium probe, Indo-1. The same cell is seen here as a differential interference contrast (A) and fluorescence (B) image. Note that fluorescence is localized entirely in the cytoplasm with no fluorescence from the vacuole.

Active cytoplasmic streaming was evident in healthy appearing cells, indicating no toxicity was associated with the low pH loading solution.

Successful Indo-1 loading was dependent upon several factors. By 3 h after protoplast isolation, Calcofluor White ST fluorescent staining indicated that cell wall regeneration had begun. Attempts to load Indo-1 in pH 4.5 suspension medium beyond this time were unsuccessful because the Indo-1 fluorescence signal became localized in association with newly regenerated cell wall materials at the cell surface. The inclusion of galactose (18) in the loading medium inhibited cell wall biosynthesis (again, as indicated by Calcofluor White ST staining) sufficiently to permit the use of older protoplasts. Cell debris, when present in the protoplast preparation, became brightly fluorescent, as did obviously injured or unhealthy cells. These factors led to erratic loading in preparations that contained significant cellular debris. Erratic loading was also obtained when protoplast density exceeded $10^5 mL^{-1}$, perhaps because of dye accumulation by debris and injured cells, typically present as a small but significant fraction of all protoplast preparations.

In choosing protoplasts for fluorescent measurement, we eliminated protoplasts damaged during protoplast isolation by selecting those with cytoplasmic streaming and cytomorphology typical of healthy protoplasts (*i.e.* having stranded rather than vesiculate cytoplasm). Because elevated cytoplasmic Ca activity can inhibit streaming and disrupt cell morphology (11, 19, 20), it is possible that this precaution eliminated protoplasts in which NaCl treatment lead to a rise in cytoplasmic Ca sufficient to produce vesiculate or non-streaming protoplasts. Two observations argue against this possibility. First, we observed no difference in the percentage of protoplasts with vigorous cytoplasmic streaming following NaCl treatment; vigorous streaming was observed in 73.3% of the protoplasts in isolation buffer medium and in 76.1% of the protoplasts in medium containing high concentrations of NaCl. Second, fluorescence ratios of individual protoplasts within a given NaCl treatment were distributed normally, whereas a truncated distribution would be expected at high NaCl if protoplasts with high cytoplasmic Ca activity were being disregarded.

In order to determine if the Indo-1 fluorescence ratio was responsive to changes in intracellular Ca activity, protoplasts in various Ca buffers were made permeable to Ca with the ionophore Br-A23187 prior to fluorescence measurement. For external Ca activities ranging from approximately 30 to 1000 nM, the fluorescence ratio for Indo-1-loaded, Ca-permeable protoplasts increased linearly with the log of external Ca activity (Fig. 2). Using the data obtained from Ca-permeable protoplasts as an approximate calibration curve (although intracellular buffering of Ca activity makes this a tenuous approximation), the mean fluorescence ratio of Indo-1-loaded protoplasts in suspension medium without ionophore (Fig. 3) corresponded to that of Ca-permeable protoplasts equilibrated at an external Ca activity of 93 nM, which is in general agreement with estimates of cytoplasmic Ca activity in other eukaryotic cells (4, 7).

High concentrations of NaCl significantly increased the Indo-1 fluorescence ratio (Fig. 3), indicating a rise in cytoplasmic Ca activity. NaCl concentrations up to 90 mM had little effect on the Indo-1 fluorescence ratio, but a sharp increase occurred between 90 and 120 mM NaCl. Between

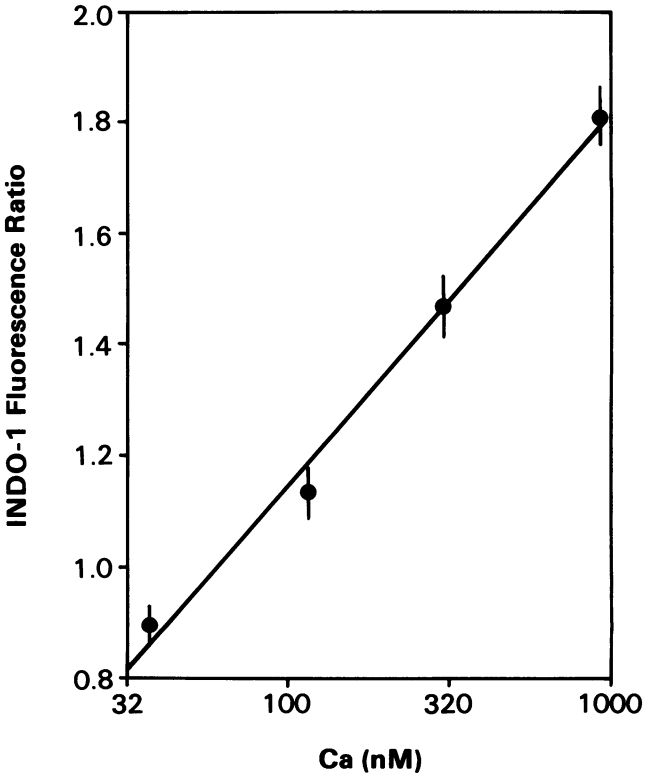

Figure 2. Indo-1 fluorescence ratios (405/480 nm) of individual maize root protoplasts made permeable to Ca by the calcium ionophore BR-A23187. Values on the abscissa represent Ca activity of Ca-EGTA buffered media. Exact media Ca activities used were 37, 115, 301, and 929 nM Ca. Each point is the mean of from 20 to 24 individual protoplasts from three separate protoplast preparations, ± SE. The r^2 for the regression of fluorescence ratio on extracellular Ca activity is 0.991, significant at $P = 0.0001$.

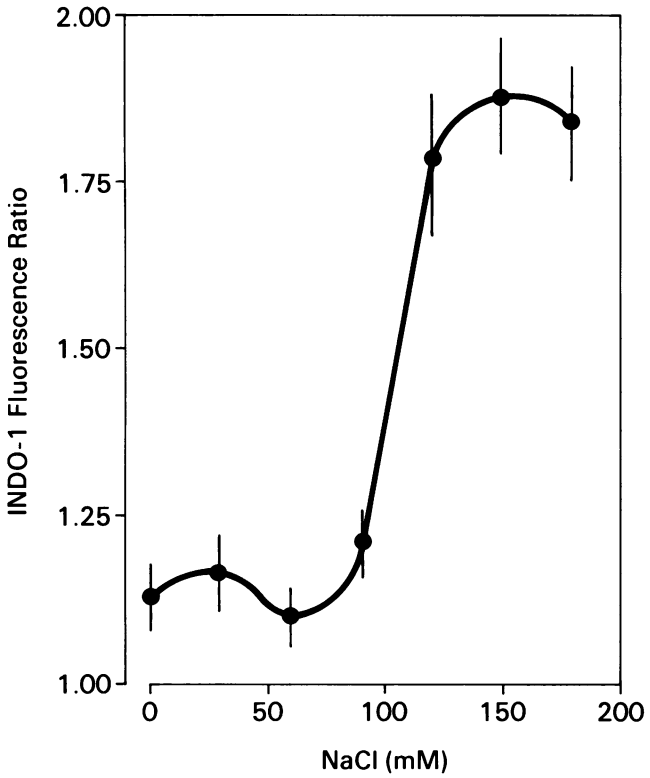

Figure 3. Response of Indo-1 fluorescence ratios (405/480 nm) of individual maize root protoplasts to media NaCl concentration. Each point is the mean of from 24 to 30 individual protoplasts from three separate protoplast preparations, ±SE. Fluorescence was measured within 10 min following medium salinization. ANOVA indicates that the effect of NaCl on Indo-1 fluorescence ratios was significant at P = 0.0001.

120 and 180 mM NaCl no further significant increases were observed. This pattern is indicative of a discrete stress signal activated by a specific level of stress perception. The mean Indo-1 fluorescence ratio for protoplasts treated with 150 mM NaCl corresponds to that of Ca-permeable protoplasts equilibrated at an external Ca activity of 1260 nM. This correspondence does not, however, imply that intracellular Ca activity had risen this high. Intracellular Ca buffering and Ca-pump activity can modulate Ca activity in the ionophore-permeabilized cells, so it is not certain that intracellular Ca activity equilibrates with that of the external medium, particularly at the extremes. Also, at this level one would expect a complete cessation of cytoplasmic streaming (11, 20) which did not occur. Thus, while it is evident that intracellular Ca activity does increase sharply in response to Na, the precise extent of this rise is not clear from these experiments.

Li inhibits inositol scavenging needed for regeneration of phosphoinositides that regulate Ca release from intracellular pools (1, 5). Li pretreatment reduced the impact of NaCl on the Indo-1 fluorescence ratio (Table I). As expected, subsequent inositol eliminates the Li effect (Table I). This evidence is consistent with, but not direct evidence for, the hypothesis that NaCl increases cytoplasmic Ca activity by activating the phosphoinositide regulatory system. Direct measurement of phosphoinositide dynamics is required to establish this relationship firmly.

Table I. *Interaction of Li and Inositol Pretreatment with the NaCl Effect on the Indo-1 Fluorescence Ratio (405/480 nm) of Maize Root Protoplasts*

Li pretreatment was applied as LiCl at 10 mM 30 min prior to NaCl application. Inositol pretreatment was at 20 mM 30 min after Li pretreatment and 30 min before NaCl treatment. Each value is the mean of from 8 to 11 individual protoplasts. ANOVA indicated that the interaction of Li, inositol, and NaCl effects on the Indo-1 fluorescence ratio was significant at P = 0.0074.

Pretreatment	Indo-1 Fluorescence Ratio (405/480 nm) NaCl Treatment		
	0	150	
	mM		%
None	1.094	1.759	161
Li	1.143	1.389	122
Inositol	1.083	1.747	161
Li + inositol	1.081	1.828	169
LSD (0.05)		0.177	

It is not clear to what extent our results apply to walled cells. The stringent osmotic requirements of protoplasts preclude consideration of the osmotic component of salinity stress. However, the present results are consistent with analysis of root hair cells in intact seedlings that show calcium homeostasis is disrupted by salinity treatment (6). In addition, evidence from intact organs and cell culture systems (20) indicate that at least one other environmental stress factor, chilling, may have similar effects. Furthermore, the low cytoplasmic Ca activity of unstressed protoplasts and the responsiveness of cytoplasmic Ca to NaCl stress suggest that the basic features of the Ca regulatory system are functional in protoplasts.

Our finding that salinity stress increased cytoplasmic Ca activity, perhaps by activating the phosphoinositide system, substantiates our previous model based on indirect evidence (14). There is extensive direct and indirect evidence that elevated cytoplasmic Ca activity in plant cells is associated with a variety of metabolic and developmental phenomena (3, 10, 11, 13). It is likely that increased cytoplasmic Ca activity following exposure to salinity stress is instrumental in determining metabolic responses to salinity, although it is presently unclear which of these consequences would be adaptive and which injurious. In the short term, elevated cytoplasmic Ca activity and activation of the phosphoinositide system may be stress signals that trigger useful metabolic changes such as synthesis of novel proteins (12), or adjustment of biosynthetic activities (13). Longer-term perturbations of intracellular Ca dynamics may be injurious (16).

ACKNOWLEDGMENTS

We thank Dr. D. S. Bush for his generous advice and assistance in loading protoplasts with Indo-1, Dr. J. M. Labavitch for advice on inhibiting cell wall regeneration, and both for their critical reading of the manuscript.

LITERATURE CITED

1. **Berridge MJ, Irvine RF** (1984) Inositol triphosphate, a novel second messenger in cellular signal transduction. Nature **312:** 315–321
2. **Bush DS, Jones RL** (1987) Measurement of cytoplasmic calcium in aleurone protoplasts using Indo-1 and fura-2. Cell Calcium **8:** 455–472
3. **Bush DS, Jones RL** (1988) Cytoplasmic calcium and α-amylase secretion from barley aleurone protoplasts. Eur J Cell Biol **46:** 466–469
4. **Carafoli E** (1987) Intracellular calcium homeostasis. Annu Rev Biochem **56:** 395–433
5. **Conrad PA, Hepler PK** (1986) The PI cycle and cytokinin-induced bud formation in the moss *Funaria* (abstract No. 315). Plant Physiol **80:** S-60
6. **Cramer GR, Läuchli A, Polito VS** (1985) Displacement of Ca by Na^+ from the plasmalemma of root cells. A primary response to salt stress? Plant Physiol **79:** 207–211
7. **Gilroy S, Hughes WA, Trewavas AJ** (1986) The measurement of intracellular calcium levels in protoplasts from higher plant cells. FEBS Lett **199:** 217–221
8. **Gronwald JW, Leonard RT** (1982) Isolation and transport properties of protoplasts from cortical cells of corn roots. Plant Physiol **70:** 1391–1395
9. **Grynkiewicz G, Poenie M, Tsien RY** (1985) A new generation of CA^{2+} indicators with greatly improved fluorescence properties. J Biol Chem **260:** 3440–3450
10. **Hanson JB** (1984) The function of calcium in plant nutrition. *In* PB Tinker, A Läuchli, eds, Advances in Plant Nutrition, Vol 1. Praeger, New York, pp 149–208
11. **Hepler PK, Wayne RO** (1985) Calcium and plant development. Annu Rev Plant Physiol **36:** 397–439
12. **Hurkman WJ, Tanaka CK** (1987) The effects of salt on the pattern of protein synthesis in barley roots. Plant Physiol **83:** 517–524
13. **Kauss H** (1987) Some aspects of calcium-dependent regulation in plant metabolism. Annu Rev Plant Physiol **38:** 47–72
14. **Lynch J, Läuchli A** (1988) Salinity affects intracellular Ca in corn root protoplasts. Plant Physiol **87:** 351–356
15. **Lynch J, Cramer GR, Läuchli A** (1987) Salinity reduces membrane-associated calcium in corn root protoplasts. Plant Physiol **83:** 390–394
16. **Minorsky PV** (1985) An heuristic hypothesis of chilling injury in plants: a role for calcium as the primary physiological transducer of injury. Plant Cell Environ **8:** 75–94
17. **Perrin DD, Sayce IG** (1967) Computer calculations of equilibrium concentrations in mixtures of metal ions and complexing species. Talanta **14:** 833–842
18. **Yamamoto R, Masuda Y** (1984) Auxin-induced modifications of cell wall polysaccharides in oat coleoptile segments. Effects of galactose. *In* WM Dugger, S Bartnicki-Garcia, eds, Structure, Function, and Biosynthesis of Plant Cell Walls. American Society of Plant Physiologists, Rockville, MD, pp 284–301
19. **Williamson RE, Ashley CC** (1982) Free Ca and cytoplasmic streaming in the alga *Chara*. Nature **296:** 647–651
20. **Woods CM, Polito VS, Reid MS** (1984) Response to chilling stress in plant cells. II. Redistribution of intracellular calcium. Protoplasma **121:** 17–24