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PHOSPHOLIPID INVOLVEMENT IN THE NaCl-INDUCED UP-REGULATION OF ANTIOXIDANT ENZYME ACTIVITY

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Abstract

The objective of this study was to examine the possible involvement of phospholipid signaling in the NaCl-induced up-regulation of antioxidant enzyme activity in cotton callus. This was accomplished by using callus tissue from a NaCl-tolerant cotton cell line in a series of time-course studies in which the tissue was subjected to one of the following treatments: no treatment (controls), 250 mM NaCl (NaCl stress), abscisic acid (ABA), myoinositol 1,4,5-triphosphate (IP_3), diacylglycerol (DAG), phosphatidylinositol (PI), 1-butanol (an inhibitor of phospholipase D), U73122 (an inhibitor of phospholipase C), a pre-treatment with 1-butanol followed by treatment with ABA or 250 mM NaCl, and pre-treatment with U73122 followed by treatment with 250 mM NaCl. Both NaCl stress and ABA treatment resulted in rapid increases in antioxidant enzyme activity. Pretreatment of both control and NaCl stressed cotton callus with 1-butanol actually resulted in increases rather than decreases in most of the antioxidant enzyme activities; however, pretreatment with 1-butanol did inhibit the NaCl-induced increase in peroxidase activity. Thus, it would appear that phospholipase D has a very limited, and perhaps no role in the NaCl-induced up-regulation of antioxidant enzyme activity. Pretreatment with U73122 decreased and/or delayed the NaCl-induced up-regulation of glutathione reductase (GR), ascorbate peroxidase (APX), and peroxidase activities and completely inhibited the NaCl-induced increase in catalase activity in the NaCl-treated cotton callus tissue, while treatment of the control with either PI, IP3, or DAG (enzymatic products of phospholipase C) resulted in rapid increases in the activities in all four of the enzymes. These findings would suggest that phospholipase C may be involved in the NaCl-induced up-regulation of antioxidant enzyme activity in cotton callus tissue.

Introduction

Phosphoinositides are membrane phospholipids that play many roles in cellular processes. It has been suggested that salinity and hyperosmotic stress may activate a phosphoinositide signaling system (Pical *et al*., 1999). Phosphoinositides can be hydrolyzed into the signaling molecules myoinositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), which trigger calcium release from intracellular stores and activate protein kinase C (PKC), respectively (Beeridge, 1993). IP_3- binding channels have been identified in plants (Allen *et al*., 1995), and more importantly, delivery of IP_3 to plant cells has been found to cause the release of intracellular calcium (Alexandre, *et al*., 1990; Franklin-Tong *et al*., 1996). It has been suggested that IP_3 causes Ca^{2+} release via endomembrane Ca^{2+} channels in response to ABA in guard cells (Gilroy *et al*., 1990; Parmar and Brearley, 1995; Staxen *et al*., 1999). It has also been recently reported that there is a correlation between synthesis of phosphatidylinositol (PI), IP_3 production, and calcium mobilization (Dewald *et al*., 2001). These studies indicate that phosphoinositide-derived second messengers are involved in calcium signaling, which has been shown to alter gene expression and plant adaptation to salinity and osmotic stress (Knight *et al*., 1997). Phospholipase C (PLC), a G-protein-mediated phospholipase found in the plasma membrane, yields phosphatidylinositol 4,5-biphosphate, which is cleaved to yield IP_3 and DAG. U-73122 is an inhibitor of phospholipase C (PLC). Phospholipase D (PLD) is activated by abscisic acid and is most likely mediated by G-protein (Ritchie and Gilroy, 2000). PLD acts upon membrane phospholipids to produce phosphatidic acid (PtdOH), which may trigger the ABA response signal transduction cascade. PLD activity can be inhibited by 1-butanol. Phosphatidic acid and is thought to function as an ionophore that allows Ca^{2+} to move freely across the plasma membrane. Increases in PtdOH can also amplify the PLC signaling pathway through second messengers. DAG can also contribute to the levels of PtdOH through a reaction catalyzed by DAG kinase. The purpose of this study was to examine the possible involvement of phospholipid signaling in the NaCl-induced up-regulation of antioxidant enzyme activity in cotton callus.

Methods and Materials

Callus Tissue

Callus tissue was generated from sterile hypocotyls of Coker 312 by the method described by Trolinder and Goodin (1987). The callus tissue was maintained on T1 media containing MS salts (Murashige and Skoog, 1962) supplemented with Gamborg's vitamins (Gamborg, 1978), 0.75 mg/L $MgCl_2$, 0.1 mg/L 2,4-D, 0.1 mg/L kinitin, 2 g/L phytogel, and 30 g/L glucose, and 150 mM NaCl adjusted to a pH of 5.8 (Trolinder and Goodin, 1987). For the time-course studies with a NaCl-tolerant cell line, a NaCl-tolerant cell line was selected as described by Gossett *et al.* (1996) and maintained on T1 amended with 150 mM NaCl.

Time-Course Studies

NaCl-tolerant callus tissue was transferred to culture tubes amended with 150 mM NaCl and subjected to following treatments: (a) control- pre-incubated for 2 hr and left untreated, (b) 250 NaCl- pre-incubated for 2 hr and then amended with NaCl to a final concentration of 250 mM at time point zero, (c) ABA- pre-incubated for 2 hr and then treated with 20 µM ABA at time point zero, (d) ABA + 1-butanol- pre-incubated for 2 hr with 0.1% 1-butanol, an inhibitor or PLD (126), and then treated with 20 µM ABA at time point zero, (e) control + 1-butanol- pre-incubated with 0.1% 1-butanol for 2 hr with no further treatment at time point zero, (f) 250 NaCl + 1-butanol- pre-incubated with 0.1% 1-butanol for 2 hr and then amended with NaCl to a final concentration of 250 mM at time point zero, (g) control + U73122- pre-incubated with 1 µM U73122 (1-(6-[([17]-3-methoxyestra-1,3,5[10]-trien-17-yl)-amino]hexyl)-1H-pyrrole-2,5,-dione) for 2 hr with no further treatment at time point zero, (h) 250 NaCl + U73122- pre-incubated with 1 µM U73122 for 2 hr and then amended with NaCl to a final concentration of 250 mM at time point zero, (i) control + phosphatidylinositol (PI)- treated with 35 µM PI (1,2-diacyl-sn-glycero-3-phospho-(1-D-myo-inositol) and harvested after 2 hr (j) control + D-myoinositol 1 ,4,5-triphosphate (IP_3)- treated with 3 µM IP_3 and harvested after 2 hr, (k) control + diacylglycerol (DAG)- treated with 50 µM DAG (1-stearoyl-2-arachidonyl-sn-glycerol(C18:0/C20:4, [cis,cis,cis,cis]-5,8,11,14)) and harvested after 2 hr. The callus tissue was harvested at 0, 0.5, 1, 2, and 4-hour intervals in treatments (a) through (h) and only at the 2 hr time point in treatments (i) through (k).

Antioxidant Enzyme Assays

Samples were prepared for SOD, catalase, peroxidase, GR and APX analyses according to the method of Anderson *et al.* (1992) as modified by Gossett *et al.* (1996). Samples were prepared by homogenizing 1 g of frozen leaf or callus tissue, 0.25 g of insoluble polyvinylpyrrolidone (PVP), and one drop of antifoam A emulsion in 2.5 mL of ice cold 50 mM Pipes buffer (pH 6.8), containing 6mM cysteine hydrochloride, 10 mM D-isoascorbate, 1 mM EDTA, 1% PVP-10, and 0.3% (v/v) Triton X-100. The homogenate was centrifuged for 20 min at 4^0C at 10,000 X g. Following centrifugation, 1 mL of the supernatant was centrifuge-desalted through a 10 mL bed of Sephadex G-25 according to the procedure outlined by Anderson *et al.* (1992). A portion of the eluate was analyzed immediately for catalase activity, and the remainder was stored at -70°C for subsequent analysis of SOD, GR, APX, and peroxidase activities. Catalase activity was determined by monitoring the disappearance of H_2O_2 at 240 nm according to the method of Beers and Sizer (1952). Peroxidase activity was measured by monitoring at 675 nm the H_2O_2-dependent oxidation of reduced 2,3',6-trichloroindophenol after the method of Nickel and Cunningham (1969). APX activity was assayed at 265 nm by monitoring the ascorbic acid-dependent reduction of H_2O_2 by the method described by Anderson *et al*. (1992). Total SOD activity was assayed at 550 nm and was measured by determining the amount of enzyme required to produce 50% inhibition of the reduction of cytochrome c by superoxide generated by xanthine oxidase according to the method of Forman and Fridovich (1973). GR activity was determined by monitoring the glutathione-dependent oxidation of NADPH at 340 nm after the method of Schaedle and Bassham (1997). One unit of catalase and peroxidase was defined as the amount of enzyme to decompose 1 µmole of substrate/min at 25^0 C, and one unit of APX and GR was defined as the amount of enzyme required to reduce 1 nmole of substrate/min at 25^0C. All data points are based on a mean of the measurements taken from a minimum of four tissue samples. All data were subjected to a one-way analysis of variance, and significance was determined at the 95% confidence limits.

Results and Discussion

Tables 1 and 5 show the GR activity measured in the different treatments. While GR activity in the NaCl-tolerant control showed a slight, but significant, increase at 4 hr, treatment with 250 mM NaCl resulted in a significant increase in GR activity within 1 hour (Table 1). Pretreatment with 1-butanol failed to inhibit the increase in GR in the callus tissue subjected to NaCl treatment (Table 1). Pretreatment of the NaCl-tolerant controls with 1-butanol also resulted in a significant increase in GR activity at the 2-hr time point (Table 1). As with the treatment with NaCl, treatment of the NaCl-tolerant controls with ABA showed a significant increase in GR activity at 1 hr (Table 1). Pretreatment of the NaCl-tolerant controls with 1-butanol prior to receiving the ABA treatment produced a significant increase rather than an inhibition in GR activity at 1 hr (Table 1). Pretreatment with U73122 significantly decreased GR activity at 2 and 4 hr in the NaCl-tolerant controls (Table 1). While U73122 pretreatment of the NaCl-treated callus did not inhibit the NaCl induced increase, it did reduce the magnitude of the response and delayed the increase in GR activity until the 2-hr time point (Table 1). Treatment with DAG, IP_3, and PI resulted in significant increases in GR activity within 2 hr after receiving the respective treatments (Table 5).

APX activity is shown in Tables 2 and 5. APX activity increased significantly within 30 min in the NaCl-treated callus, while it remained constant throughout the 4-hour time period in the controls (Table 2). Pre-treatment with either 1-butanol or U73122 did not have any effect on the NaCl-tolerant controls, nor did either treatment inhibit the NaCl-induced increase in APX activity; however the 1-butanol treatment did delay the NaCl-induced APX response to the 1 hr time point, and U73122 delayed the increase to the 2 hr time point (Table 2). As with the NaCl treatment, ABA resulted in a significant increase in APX activity within 30 min, and while pre-treatment with 1-butanol did not inhibit the ABA-induced increase, it did delay the response until the 1 hr time point (Table 2). The DAG, IP_3 and PI treatments all resulted in significant increases in APX activity within 2 hr after receiving the respective treatment (Table 5).

Tables 3 and 5 show peroxidase activity. The NaCl treated callus tissue showed a significant increase in peroxidase activity within 1 hr, whereas the NaCl-tolerant control callus tissue showed no significant change in peroxidase activity over the 4-hr time course (Table 3). Other than a slight but significant decrease at time 0, pretreatment with 1-butanol did not significantly change peroxidase activity in the NaCl-tolerant control callus tissue (Table 3). Pretreatment of the NaCl treated tissue with 1-butanol, however, did inhibit the NaCl-induced increase in peroxidase activity (Table 3). The NaCl-tolerant control callus tissue treated with ABA also showed a significant increase in peroxidase activity at the 1-hr time point, and pretreatment with1-butanol did not inhibit this ABA-induced increase in peroxidase activity (Table 3). U73122 pretreatment of NaCl-tolerant control callus tissue did not significantly change peroxidase activity (Table 3). While Pretreatment of NaCl treated callus tissue with U73122 did not inhibit the NaCl-induced increase in peroxidase activity, it did significantly delay the response from the 1-hr time point to the 2-hr time point (Table 3). Like GR and APX, treatment with DAG, IP_3, and PI resulted in significant increases in peroxidase activity within 2 hr after receiving the respective treatments (Table 5).

Catalase activity is shown in Tables 4 and 5. While catalase activity remained constant throughout the 4-hr time period in the control, it increased significantly within 2 hr following the NaCl treatment (Table 4). Pre-treatment with either 1-butanol or U73122 did not have any effect on the NaCl-tolerant controls, and pre-treatment with 1-butanol did not inhibit the NaCl-induced increase in catalase activity (Table 4). On the other hand, pre-treatment with U73122 completely blocked the NaCl-induced increase in catalase activity (Table 4). The ABA treatment, like the NaCl treatment, resulted in a significant increase in catalase activity within 2 hr, and pre-treatment with 1-butanol did not inhibit this increase (Table 4). As with the other antioxidant enzymes, the DAG, IP_3 and PI treatments all resulted in significant increases in APX activity within 2 hr after receiving the respective treatment (Table 5).

Pretreatment of both control and NaCl stressed cotton callus with 1-butanol actually resulted in increases rather than decreases in GR (Table 1) and most of the other antioxidant enzyme activities; however, pretreatment with 1-butanol did inhibit the NaCl-induced increase in peroxidase activity (Table 3). Thus, it would appear that phospholipase D has a very limited, and perhaps no role in the NaCl-induced up-regulation of antioxidant enzyme activity. Pretreatment with 1-butanol also failed to inhibit the ABA-induced responses (Tables 1-4), suggesting that ABA is involved in the up-regulation of antioxidant enzyme activity as has been previously shown, and that the signal pathway does not involve PLD. Perhaps it could be argued that the concentration of 1-butanol used in this study, 1%, was not sufficient to cause inhibition, or the inhibitor was not taken up by the callus tissue. While either of these arguments is possible, it should be noted that 1% 1-butanol is sufficient to inhibit other plant responses such as the ABA-induced closure of the stomata when applied to the leaf surface (Ritchie and Gilroy, 2000). Pretreatment with U73122 decreased and/or delayed the NaCl-induced up-regulation of GR (Table 1), APX (Table 2), and peroxidase (Table 3) activities and completely inhibited the NaCl-induced increase in catalase activity (Table 4) in the NaCl-treated cotton callus tissue, while treatment of the control with either PI, IP3, or DAG resulted in rapid increases in the activities in all four of the enzymes (Table 5). These findings would suggest that phospholipase C may be involved in the NaCl-induced up-regulation of antioxidant enzyme activity in cotton callus tissue. Inhibition of PLC resulted in significant delays and/or reductions in the NaCl-induced increases in the activities all four enzymes and treatment with the enzymatic products of PLC caused significant rapid increases in antioxidant activity, even in the control tissue. While phosphoinositide signaling pathways have not been well characterized in plants, recent studies with *Arabidopsis* have also suggested a role for PLC in response to NaCl stress (Dewald *et al*., 2001). These researchers showed that plant cells rapidly increase phosphatidylinositol 4,5-bisphosphate synthesis and accumulation of its hydrolytic product, IP_3 in response to NaCl stress. Furthermore, they demonstrated that the NaCl-induced accumulation of IP_3 was dramatically reduced when the plants were pretreated with the inhibitor U73122. In animals, IP_3 is associated with calcium mobilization, and DeWald *et al.* (2001) showed not only that NaCl stress increases calcium mobilization, but also that inhibition of IP_3 accumulation with U73122 inhibited the mobilization of calcium.

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Table 1. GR activity (units/g fresh weight ±SE) in NaCl-tolerant callus tissue measured at 0, 0.5, 1, 2, and 4 hours after being subjected to the following treatments: Control, pre-treatment with 0.1 % 1-butanol, pre-treatment with 1 µM U73122, 20 µM ABA, 20 µM ABA following pre-treatment with 0.1% 1-butanol, 250 mM NaCl, 250 mM NaCl following pre-treatment with 0.1% 1-butanol, or 250 mM NaCl following pre-treatment with 1 µM U73122.

	Time (hrs)				
Treatment	**0**	**0.5**	**1**	**2**	**4**
Control	46±3	63±6	48±9	44±4	78±9*
1-butanol	51±8	66±4	71±7	239±21*	93±8*
U73122	42±4	31±3	32±2	24±12	17±3
ABA	44±6	55±5	86±6*	45±4	55±7
ABA + 1-butanol	44±6	49±5	269±17*	72±5*	25±3
NaCl	47±2	79±5	147±12*	161±8*	220±13*
NaCl + 1-butanol	50±5	56±6	256±24*	96±7*	99±4*
NaCl + U73122	42±6	40±3	44±13	139±7*	45±5

* denotes a significant increase.

Table 2. APX activity (units/g fresh weight ±SE) in NaCl-tolerant callus tissue measured at 0, 0.5, 1, 2, and 4 hours after being subjected to the following treatments: Control, pre-treatment with 0.1 % 1-butanol, pre-treatment with 1 µM U73122, 20 µM ABA, 20 µM ABA following pre-treatment with 0.1% 1-butanol, 250 mM NaCl, 250 mM NaCl following pre-treatment with 0.1% 1-butanol, or 250 mM NaCl following pre-treatment with 1 µM U73122.

	Time (hrs)				
Treatment	**0**	**0.5**	**1**	**2**	**4**
Control	511±40	482±24	462±19	460±20	501±35
1-butanol	521±35	464±18	500±37	526±24	572±45
U73122	511±40	501±25	558±32	542±15	509±38
ABA	526±43	1186±24*	921±41*	979±80*	623±45
ABA + 1-butanol	526±43	458±35	980±41*	505±14	190±20
NaCl	582±42	982±12*	1003±12*	1031±45*	870±33*
NaCl + 1-butanol	563±30	467±22	883±56*	474±19	149±13
NaCl + U73122	511±40	552±44	698±69	1009±25*	892±24*

* denotes a significant increase

Table 3. Peroxidase activity (units/g fresh weight ±SE) in NaCl-tolerant callus tissue measured at 0, 0.5, 1, 2, and 4 hours after being subjected to the following treatments: Control, pre-treatment with 0.1 % 1-butanol, pre-treatment with 1 µM U73122, 20 µM ABA, 20 µM ABA following pre-treatment with 0.1% 1-butanol, 250 mM NaCl, 250 mM NaCl following pre-treatment with 0.1% 1-butanol, or 250 mM NaCl following pre-treatment with 1 µM U73122.

	Time (hrs)				
Treatment	**0**	**0.5**	**1**	**2**	**4**
Control	132±4	151±11	109±7	140±8	138±14
1-butanol	68±7	135±4	145±8	153±5	153±9
U73122	122±8	155±7	148±1	114±12	137±13
ABA	136±5	155±6	244±13*	266±12*	124±10
ABA + 1-butanol	148±9	157±6	282±16*	278±10*	168±15
NaCl	131±1	153±11	269±16*	217±6*	148±8
NaCl + 1-butanol	129±5	130±7	117±10	119±11	118±17
NaCl + U73122	143±13	126±10	128±6	297±28*	353±19*

* denotes a significant increase

Table 4. Catalase activity (units/g fresh weight ±SE) in NaCl-tolerant callus tissue measured at 0, 0.5, 1, 2, and 4 hours after being subjected to the following treatments: Control, pre-treatment with 0.1 % 1-butanol, pre-treatment with 1 μM U73122, 20 μM ABA, 20 μM ABA following pre-treatment with 0.1% 1-butanol, 250 mM NaCl, 250 mM NaCl following pre-treatment with 0.1% 1-butanol, or 250 mM NaCl following pre-treatment with 1 μM U73122.

	Time (hrs)				
Treatment	**0**	**0.5**	**1**	**2**	**4**
Control	15±1	16±2	13±2	18±1	15±1
1-butanol	17±1	16±1	16±2	16±1	15±2
U73122	16±1	18±1	16±1	12±1	14±1
ABA	17±1	18±1	15±1	59±5*	15±2
ABA + 1-butanol	17±1	17±1	15±1	35±1*	13±1
NaCl	15±1	15±1	13±2	53±7*	16±2
NaCl + 1-butanol	15±2	16±2	39±4*	15±1	16±1
NaCl + U73122	17±2	14±2	13±1	11±1	12±1

* denotes a significant increase

Table 5. GR, APX, peroxidase, and catalase activities (units/g fresh weight ±SE) in NaCl-tolerant callus tissue measured at 0 and 2 hrs after the following treatments: Controls. 35 μM phosphatidylinositol (PI), 3 μM myo-inositol 1,4,5 -triphosphate (IP_3), or 50 μM diacylglycerol (DAG).

	Enzyme							
	GR		**APX**		**Peroxidase**		**Catalase**	
Treatment	**0**	**2 hr**	**0**	**2 hr**	**0**	**2 hr**	**0**	**2 hr**
Control	46±3	44±4	511±40	460±20	132±4	140±8	15±2	18±1
PI	47±2	153±5*	520±33	1850±65*	128±6	380±6*	16±1	58±1*
IP_3	44±4	139±9*	544±23	1456±78*	135±4	285±35*	15±2	56±6*
DAG	45±3	188±7*	535±41	1110±19	*131±5	290±13	*17±1	40±2*

* denotes a significant increase