INVOLVEMENT OF CALCIUM IN THE NACL–INDUCED UP-REGULATION OF ANTIOXIDANT ENZYME ACTIVITY IN COTTON CALLUS

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Abstract

Numerous investigations have shown that elevated levels of NaCl induce an increase in the activities of several antioxidant enzymes in cotton callus. The signal transduction pathway responsible for this increase is currently under investigation in our laboratory. A series of experiments was carried out to determine if calcium is involved in the NaCl induced up-regulation of antioxidant enzymes in salt-tolerant callus derived from the cotton cultivar Coker 312. The activities of four antioxidant enzymes, was measured in callus exposed to three calcium channel blockers, (Lanthanum Chloride, ruthenium red and TMB-8), and one calcium ionophore (A23187). Following a 2 hour pre-treatment with one of the Ca^{2+} channel blockers or A23187, antioxidant enzyme activity was measured over an 8-hour (ruthenium red and lanthanum chloride) or 2-hour (TMB-8 and A23187) periods in the absence (control) or presence of 250mM NaCl (salt stressed). The three calcium channel blockers, abolished the NaCl induced increases in the activities of glutathione reductase, catalase, peroxidase and ascorbate peroxidase. However, the Ca^{2+} ionophore, A23187 induced an increase in the activities of glutathione reductase, peroxidase and ascorbate peroxidase but not catalase. In addition, an increase in the activity of all four enzymes was observed in the callus under NaCl stress in the presence of TMB-8 and A23187. With control callus, the TMB-8 and A23187 combination induced an up-regulation of peroxidase, glutathione reductase and ascorbate peroxidase activites but no up-regulation of catalase was observed under these conditions. The results of these experiments supports the hypothesis that calcium is most likely involved as a secondary messenger in the NaCl-induced signal transduction pathway.

Introduction

It has been inferred from numerous studies the that NaCl stress contributes to oxidative stress in whole plants and callus tissue and that this stress leads to an up-regulation in the activity of antioxidant enzyme activities. (Gossett, *et al*., 1994a,b,c; Gossett, *et al*., 1996; Hernandez, *et al*., 1993). Recent results have demonstrated that in cotton callus, NaCl stress does result in the production of superoxide (Banks *et al.,* 2000, Bellaire *et al.,* 2000) which induces an up-regulation in the activity of the antioxidant enzymes, glutathione reductase (GR), ascorbate peroxidase (APX), catalase, glutathione S-transferase (GST) peroxidase and superoxide dismutase (SOD), (Gossett *et al*. 1994a, 1994b, 1996) and that these increases are transcriptionally regulated, (Banks *et al.,* 1998, Manchandia *et al.,* 1999). In addition, results from experiments using a terpene synthesis inhibitor, fluridone, have revealed that superoxide and H_2O_2 may signal the up-regulation of antioxidant enzyme activity through abscisic acid-dependant and abscisic acid-independent pathways (Bellaire *et al.,* 2000). Indeed, contributions from several investigators draw attention to the fact that abscisic acid, (Leung and Geraudat 1998), superoxide, (Doke *et al.,* 1994) hydrogen peroxide (Foyer et al., 1997) both Ca^{2+} dependent and Ca^{2+} independent protein kinases, (Ohto and Nakamura 1995, Gossett *et al.,* 2001) and Ca^{2+} ions (Cormier *et al.,* 1985) may be involved in the signal transduction pathway. Although there is very little information about second messenger activation in plants, there is a possibility that Ca^{2+}-independent and Ca^{2+}-dependent protein kinases can induce gene expression (Gossett *et al.,* 2001). Clearly, the mechanism by which plants can perceive increased reactive oxygen intermediates and compensate by increasing the level of oxidative protection are basic to the understanding of the functioning of plants in saline conditions. Special significance has been placed on the relationship between abscisic acid and environmental stressors such as salinity that up-regulate the expression of several genes which encode antioxidant enzymes. Little is known about the relationship between second messenger activation and the up-regulation of antioxidant enzymes. The purpose of this study was to determine if Ca^{2+} is necessary for the NaCl-induced up-regulation of antioxidant enzyme activity. This objective was addressed by performing a series of time-course experiments in which antioxidant enzyme activity was measured over an 8–hour period with a NaCl-tolerant cotton cell line derived from Coker 312 subjected to NaCl-stress after a 2–hour pre-incubation period with two calcium-blockers, ruthenium red and lanthanum chloride. It was also addressed by performing a series of time-course experiments in which antioxidant enzymes activity was measured over a 2-hour period with a NaCl-tolerant cell line subjected to NaCl-stress following a 2-hour pre-incubation period with TMB-8, a calcium-blocker, and A2318, a Ca^{2+} ionophore.

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Materials and Methods

Callus Growth and Maintainance

NaCl-adapted callus (150mM NaCl) from the cotton cultivar Coker 312-17 was generated by the method established by Trolinder and Goodin (1987) as modified by Gossett *et al,* (1994b). Since it was established in previous studies that antioxidant levels increased to a greater extent in NaCl-tolerant callus (Bellaire, 1998), only NaCl-tolerant cell lines were used in these experiments. The NaCl-adapted cell line was established by progressively growing portions of the callus on media with increasing concentrations of NaCl until the NaCl adapted callus of the selected cell line grew on the 150mM NaCl media as well as on NaCl-sensitive (0mM NaCl) media. In order to maintain viability, the callus tissue was sub-cultured every 3 to 5 weeks. NaCl-tolerant callus which was grown on media amended with 150mM NaCl was used in these time course studies.

Time Course Studies

Approximately 4 grams of NaCl-tolerant callus was added to each of the series of culture tubes containing liquid media amended with 150mM NaCl or 150mM NaCl + Ca^{2+}-blocker ($LaCl_3$ or ruthenium red). After each culture tube was connected to an aerator to allow for constant aeration, they were pre-incubated for two hours. Following the pre-incubation, the salt stress experiment culture tubes were treated with NaCl to a final concentration of 250mM NaCl. No additional NaCl was added to the culture tubes used as controls. The callus tissue was extracted at 0, 0.5, 1-, 2-, 4-, and 8–hours intervals and stored at -70°C for subsequent antioxidant analysis. Since it was established in previously described time-course studies that antioxidant enzyme activity was up-regulated within 2-hours, only a 2-hour time-course was performed with the TMB-8 and A23187 studies. However, the aerated culture tubes were subjected to the same 2-hour pre-incubation and to 250mM NaCl treatment, while no additional NaCl was added to tubes used as controls. The callus was extracted at 1, and 2-hour intervals and stored at -70°C for subsequent analysis.

Calcium Blockers

The Ca^{2+}-blockers used in the experiment were lanthanum chloride ($LaCl_3.7H_2O$), TMB-8, [(8-*NN*-diethylamino)-octyl-3,4,5-trimethoxybenzoate] and ruthenium red (ammoniated ruthenium oxychloride). The calcium ionophore used was calimycin also known as A23187 (Kojima, *et al.,* 1985). In a previous study (Price, *et al.,* 1994), lanthanum chloride at 1mM was found to decrease cytoplasmic [Ca^{2+}] to approximately one-half, and for total abolition, the amount of lanthanum chloride had to be increased to 10 mM. For ruthenium red, 20μmM resulted in total abolition of cytoplasmic [Ca^{2+}]. In the time course studies reported here, the callus was subjected to either 20μmM ruthenium red, 10mM lanthanum chloride or 5μmM TMB-8. TMB-8 [(8-*NN*-

diethylamino)octyl-3,4,5–trimethoxybenzuate] is a compound frequently used to assess the involvement of intracellular calcium pools. Inhibition caused by TMB-8 is often reversed by either the addition of a calcium ionophore or by an elevation in extracellular calcium concentration (Kojima, *et al,* 1985). Both lanthanum chloride and ruthenium red were obtained from the Sigma Chemical Company and were dissolved in water. TMB-8 and A23187 was donated to us by Dr. Steve Alexander at the LSU Health Science Center, Shreveport, LA. The TMB-8 was dissolved in DMSO. The Ca^{2+} ionophore, A23187, is also known as calimycin (Kojima, *et al,* 1985). A23187 was dissolved in DMSO, to a final concentration of 100nM.

Enzyme Extraction and Analysis

Samples were prepared for antioxidant enzyme analyses by the methods previously described by Anderson *et al.,* (1992) as modified by Gossett *et al.,* (1994b), Samples were prepared for the following analyses: ascorbate peroxidase (APX); catalase, and glutathione reductase (GR). Enzymes were extracted by homogenizing approximately one gram of frozen callus tissue, 0.25 gram of insoluble PVP, and one drop of antifoam A emulsion in 3.0 ml of ice cold PIPES buffer (pH 6.8). The PIPES buffer contained 6mM cysteine hydrochloride, 10mM D-isoascorbate, 1mM EDTA, 1% PVP-10, and 0.3% Triton X-10. The homogenate was centrifuged for 20 minutes at 4°C at 15,000 rpm. After centrifugation and the recording of volumes of supernatant, 1 ml of the supernatant was desalted and centrifuged through a 10 ml bed of Sephadex G-25 according to the method outlined by Anderson *et al.,* (1992). The eluate volume was measured and immediately analyzed for catalase activity. The remaining eluate was stored in microfuge tubes at -70°C for subsequent analysis of ascorbate peroxidase(APX), glutathione reductase (GR), and peroxidase activities. Catalase activity was determined by measuring the disappearance of H_2O_2 at 240 nm in a reaction mixture containing 1.9 ml H_2O, 1.0m1 0.059 M H_2O_2 in KPO4 buffer (pH 7.0) with 0.1 ml extract (Beers and Sizer, 1952). APX activity was assayed at 265 nm by measuring the ascorbic acid-dependent reduction of H_2O_2 in a reaction mixture containing 100ul each of 1.5mM Na ascorbate, 1mM EDTA, 1mM H_2O_2, 500 μml of 166 mM HEPES-KOH (pH 7.0), and 100 ul of sample (Anderson *et al,* 1992). Peroxidase activity was determined at 675nm by measuring the H_2O_2-dependent oxidation of reduced 2,3',6–trichloroindophenol in a reaction mixture with 950ul of 17mM $Na_2S_2O_3$, 120mM H_2O_2 and 0.3mM 2,3', 6–trichloroindophenol in 40mM $NaPO_4$ buffer (pH 6.0), and 50ul of sample (Nickel and Cunningham, 1969). Glutathione reductase activity was measured at 340nm and was determined by monitoring glutathione–dependent oxidation of NADPH. The reaction mixture consisted of 950ul of 0.5 nM GSSG, 0.15mM NADPH, and 3mM $MgCl_2$ in 50mM Tris (pH 7.5) and 50ul of sample (Schaedle and Bassham, 1977). One unit of catalase and peroxidase activity was defined as the amount necessary to decompose 1 mmole of substrate/min at 25°C. One unit of ascorbate peroxidase and glutathione reductase activity was defined as the amount of enzyme necessary to reduce 1μmmole of substrate/min at 25°C (Gossett *et al.,* 1994b). All data were subjected to a one-way analysis of variance and significance was determined at a 95% confidence limit. Data points were derived from a mean of a minimum of three replicates.

Results and Discussion

Ascorbate Peroxidase Activity

NaCl-stress resulted in a significant increase in APX activity within 30 minutes (Table 1). No significant change in APX activity was observed in either the controls or NaCl-stressed tissue pre-treated with $LaCl_3$ during the entire 8-hour time-course. Except for a slight decrease in APX activity at the 8-hour point, pretreatment with ruthenium red did not cause a significant change in APX activity in either the controls or NaCl-stressed tissue during the entire time-course. Similarly, no significant change in APX activity was observed in controls or NaCl-stressed tissue throughout the 2-hr time period when pretreated with TMB-8 (Table 5). When compared with the untreated control (150mM NaCl), pretreatment with the ionophore, A23187, resulted in a significant up-regulation in APX activity at the one-hour time point. Pretreatment with TMB-8 + A23187 caused a significant up-regulation in APX activity in the controls and NaCl-stressed tissue at both the 1-hr and 2-hr time points.

Catalase Activity

Catalase activity increased significantly at both the 1-hour and 2-hour time points when the tissue was subjected to NaCl stress (Table 2). No significant change in catalase activity was observed in either the controls or NaCl-stressed tissues when subjected to pretreatment with either $LaCl_3$ or ruthenium red. Pretreatment with TMB-8 resulted in no significant up-regulation in catalase activity in the control or NaCl-stressed tissues (Table 6). Similar results were observed with preteatment with the ionophore, A23187. Although pretreatment with TMB-8 + A23187 caused no significant change in catalase activity in the controls, the TMB-8+A23187 pretreatment resulted in a significant up-regulation in catalase activity at the 1-hour and 2-hour time point in the NaCl-stressed tissue.

Glutathione Reductase Activity

Significant increases in glutathione reductase activity were observed at both the 1-hour and 2-hour time points when the tissue was subjected to NaCl-stress (Table 4). Pretreatment with $LaCl_3$ and ruthenium red resulted in no significant change in glutathione reductase activity during the entire 8-hour time course in either the controls or NaCl-stressed tissue. No significant change in GR activity was observed in either the controls or NaCl-stressed tissue pretreated with TMB-8 during the 2-hour time-course (Table 8). However, a significant increase in GR activity occurred in controls pretreated with A23187 and with TMB-8 + A23187 at the 1-hr and 2-hr time points. In the NaCl-stressed tissue pretreated with TMB-8 + A23187, a 10-fold increase occurred at the 2-hour time point when compared with the untreated control.

Peroxidase Activity

Peroxidase activity increased significantly within 1-hour in the callus tissue subjected to NaCl-stress (Table 3). Pretreatment with $LaCl_3$ and ruthenium red (Figure 17) completely suppressed any increase in peroxidase activity in both the control and NaCl-stressed tissue. Pretreatment of the control and NaCl-stressed tissue with TMB-8 resulted in no significant change in peroxidase activity during the 2-hour time-course (Table 7). However, a significant increase in peroxidase activity occurred at the 1-hr time point when the control tissue was pretreated with the ionophore, A23187. A significant increase in peroxidase activity occurred at the 1-hr and 2-hr time points in both the control and NaCl-stressed tissue pretreated with TMB-8 + A23187.

The three calcium channel blockers, Lanthanum Chloride, ruthenium red and TMB-8, effectively abolished the NaCl induced increases in the activities of glutathione reductase, catalase, peroxidase and ascorbate peroxidase. However, the Ca^{2+} ionophore, A23187 induced an increase in the activities of glutathione reductase, peroxidase and ascorbate peroxidase but not catalase. In addition, an increase in the activity of all four enzymes was observed in the callus under NaCl stress in the presence of TMB-8 and A23187. With control callus treated with the TMB-8 and A23187 combination, an up-regulation of peroxidase, glutathione reductase and ascorbate peroxidase activites were recorded, but no up-regulation of catalase was observed under these conditions. The results of these experiments when combined with those of Gossett, *et al.,* 2001, support the hypothesis that calcium is involved, possibly by activating protein kinases in the NaCl-induced signal transduction pathway.

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Table 1. Ascorbate Peroxidase activity (units/g fresh weight ± SE) in salt-tolerant callus tissue pre-treated for 2 hours with $LaCl_3$ at 10mM, ruthenium red at 20mM and 150mM NaCl. At the start of the 8 hour time period controls received no further NaCl, salt. Stressed received additional NaCl to bring the final concentration to 250mM NaCl.

Time (hr)	0	0.5	1	2	4	8
Control	434±16	446±51	374±36	542±61	400±43	379±23
Control+ $LaCl_3$	548±33	391±52	200±46*	249±46*	176±8	217±25
Control+ RuRed	440±41	213±19*	243±11	185±40	217±17	52±15*
NaCl Stress	455±50	1671±139*	1071±65	601±101	651±13	354±44
NaCl Stress+ $LaCl_3$	503±39	466±28	148±15*	176±38	117±31	104±17
NaCl Stress+ RuRed	452±19	482±57	556±40	532±4	564±33	586±58

Table 2. Catalase activity (units/g fresh weight ± SE) in salt-tolerant callus tissue pre-treated for 2 hours with $LaCl_3$ at 10mM, ruthenium red at 20mM and 150mM NaCl. At the start of the 8 hour time period controls received no further NaCl, stressed received additional NaCl to bring the final concentration to 250mM NaCl.

Time(hr)	0	0.5	1	2	4	8
Control	30±3	26±2	36±2	28±2	32±2	26±2
Control+$LaCl_3$	28±1	28±4	39±4	45±8	28±3	15±2
Control+RuRed	15±3	14±4	18±1	16±3	17±1	18±2
NaCl Stress	31±6	37±4	114±13*	189±14	43±7	32±8
NaCl Stress+$LaCl_3$	26±2	14±2	12±2	15±3	12±2	8±1
NaCl Stress+RuRed	15±2	16±2	19±1	19±2	16±2	14±1

Table 3. Peroxidase activity (units/g fresh weight ± SE) in salt-tolerant callus tissue pre-treated for 2 hours with $LaCl_3$ at 10mM, ruthenium red at 20mM and 150mM NaCl. At the start of the 8 hour time period controls received no further NaCl, salt stressed received additional NaCl to bring the final concentration to 250mM NaCl.

Time (hr)	0	0.5	1	2	4	8
Control	319±42	364±45	329±25	331±15	379±39	344±20
Control+ $LaCl_3$	271±32	263±38	311±75	322±12	295±6	397±32
Control+ RuRed	315±33	282±35	313±69	392±48	381±10	457±46
NaCl Stress	459±21	483±8	1147±58*	468±49	406±93	254±19
NaCl Stress +$LaCl^3$	473±34	312±40	266±32	315±8	301±67	387±67
NaCl Stress+ RuRed	296±25	318±16	283±49	279±32	307±45	302 ±33

Table 4. Glutathione reductase activity (units/g fresh weight ± SE) in salt-tolerant callus tissue pre-treated for 2 hours with $LaCl_3$ at 10mM, ruthenium red at 20mM and 150mM NaCl. At the start of the 8 hour time period controls received no further NaCl, salt stressed received additional NaCl to bring the final concentration to 250mM NaCl.

Time (hr)	0	0.5	1	2	4	8
Control	31±2	41±4	57±5	56±5	59±6	49±4
Control+$LaCl_3$	49±7	42±4	43±5	54±7	31±4	35±3
Control+ RuRed	35±4	23±3	30±4	28±3	58±6	40±3
NaCl Stress	44±4	49±4	140±4*	116±4*	34±6	35±5
NaCl Stress+ $LaCl_3$	44±5	33±5	29±5	32±6	25±4	23±1
NaCl Stress+ RuRed	41±4	48±5	45±5	29±7	36±2	15±1

Table 5. Ascorbate Peroxidase activity (units/g fresh weight ± SE) in salt-tolerant callus tissue pre-treated for 2 hours with TMB-8 at 5mM, A23187 at 100nM and 150mM NaCl. At the start of the 2 hour time period controls received no further NaCl, salt stressed received additional NaCl to bring the final concentration to 250mM NaCl.

Time (hr)	0	1	2
Control	434±16	374±36	342±31
Control+TMB-8	475±43	375±37	322±27
Control+A23187	405±21	660±30*	548±19
Control+TMB-8&A23187	403±39	726±81	739±86
NaCl-stressed	455±50	1071±65*	601±37*
NaCl-stressed+TMB-8	447±65	401±26	411±21
NaCl-stressed+TMB-8&A23187	311±70	1069±45*	917±58*

Table 6. Catalase activity (units/g fresh weight ± SE) in salt-tolerant callus tissue pre-treated for 2 hours with TMB-8 at 5mM, A23187 at 100nM and 150mM NaCl. At the start of the 2 hour time period controls received no further NaCl, salt stressed received additional NaCl to bring the final concentration to 250mM NaCl.

Time (hr)	0	1	2
Control	30±3	36±2	28±2
Control+TMB-8	38±10	25±5	26±4
Control+A23187	28±1	24±7	13±1
Control+TMB-8 &A23187	26±2	22±3	20±3
NaCl-stressed	31±6	114±13*	189±14
NaCl-stressed+TMB-8	36±6	23±4	30±7
NaCl-stressed+TMB-8&A23187	31±3	118±12*	104±11*

Table 7. Peroxidase activity (units/g fresh weight ± SE) in salt-tolerant callus tissue pre-treated for 2 hours with TMB-8 at 5mM, A23187 at 100nM and 150mM NaCl. At the start of the 2 hour time period controls received no further NaCl, salt stressed received additional NaCl to bring the final concentration to 250mM NaCl.

Time (hr)	0	1	2
Control	319±42	329±25	331±15
Control+TMB-8	447±58	371±67	381±36
Control+A23187	354±28	633±33*	463±27
Control+TMB-8&A23187	315±33	642±33*	904±65*
NaCl-stressed	459±21	1147±58*	468±49
NaCl-stressed+TMB-8	301±20	378±71	369±55
NaCl-stressed+TMB-8&A23187	473±74	936±41*	1000±46*

Table 8. Glutathione reductase activity (units/g fresh weight ± SE) in salt-tolerant callus tissue pre-treated for 2 hours with TMB-8 at 5μM, A23187 at 100nM and 150mM NaCl. At the start of the 2 hour time period controls received no further NaCl, salt stressed received additional NaCl to bring the final concentration to 250mM NaCl.

Time (hr)	0	1	2
Control	31±2	37±5	26±5
Control+TMB-8	30±4	23±6	28±5
Control+A23187	32±5	47±2*	52±3*
Control+TMB-8&A23187	32±5	56±4*	52±3*
NaCl-stressed	34±4	140±4*	116±4*
NaCl-stressed+TMB-8	41±4	35±5	27±4
NaCl-stressed+TMB-8&A23187	44±5	76±6	224±7*