INVOLVEMENT OF PROTEIN KINASES IN THE NaCl-INDUCED UPREGULATION OF ANTIOXIDANT ENZYME ACTIVITIES IN COTTON CALLUS

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Abstract

Antioxidant enzyme activity was measured in salt tolerant callus tissue derived from the cultivar Coker 312 over an 8 hour period following treatment with either 250 mM NaCl, 2 µM staurosporine, 2µ M K252a, 5 µM TMB-8, 100 nM A23187, 250 mM NaCl + 2µ M staurosporine, 250 mM NaCl + 2µ M K252a, 250 mM NaCl + 5 µM TMB-8, and 250 mM NaCl + 5 µM TMB-8 + 100 nM A23187. Staurosporine and K252a have been reported to inhibit plant protein kinase activity. TMB-8 is a putative calcium channel blocker, and A23187 is a calcium ionophore. NaCl induced an up-regulation of catalase, peroxidase, ascorbate peroxidase, and glutathione reductase activities within 1 hour after treatment. The K252a and TMB-8 treatments completely inhibited the NaCl stress-induced increases in the activities of all four enzymes. At the concentration used in this experiment, staurosporine did not completely inhibit the NaCl-induced increases in antioxidant activity; however, it either reduced the responses of ascorbate peroxidase, glutathione reductase, and peroxidase or delayed the response of catalase. Treatment with A23187 increased antioxidant activity in the NaCl-tolerant controls, and treatment with TMB-8 + A23187 increased antioxidant activity in both the controls and the NaCl-stressed callus. These data support the hypothesis that protein kinases are involved in the signal transduction pathway associated with the NaCl-induced up-regulation of antioxidant activity and that these protein kinases may be regulated by calcium.

Introduction

Environmentally-induced physiological stress results in a cascade of stress responses, one of which is the up-regulation of the antioxidant defense system. Results from on-going research in our laboratory have shown that NaCl stress results in an up-regulation of ascorbate peroxidase (APX), catalase, glutathione reductase (GR), glutathione S-transferase (GST), peroxidase, and superoxide dismutase (SOD) (Gossett *et al.*, 1994a, 1994b, 1996). Since these enzymes appear to be up-regulated by NaCl-induced oxidative stress, there is a question as to which molecule or molecules may be involved in the signal transduction process. The literature has suggested that abscisic acid (ABA) (Leung and Giraudat, 1998), superoxide (Doke *et al.*, 1994), H_2O_2 (Foyer *et al.*, 1997), Ca^{2+} (Cormier *et al.*, 1985), and protein kinases (Ohto and Nakamura, 1995) may be involved in the signal transduction pathway. Recent results from our laboratory have shown that reactive oxygen intermediates such as superoxide and H_2O_2 may signal the up-regulation of antioxidant enzyme activity through both ABA-dependent and ABA-independent pathways (Bellaire *et al.*, 2000). Other recent studies (Banks *et al.*, 2001) have shown that Ca^{2+} plays a vital role in the up-regulation of antioxidant enzyme activity.

Several studies have shown an increase in protein kinase activity in response to external stimuli. These studies include tomato tissue culture cells exposed to elicitor (Felix *et al.*, 1991), soybean exposed to phytophora infection (Feller, 1989), and tomato exposed to citrus exocortis viroid infection (Vera and Conejero, 1990). Other studies have described protein kinases in plants homologous to MAP-kinases found in animals and yeast (Seo *et al.*, 1995; Suzuki and Shinshi, 1995; Usami *et al.*, 1995; Bogre *et al.*, 1997). Such protein kinases are activated in response to stress stimuli such as wounding or elicitors. These studies have proved a link between protein phosphorylation and the activation of defense responses in plants. Ohto and Nakamura (1995) affirmed that protein kinases can play an important function in the regulation of gene expression by phosphorylating transcription factors that regulate the expression of target genes.

Calcium dependent protein kinase (CDPK) is the prototype for a family of calcium regulated protein kinases in which kinase activity is regulated by direct binding of calcium (Harmon et al., 1987). In plant plasma membranes, a predominant protein kinase activity is stimulated by calcium (Klimczak and Hind, 1990; Ladror and Zielinski, 1989; Schaller and Sussman, 1987). Both the zucchini plasma membrane protein kinases (Verhey *et al.*, 1993) and the soybean CDPKs (Harmon *et al.*, 1987; Putnam-Evans *et al.*, 1990) have been shown to require calcium for autophosphorylation. In the presence of 10 mM free calcium, soybean CDPK displayed a 100-fold activation, while the zucchini plasma membrane protein kinase required 11 mM free calcium for activation. CDPKs have been shown to be induced by cold, drought, and ABA (Assmann, 1993; Braam, 1992; Knight *et al.*, 1997; McAinsh *et al.*, 1992). Sheen (1996) demonstrated that two related CDPKs induced a stress and ABA-responsive promoter.

The purpose of this study was to determine whether protein kinases are involved in the NaCl-induced up-regulation of GR, peroxidase, APX, and catalase activities and whether calcium is required for such up-regulation by examining the effects of two protein kinases inhibitors (staurosporine and K252a), a calcium channel blocker (TMB-8), and a calcium ionophore (A23187) on the NaCl stress-induced response. Staurosporine and K252a have been shown to markedly inhibit protein kinases in both animals (Tamaoki *et al.*, 1985) and plants (Ohto and Nakamura, 1995; Yan *et al.*, 1997). In animal cells, TMB-8 has been shown to inhibit the activity of protein kinase C in a dose-dependent manner, and TMB-8 is frequently used to assess the involvement of intracellular calcium pools in a variety of cellular processes (Kojima *et al.*, 1985). The inhibitory effect of TMB-8 on calcium flux has been proven to be reversed by the effect of calcium ionophores such as A23187 (Kojima *et al.*, 1985)

Methods and Materials

Approximately 4 grams of salt-tolerant cotton callus tissue was transferred to culture tubes containing 25 ml of media amended with 150 mM NaCl. Each culture tube was connected to an aerator for constant aeration. The tubes were pre-incubated for 2 hours as controls or treated with 2 µM staurosporine, 2 µM K252a, 5 µM TMB-8, 100 nM A23187, or 5 µM TMB-8 + A23187. After the pre-incubation period, the tubes were left untreated as controls or amended to a final concentration of 250 mM NaCl. The callus tissue was harvested at 0, 30 min, 1 hr, 2 hr, 4 hr, and 8 hr intervals for the staurosporine and K252a treatments. Since previous studies (Bellaire *et al.*, 2000) as well as these studies have shown a rapid increase in antioxidant enzyme activities that level off at or before the 2-hour period, the TMB-8, A23187, and TMB-8 + A23187 treatments were harvested at 0, 1, and 2 hours. The callus tissue was stored at -70ºC for subsequent antioxidant enzyme analyses.

Samples were prepared for antioxidant analyses by the method previously described by Gossett *et al.* (1996). Catalase activity was determined by monitoring the disappearance of H_2O_2 according to the method of Beers and Sizer (1952). Peroxidase activity was measured by monitoring the H_2O_2-dependent oxidation of 2,3',6-trichloroindophenol after the method of Nickel and Cunningham (1969). GR activity was measured by monitoring the glutathione-dependent oxidation of NADH as described by Schaedle and Bassham (1977). APX activity was assayed by monitoring the ascorbate-dependent reduction of H_2O_2 by the method described by Anderson *et al.* (1992).

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Results and Discussion

Enzyme activities are shown in Tables 1-4. Catalase activity (Table 1) remained relatively constant throughout the treatment period in the controls and the controls treated with staurosporine, K252a, TMB-8, and TMB-8, or A23187. Treatment with 250 mM NaCl caused catalase activity to increase significantly within 1 hour, and the activity returned to control levels within 4 hours. Pre-treatment with staurosporine significantly delayed the NaCl-induced increase in catalase activity, and both K252a and TMB-8 completely suppressed the increase in catalase activity in the NaCl-stressed callus. Pre-treatment with TMB-8 + A23187 produced and increase in catalase activity within one hour in the NaCl-treated tissue. In the controls and the controls pre-treated with staurosporine, K252a, or TMB-8, peroxidase activity did not change significantly throughout the treatment period (Table 2). The NaCl treatment resulted in a significant increase in peroxidase activity within 1 hour after treatment. Staurosporine pre-treatment significantly reduced the peroxidase response and both K252a and TMB-8 completely abolished the NaCl-induced increase in peroxidase activity. Pre-treatment with A23187 resulted in an increase in peroxidase activity within 1 hour in the controls, and pre-treatment with TMB-8 + A23187 resulted in an increase in peroxidase activity within 1 hour in the NaCl-stressed callus.

APX activity remained somewhat constant throughout the treatment period in controls and the controls treated with staurosporine, K252a, and TMB-8 (Table 3). Treatment with 250 mM NaCl resulted in a significant increase in APX activity within 30 minutes. Pre-treatment with staurosporine significantly reduced the NaCl-induced increase in APX activity. The K252a and TMB-8 pre-treatments completely suppressed the increase in APX activity in the NaCl-stressed callus. There was a significant increase in APX activity within 1 hour in the controls pre-treated with A23187 and in the NaCl-stressed tissue pre-treated with TMB-8 + A23187. GR activity did not change significantly throughout the treatment period in the controls and the controls pre-treated with staurosporine, K252a, or TMB-8 (Table 4). The NaCl treatment resulted in a significant increase in GR activity within 1 hour after treatment. Staurosporine pre-treatment significantly diminished the GR response and both K252a and TMB-8 completely abolished the NaCl-induced increase in GR activity. Pre-treatment with A23187 resulted in an increase in GR activity within 1 hour in the controls, and pre-treatment with TMB-8 + A23187 resulted in an increase in GR activity within 1 hour in the NaCl-stressed callus.

Staurosporine treatment resulted either in a decrease in the NaCl-induced up-regulation of the activities of GR, peroxidase, and APX or in a significant delay in the increase in catalase activty in the NaCl-tolerant callus tissue subjected to NaCl stress. On the other hand, the increase in all four of the antioxidant enzyme activities generated by the NaCl treatment were negated when the callus tissue was subjected to pre-incubation with K252a. The same results were observed in callus tissue pre-incubated with TMB-8. The fact that staurosporine and K252a treatment did not produce the same results was contradictory to our expectations. There are several possibilities for this observation. One possibility is that staurosporine was not completely assimilated by the cotton callus tissue. Another possibility is that the staurosporine concentration used in this experiment was not sufficient to produce a complete inhibitory effect on the protein kinases.

A previous study (Manchandia *et al.,* 1999) suggested that the up-regulation of antioxidant enzymes in cotton callus is transcriptionally regulated. The results from the present study are in concordance with this suggestion, since it has been proven that protein kinases play an important function in the regulation of gene expression by phosphorylating transcription factors that regulate the expression of target genes (Ohto and Nakamura, 1995). Further research, namely Northern blot analysis, is required to prove that the up-regulation of antioxidant enzymes is regulated at the transcription level. Despite the level of control, this study strongly suggests that protein kinases are involved in the signal transduction pathway for the NaCl-induced up-regulation of antioxidant enzyme activities in cotton callus tissue. Moreover, the results from the experiments using TMB-8 and A23187, in which A23187 abolished the inhibitory effect of TMB-8 on the increase in antioxidant activity, suggest that calcium may play a second messenger role by activating protein kinases in the signal transduction pathway.

Summary

The protein kinase inhibitors, staurosporine and K252a, and the calcium channel blocker, TMB-8, inhibited the NaCl-induced increases in the activities of APX, catalase, GR, and peroxidase. This strongly suggests that protein kinases are involved in the signal transduction pathway associated with the NaCl-induced up-regulation of antioxidant enzyme activity.

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Table 1. Catalase activity (units/g fresh weight ±SE) in salt-tolerant callus tissue treated for 0, 0.5, 1, 2, 4, or 8 hrs with 150 mM NaCl (control), 2 μM staurosporine (S), 2 μM K252a (K), 5 μM TMB-8 (T), 100 nM A23187 (A), 5 μM TMB-8 + 100 nM A23187 (T+A), 250 mM NaCl (NC), 250 mM NaCl + 2 μM staurosporine (NC+S), 250 mM NaCl + 2 μM K252a (NC+K), 250 mM NaCl + 5 μM TMB-8 (NC+T), and 250 mM NaCl + 5 μM TMB-8 + 100 nM A23187 (NC+TA)

	Time					
Treatment	**0**	**0.5 h**	**1 hr**	**2 hr**	**4 hr**	**8 hr**
Control	30±3	26±2	36±2	28±2	32±2	26±2
S	38±10	36±3	38±4	34±5	39±3	37±5
K	16±2	17±1	16±1	15±1	15±1	16±1
T	38±10		25±5	26±4		
A	28±1		24±7	13±1		
NC	31±6	37±4	114±13	189±14	43±7	32±8
NC+S	36±6	3 4±3	35±6	24±3	63±7	83±4
NC+K	15±1	13±1	15±2	16±3	24±4	16±3
NC+T	36±6		23±4	30±7		
NC+TA	31±3		118±12	104±11		

Table 2. Peroxidase activity (units/g fresh weight ±SE) in salt-tolerant callus tissue treated for 0, 0.5, 1, 2, 4, or 8 hrs with 150 mM NaCl (control), 2 µM staurosporine (S), 2 µM K252a (K), 5 µM TMB-8 (T), 100 nM A23187 (A), 5 µM TMB-8 + 100 nM A23187 (T+A), 250 mM NaCl (NC), 250 mM NaCl + 2 µM staurosporine (NC+S), 250 mM NaCl + 2 µM K252a (NC+K), 250 mM NaCl + 5 µM TMB-8 (NC+T), and 250 mM NaCl + 5 µM TMB-8 + 100 nM A23187 (NC+TA)

	Time					
Treatment	**0**	**0.5 h**	**1 hr**	**2 hr**	**4 hr**	**8 hr**
Control	319±42	364±45	329±25	331±15	379±39	334±20
S	447±58	518±92	520±81	500±67	520±120	509±67
K	354±28	293±52	281±12	307±26	297±60	368±49
T	447±58		371±67	381±36		
A	354±28		633±33	904±65		
NC	459±21	483±8	1147±58	468±49	406±93	254±19
NC+S	282±40	398±84	996±112	455±85	536±88	477±21
NC+K	301±20	287±25	322±28	251±28	307±36	369±27
NC+T	301±20		387±71	369±55		
NC+TA	473±34		936±41	999±46		

Table 3. APX activity (units/g fresh weight ±SE) in salt-tolerant callus tissue treated for 0, 0.5, 1, 2, 4, or 8 hrs with 150 mM NaCl (control), 2 µM staurosporine (S), 2 µM K252a (K), 5 µM TMB-8 (T), 100 nM A23187 (A), 5 µM TMB-8 + 100 nM A23187 (T+A), 250 mM NaCl (NC), 250 mM NaCl + 2 µM staurosporine (NC+S), 250 mM NaCl + 2 µM K252a (NC+K), 250 mM NaCl + 5 µM TMB-8 (NC+T), and 250 mM NaCl + 5 µM TMB-8 + 100 nM A23187 (NC+TA)

	Time					
Treatment	**0**	**0.5 h**	**1 hr**	**2 hr**	**4 hr**	**8 hr**
Control	434±16	446±51	374±36	542±61	400±43	379±23
S	475±43	464±61	582±51	464±60	482±12	608±44
K	405±21	362±33	322±21	303±34	290±55	505±24
T	475±43		660±30	548±19		
A	405±21		726±81	739±86		
NC	455±50	1671±139	1071±65	601±101	651±13	354±44
NC+S	447±65	1420±56	920±111	940±34	994±89	558±83
NC+K	311±70	269±45	440±78	290±38	300±52	326±23
NC+T	447±65		401±26	411±21		
NC+TA	311±70		1069±45	917±58		

Table 4. GR activity (units/g fresh weight ±SE) in salt-tolerant callus tissue treated for 0, 0.5, 1, 2, 4, or 8 hrs with 150 mM NaCl (control), 2 µM staurosporine (S), 2 µM K252a (K), 5 µM TMB-8 (T), 100 nM A23187 (A), 5 µM TMB-8 + 100 nM A23187 (T+A), 250 mM NaCl (NC), 250 mM NaCl + 2 µM staurosporine (NC+S), 250 mM NaCl + 2 µM K252a (NC+K), 250 mM NaCl + 5 µM TMB-8 (NC+T), and 250 mM NaCl + 5 µM TMB-8 + 100 nM A23187 (NC+TA)

	Time					
Treatment	**0**	**0.5 h**	**1 hr**	**2 hr**	**4 hr**	**8 hr**
Control	31±2	41±4	57±5	56±5	59±6	49±4
S	40±4	41±7	44±4	41±5	44±4	29±4
K	32±5	29±4	29±4	28±3	36±5	33±2
T	30±4		23±6	28±5		
A	32±5		47±2	52±3		
NC	44±4	49±4	140±4	116±4	34±6	35±5
NC+S	47±5	42±3	86±7	40±3	40±3	40±9
NC+K	52±6	57±5	31±1	18±2	18±3	15±1
NC+T	41±4		35±5	27±4		
NC+TA	44±5		76±6	224±7		