Okadaic Acid, a Protein Phosphatase Inhibitor, Blocks Calcium Changes, Gene Expression, and Cell Death Induced by Gibberellin in Wheat Aleurone Cells

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The cereal aleurone responds to both hormonal and environmental stimuli during seed germination. Gibberellic acid (GA) acts as a positive stimulus for hydrolase production by aleurone and promotes seed germination, whereas abscisic acid (ABA) blocks the effects of GA and retards germination (Jones and Jacobsen, 1991). In addition to these hormonal stimuli, seed germination and aleurone function are also regulated by environmental stimuli, such as osmotic stress, hypoxia, and temperature (Fadeel et al., 1980; Moll and Jones, 1982; Hanson and Jacobsen, 1984; Perata et al., 1993). All of these stimuli are perceived by individual aleurone cells, and in the case of hypoxia, GA and ABA have been shown to induce highly characteristic changes in cytosolic Ca²⁺ (Wang et al., 1991; Gilroy and Jones, 1992) and subsequently changes in gene expression (Hanson and Jacobsen, 1984; Nolan and Ho, 1988). Progress has been made in understanding the signal transduction pathways for ABA and hypoxia in other cell types; however, little is known about the intermediates in transduction pathways for GA or how multiple transduction pathways are integrated in a single cell type. Cereal aleurone cells respond to GA with alterations in membrane transport (Bush et al., 1989), gene expression (Chrispeels and Varner, 1987; Jones and Jacobsen, 1991), and cellular structure (Bush et al., 1986). The earliest events in the signal transduction pathway are only beginning to be understood, but there is accumulating evidence that the receptor for GA is at the plasma membrane (Hooley et al., 1991; Gilroy and Jones, 1994) and that intracellular mediators, including Ca²⁺, are involved in subsequent transduction events (Gilroy and Jones, 1992; Bush, 1995). Previously, we have shown that changes in cytosolic Ca²⁺ occur rapidly in response to GA and are apparently the earliest response to GA that has been reported (Bush, 1996). These changes in cytosolic Ca²⁺ are highly correlated with the GA response but are not sufficient by themselves to induce the full GA response. Evidence for the participation of other intracellular mediators or indeed for any other component of the transduction pathway is meager. Early evidence that CAMP activates GA responses has led to the suggestion that cyclic nucleotides and their associated kinases are involved in GA transduction, but this has remained somewhat controversial (Brown and Newton, 1981). Similarly, the ability of fatty acids to inhibit GA-induced amylase production may indicate that lipases function in the GA or ABA transduction pathways (Gilroy and Trewavas, 1990), and evidence for a role of heterotrimeric G proteins has also been obtained (Wang et al., 1993). These numerous observations prompted us to look more closely for evidence that signaling components found in many transduction pathways, such as lipases, G proteins, kinases, and phosphatases, are important in GA signaling in the cereal aleurone.
A valuable approach to elucidating the components of a transduction pathway is identifying pharmacological agents that inhibit or promote stimuli–response coupling. This approach has led, for example, to the identification of heterotrimeric G proteins as probable components of the phytochrome response pathway and the role of protein phosphatases in guard cell opening (Luan et al., 1993; Neuhaus et al., 1993). Pharmacological agents that selectively affect stimulus–response coupling are particularly valuable in identifying the unique features of a transduction pathway. Multiple transduction pathways exist in aleurone cells, as in all cells, and these pathways often interact. In aleurone cells, GA, ABA, and hypoxia induce unique responses, but ABA and hypoxia also modulate the GA response. In an effort to understand the unique features of the GA transduction pathway, we tested the effect of various pharmacological agents on the GA response. We found that a protein phosphatase inhibitor, okadaic acid (OA; Stone et al., 1994), completely blocks every aspect of the GA response that we have measured. OA reduced but did not completely block the response of aleurone cells to ABA, and OA had no effect on their response to hypoxia.

We conclude that OA-sensitive protein phosphatases play a crucial role early in the GA transduction pathway. Our data also indicate that hypoxia and ABA do not block the GA response by altering the activity of this OA-sensitive phosphatase but act at some other point in the GA transduction pathway. Although we have not identified the point at which multiple transduction pathways intersect, we speculate that levels of cytosolic Ca²⁺ are a key point of convergence in the response to ABA, GA, and hypoxia.

RESULTS

OA Blocks the GA Response of Wheat Aleurone Cells

To identify important intermediates in the signal transduction pathway for GA, we surveyed a number of pharmacological agents known to affect the activity of proteins that are commonly part of transduction pathways in plants and animal cells. Inhibitors and activators of heterotrimeric G proteins (Sukumar and Higashijima, 1992; Seo et al., 1995), protein kinases (Conrath et al., 1991; Tamaoki, 1991), and A2 lipase (AbuBakar et al., 1990) had no effect on GA-induced amylase production (Table 1). None of these agents was able to induce the production of amylase in the absence of GA (Table 1). Of the pharmacological agents we tested, only OA was effective at blocking GA-induced amylase production (Table 1). At a concentration of 1 μM in the extracellular solution, OA reduced amylase production to 3% of the GA-treated tissue, an amount approximately equal to amylase production in tissue that had not been treated with GA (Table 1).

OA at submicromolar concentrations is an inhibitor of serine/threonine protein phosphatases PP1 and PP2B. Higher concentrations of OA are required to inhibit PP2A, whereas PP2C is not inhibited by OA (Hardie et al., 1991). A dose–response curve for inhibition of GA-induced amylase production showed that OA was effective at concentrations in the incubation medium between 10 and 100 nM (Figure 1); 10 nM OA had no detectable effect on amylase production, whereas 100 nM OA completely blocked GA-induced amylase production (Figure 1). A similar effect of OA on amylase production was observed in aleurone layers that were not treated with GA. Without GA, however, there is very little amylase production and the effects of OA are correspondingly small (Figure 1).

The ability of OA to inhibit GA-induced amylase production did not change during the course of incubation. The relative amount of inhibition of amylase production was as great at 40 hr as at 26 hr of incubation (Figure 2). Moreover, incubation of aleurone layers in OA for long periods of time indicated that OA did not merely delay the action of GA but instead blocked the GA response for the entire time it was present. Amylase production in GA-treated layers was usually detectable after 12 to 17 hr and increased for up to 50 hr (Figure 2A). In the presence of OA, amylase production was blocked for at least 175 hr. After 50 to 60 hr of incubation, aleurone layers that had not been treated with GA or OA began to produce amylase, indicating that amylase production no longer required exogenous GA (Figure 2A).

The inhibitory effects of OA on amylase production could only be reversed by washing the OA-treated layers in OA-free medium. Incubation of aleurone layers with both OA and the protein kinase inhibitor staurosporine did not reverse the ef-
fects of OA (data not shown). However, if aleurone layers that were preincubated in 100 nM OA plus GA for 17 hr were extensively washed in medium containing GA but free of OA, they began to produce amylase 50 hr after the OA had been removed (Figure 2B). This ability to wash out the effects of OA was observed only if the layers were pretreated with low concentrations (100 nm) of OA. Aleurone layers that had been pretreated with 1 μM OA did not produce amylase even after 175 hr of incubation in OA-free medium (Figure 2B). The increase in amylase production after OA removal occurred with a similar time course to amylase production that did not depend on exogenous GA (compare Figures 2A and 2B).

Amylase production is the terminal step in a complex response pathway that leads from gene expression to secretion. To determine where OA acts in this pathway, we investigated the effects of OA on gene expression. Measurements of amylase mRNA levels for a GA-inducible, high-pl isoform of amylase (Rogers and Milliman, 1984; Huttly et al., 1988) showed that OA completely blocked accumulation of amylase RNA in both wheat and barley aleurone (Figure 3). Barley cDNA 1-28 hybridized to wheat RNA at the same location as did the barley high-pl amylase, and the amount of hybridizing RNA was increased by GA treatment in wheat as it was in barley (Figure 3 and Table 2). Thus, 1-28 is a reliable probe for wheat amylase RNA.

As expected, RNA extracted after 17 hr of incubation in GA showed high levels of amylase mRNA accumulation compared with layers incubated in calcium alone (Figure 3). In the presence of OA, however, GA-inducible amylase mRNA was greatly reduced (Figure 3). In several experiments, GA, ABA, and OA were all found to have no detectable, consistent effect on actin RNA levels. Therefore, actin was used as an internal standard to account for variability in the total amount of mRNA present in each lane (Table 1). Using the ratio of amylase-to-actin RNA levels, we found that aleurone layers treated with OA plus GA had only 4% of amylase RNA levels found in layers treated with GA alone (Figure 3 and Table 2). Thus, the effect of OA on amylase RNA levels is quantitatively similar to the effect of OA on amylase production (Table 2). Treatment with OA by itself also reduced amylase RNA levels compared with those found in layers incubated in calcium alone (Figure 3 and Table 2). Indeed, OA was as effective as ABA in reducing α-amylase RNA levels (Table 2).

In addition to blocking amylase mRNA accumulation, OA also inhibited GA-induced cell death (Figure 4). We observed that an important effect of GA on aleurone cell fate is accelerated cell death. Aleurone cells incubated in ABA or in CaCl₂ alone can survive for more than 4 days without significant changes in cell morphology or viability (Table 3). In the presence of GA, however, a program of cell death is initiated that

![Figure 1](image1.png)

**Figure 1.** Effect of OA on Amylase Production.

Aleurone layers were incubated with GA (+ GA; solid symbols) or without GA (− GA; open symbols) and with different levels of OA. Amylase production was measured after 26 hr (solid circles) and 40 hr (open circles and solid squares) of incubation.

![Figure 2](image2.png)

**Figure 2.** Effect of GA, OA, and the Removal of OA on the Time Course of Amylase Production.

(A) Aleurone layers were incubated with GA (solid circles), without GA (solid squares), or GA plus 100 nM OA (open circles), and amylase production was measured.

(B) Aleurone layers were incubated with GA plus 100 nM OA (solid circles) or GA plus 1 μM OA (open circles) for 17 hr. The incubation medium was then removed and replaced with GA alone; amylase production was then measured.
Figure 3. Effect of GA, OA, and ABA on Amylase RNA Levels.

Wheat (ABA, OA, and calcium) and barley aleurone layers were treated for 17 hr with GA in the presence or absence of 100 nM OA or with ABA, or with calcium alone. RNA was extracted, blotted onto a nylon membrane, and hybridized to a fragment of 1-28, a cDNA that codes for a high-pl form of barley α-amylase (Amylase). The nylon membrane was then stripped of 1-28 and hybridized to a cDNA for actin (Actin).

results in nearly 100% death in <48 hr (Figure 4A). Like other responses to GA in aleurone cells, ABA was able to block GA-induced cell death (Table 3). The morphology of cells that die as a result of GA treatment is highly distinctive. The contents of the cells appeared to coalesce into a compact, opaque ball in the center of the cell. This condensed cellular mass was separated from the cell wall by 30 to 40% of the initial cell diameter (Figures 4D and 4E). In contrast, after 48 hr of treatment with OA and GA, aleurone cells appeared healthy and were indistinguishable from aleurone cells that had not been treated with GA (Figure 4C). OA-treated cells were fully turgid, filled with protein bodies, and in every respect resembled aleurone cells that had been freshly isolated (Figures 4B and 4C). These data indicate that OA does not block amylase production by nonspecifically injuring aleurone cells; rather, the data show that the terminal step in the GA response, cell death, is also inhibited by OA.

Effect of OA on the Response to ABA and Hypoxia

To evaluate the significance of the ability of OA to block GA action, we investigated the effects of OA on the response of aleurone cells to ABA and hypoxia. Both of these stimuli induce the expression of a distinct set of genes, and both block GA-induced amylase production in aleurone cells (Hanson and Jacobsen, 1984; Hong et al., 1988; Perata et al., 1993). A cDNA for an ABA-induced mRNA in barley, PHAV1, hybridized to wheat RNA of the same size as barley, and accumulation of this RNA in wheat was greatly increased by ABA and reduced by GA (Figure 5). Therefore, we used PHAV1 RNA levels to quantitate the ABA response of wheat aleurone cells. When aleurone layers were incubated in CaCl₂ alone, PHAV1 levels were 25% of the level induced by ABA (Table 2). Treatment with OA or GA alone reduced PHAV1 to undetectable levels, and treatment with ABA plus OA resulted in PHAV1 levels that were 35% of that found in layers treated with ABA alone (Figure 5 and Table 2). Thus, at concentrations that completely inhibit the GA response, OA greatly reduced, but did not completely inhibit, the ability of aleurone cells to respond to ABA with changes in gene expression.

The results with PHAV1 indicate that OA inhibits the ABA response in wheat aleurone cells. To determine whether OA affected all stimulus–response coupling, we examined the effect of OA on a nonhormonal stimulus, hypoxia, whose signal transduction pathway is unlikely to intersect the GA pathway at the same point of intersection as ABA. The effects of hyp-

<table>
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<th>Treatment</th>
<th>Amylase (cpm)</th>
<th>Actin (cpm)</th>
<th>Actin (cpm)</th>
<th>PHAV1 (cpm)</th>
<th>Actin (cpm)</th>
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<tr>
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<td>1.4</td>
<td>1,042</td>
<td>1,738</td>
<td>25</td>
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Message levels were determined from RNA blots (Figures 3 and 5) of total RNA extracted from aleurone layers after 17 hr of incubation in medium with CaCl₂ (5 mM) and with or without GA (5 µM), ABA (50 µM), and OA (100 nM). Blots were probed with cDNA for α-amylase (Amylase) and actin or for PHAV1 and actin. Data for PHAV1 levels in layers treated with OA plus GA and for α-amylase levels in layers treated with OA plus ABA are from gels that are not shown.

Ratios were calculated as RNA levels for PHAV1 or for α-amylase divided by the amount of actin RNA on the same RNA blot. Ratios are expressed as a percentage of the maximum ratio obtained in either ABA treatment (for PHAV1) or GA treatment (for α-amylase).
Figure 4. Effect of GA and OA on Accelerated Cell Death.

(A) Aleurone layers were incubated in GA with (+ OA) or without (− OA) 100 nM OA. The percentage of dead cells was determined by staining with methylene blue.

(B) Light micrograph of freshly isolated aleurone cells. Bar = 50 μm.

(C) Light micrograph of aleurone cells after 41 hr of treatment with GA plus 100 nM OA. Bar = 50 μm.

(D) Light micrograph of aleurone cells after 41 hr of treatment with GA. The cell contents have coalesced into a dense body in the middle of the cell. Bar = 50 μm.

(E) Light micrograph of aleurone cells after 41 hr of treatment with GA. Bar = 12 μm.

Table 3. Effect of GA, ABA, and OA on Cell Death in Wheat Aleurone Layers

<table>
<thead>
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<th>Treatment</th>
<th>19 Hr</th>
<th>42 Hr</th>
<th>142 Hr</th>
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<td>17.2 ± 3.4</td>
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<tr>
<td>GA</td>
<td>7.0 ± 1.4</td>
<td>85 ± 4.2</td>
<td>100 ± 0.1</td>
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<td>GA + OA</td>
<td>0.9 ± 0.5</td>
<td>9.1 ± 2.3</td>
<td>12.4 ± 1.6</td>
</tr>
<tr>
<td>ABA</td>
<td>3.7 ± 0.6</td>
<td>5.3 ± 1.4</td>
<td>6.2 ± 2.1</td>
</tr>
<tr>
<td>ABA + GA</td>
<td>2.3 ± 0.3</td>
<td>7 ± 0.8</td>
<td>11.6 ± 4.8</td>
</tr>
<tr>
<td>OA</td>
<td>3.6 ± 1.0</td>
<td>9.1 ± 1.7</td>
<td>10.2 ± 2.3</td>
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</table>

* Aleurone layers were incubated in various media, and the percentage of dead cells, as indicated by methylene blue staining, was measured after 19, 42, and 142 hr of incubation. OA concentrations were 100 nM.

Stimulus-Response Coupling in Wheat Aleurone

oxia in maize and barley are well documented; among these effects is the induction of alcohol dehydrogenase (ADH). Wheat aleurone cells constitutively expressed high levels of ADH activity. Indeed, the mean value of ADH activity in cellular extracts from layers treated under aerobic conditions was 27.3 ± 3.1 nmol of NADH mg⁻¹ of protein min⁻¹ (n = 6) compared with 24.3 ± 3.7 nmol NADH mg⁻¹ of protein min⁻¹ (n = 6) under hypoxic conditions, values that are not statistically different from one another (P > 0.05%).

Visualization of ADH activity after PAGE showed that most of the ADH activity was present in isoforms that were not affected by hypoxia (Figure 6A). However, hypoxia induced at least four unique bands of activity, and this induction by hypoxia was not affected by OA (Figure 6A). To test whether the level of ADH RNA was affected by hypoxia and OA, we designed a 40-bp oligonucleotide that would specifically recognize ADH2 and ADH3, RNA that are induced by hypoxia in barley. Incubation of layers under hypoxic conditions greatly increases the accumulation of ADH2 and ADH3 RNA, which confirmed that these isozymes are induced by hypoxia in wheat aleurone cells (Figure 6B). Moreover, these experiments showed that OA did not have any effect on the increase in ADH2 and ADH3 RNA levels induced by hypoxia (Figure 6B).

GA, ABA, and hypoxia have all been shown to alter levels of cytosolic Ca²⁺ in aleurone cells or protoplasts. GA and hypoxia increase cytosolic Ca²⁺ in wheat aleurone cells (Bush, 1996), whereas ABA has been shown to decrease cytosolic Ca²⁺ in barley aleurone protoplasts (Wang et al., 1991; Gilroy and Jones, 1992). These alterations in cytosolic Ca²⁺ are thought to play an integral role in stimulus-response coupling for all three stimuli. Therefore, we examined the effect of OA on increases in cytosolic Ca²⁺ levels that are induced by GA and hypoxia. We have found that OA blocks the rapid steady state rise in cytosolic Ca²⁺ that is normally induced by GA (Figure 7; Bush, 1996). Hypoxia normally induces large changes in cytosolic Ca²⁺ (Subbaiah et al., 1994; Bush, 1996), and OA did not block this increase. In addition, OA did not
Figure 5. Effect of ABA, OA, and GA on PHAV1 RNA Levels.

Wheat aleurone layers were treated with ABA, ABA plus 100 nM OA, 100 nM OA alone, calcium alone, or GA, and barley aleurone layers were treated with calcium (barley). RNA was extracted after 15 hr, blotted onto a nylon membrane, and hybridized with a cDNA for the ABA-inducible RNA PHAV1 (PHAV1). The nylon membrane was then stripped of PHAV1 and hybridized with a cDNA for actin (Actin).

Figure 6. Effect of Hypoxia and OA on ADH Levels.

(A) ADH activity in aleurone cell extracts visualized after electrophoresis on native polyacrylamide gels. Aleurone layers were treated under hypoxic or aerobic conditions in the presence (+) or absence (−) of 100 nM OA. Major bands of activity correspond to ADH1 isozymes. Minor bands of activity (Induced) correspond to hetero- and homodimers of ADH2 and ADH3.

(B) ADH2 and ADH3 RNA levels in aleurone cells treated under hypoxic (Hypoxic +) or aerobic (Hypoxic −) conditions for 15 hr in the presence (+) or absence (−) of 100 nM OA. RNA was hybridized to an oligonucleotide probe designed to hybridize only with ADH2/ADH3 and not with ADH1 RNA.

it is likely that these agents block the response to many stimuli. We conclude from these observations that protein phosphatases act early in the transduction pathway for GA and are responsible for rapid changes in cytosolic Ca$^{2+}$, which are the earliest responses to GA that have been observed (Bush, 1996).

OA has been found specifically to inhibit protein phosphatases from a wide range of cells. In cellular extracts from both plants and animals, OA at nanomolar concentrations blocks protein phosphatases belonging to two classes, PP1A and PP2A (MacKintosh and Cohen, 1989). PP2A activity is almost completely inhibited at 1 nM OA, whereas similar inhibition of PP1A activity requires 100 nM OA (MacKintosh and Cohen, 1989). OA at nanomolar concentrations does not effectively inhibit the other two classes of protein phosphatases, PP2B and PP2C, both of which are also distinguished from the OA-sensitive groups by a requirement for divalent metal cations. The ability of 100 nM OA to inhibit GA-induced amylase production indicates that PP1 or PP2A protein phosphatases are involved in the early steps of GA action in aleurone cells. Based on the dose–response curve for inhibition of amylase, how-
Figure 7. Effect of OA on GA- and Hypoxia-Induced Changes in Cytosolic Ca\(^{2+}\).

An aleurone layer was loaded with the Ca\(^{2+}\)-sensitive dye fluo-3 and perfused with a solution containing 5 mM CaCl\(_2\), 25 mM Mops-Hepes, pH 5.6, and OA (100 nM). GA was added to the perfusion medium (GA, arrow), and subsequently, hypoxia was induced by stopping the flow of aerated solution (Hypoxia, arrow). Normal oxygen levels were restored to the layer by reestablishing the flow of aerated solution past the layer (Normoxia, arrow).

However, it is not possible to determine which of these protein phosphatases is the principal target for OA in the GA response, because the amount of PP1 and PP2A in aleurone cells is not known. PP1 and PP2A activity is abundant in plant cell extracts, and they exist at micromolar concentration in animal cells (Hardie et al., 1991). Complete inhibition of even the most OA-sensitive phosphatase, PP2A, may therefore require relatively high concentrations of OA (Hardie et al., 1991). Indeed, our data do not exclude the possibility that phosphatases of both classes are involved in GA action.

Multiple forms of OA-sensitive protein phosphatases have now been identified in several plants, including Arabidopsis (Anño et al., 1993) and Brassica (Rundle and Nasrallah, 1992). However, the catalytic subunits of these abundant phosphatases are usually associated with other proteins that control the cellular location and activity of the phosphatases (Hubbard and Cohen, 1991). Therefore, it is likely that multiple forms of PP1/2A exist in aleurone cells and perform a variety of functions. In view of this probable diversity, it is remarkable that OA did not appear to alter normal cellular function or viability. OA induces dramatic changes in root hair cell structure at micromolar concentrations (Smith et al., 1994). However, aleurone cells treated with 100 nM OA for 48 hr did not show any abnormalities in their structure and were healthy, as evidenced by their ability to reduce methylene blue, which is a test for cell viability. Aleurone cells treated with OA were able to respond to hypoxia and, to a lesser extent, to ABA with appropriate changes in gene expression. Moreover, OA greatly reduced cell death that is induced by GA. These observations indicate that the GA response is more sensitive to blockade by OA than are other cellular processes. This sensitivity could be the result of a lack of redundancy in the function of the OA-sensitive protein phosphatase or due to a low abundance of this protein in aleurone cells.

The ability of OA to block rapid changes in cytosolic Ca\(^{2+}\) that are induced by GA indicates that ion transporters are regulated, either directly or indirectly, by protein phosphatases. The activity of ion channels has been shown to be regulated by OA in barley aleurone vacuoles (Bethke and Jones, 1994; PC. Bethke and R.L. Jones, personal communication) and in guard cells (Luan et al., 1993). The slow vacuolar (SV) channel found in aleurone vacuoles is stimulated by Ca\(^{2+}\)/calmodulin and is blocked by OA (Bethke and Jones, 1994). It is unlikely, however, that OA inhibits changes in cytosolic Ca\(^{2+}\) by directly inactivating the aleurone SV channel. Although the SV channel has been found to carry Ca\(^{2+}\) in guard cells (Ward and Schroeder, 1994), it functions as a K\(^+\) channel in aleurone vacuoles (Bethke and Jones, 1994). Moreover, the changes in cytosolic Ca\(^{2+}\) that are induced by GA require extracellular Ca\(^{2+}\); therefore, OA must alter membrane transport events at the plasma membrane. It is possible, however, that the OA-sensitive SV channels are indirectly involved in regulating GA-induced changes in cytosolic Ca\(^{2+}\).

OA is unique among the pharmacological agents that we tested because it is able to block the GA response. Blockers or activators of heterotrimeric G proteins, lipases, and protein kinases had no effect on GA-induced amylase production. These negative data do not eliminate the possibility that G proteins, kinases, and lipases are involved in GA action because the inhibitors may be ineffective against proteins in aleurone cells or because they did not permeate the cell or are required at different concentrations than we used. Indeed, our data are in conflict with recent reports that a mastoparan-like activator of G proteins, Mas7, is able to stimulate amylase production in oat protoplasts (H.D. Jones, R. Deskilan, S. Plakidou-Dymock, and R. Hooley, personal communication).

It is possible that protoplasts are more sensitive to pharmacological agents because the permeability barrier of the wall has been removed. Nevertheless, the inability of protein kinase inhibitors to mimic the GA response is particularly interesting, because it raises the possibility that regulation of protein phosphorylation/dephosphorylation by itself is not sufficient to initiate the full GA response. This interpretation must be treated with some caution because staurosporine and K-252a inhibit a broad range of serine/threonine protein kinases and staurosporine is a potent inhibitor of tyrosine kinases (Tamaoki, 1991). Therefore, these compounds may have multiple effects on cellular processes, which could account for their inability to mimic GA or to reverse the effects of OA. Nevertheless, our data indicate that there are multiple levels of regulation of the GA response in wheat aleurone cells and that, beyond receptor binding, the pathway is not initiated by a single intracellular event but rather by multiple parallel events, one of which is activation of a protein phosphatase.
OA Differentially Affects GA- and Hypoxia-Induced Stimulus-Response Coupling

Multiple regulators of the GA response undoubtedly function during seed germination. Environmental conditions that are unfavorable to growth, such as osmotic stress and hypoxia, block the GA response in isolated aleurone layers. In addition, ABA and hypoxia each induce the expression of a unique set of genes that promote cell survival under unfavorable conditions. Our observation that OA does not interfere with the response to hypoxia is significant for two reasons. First, it indicates that the effects of OA are at least somewhat specific to the GA response, and second, it indicates that hypoxia does not block the GA response through regulation of an OA-sensitive protein phosphatase. OA by itself was not able to induce either hypoxia-specific or ABA-specific genes. Indeed, OA had no effect on either of the two responses to hypoxia that we measured: changes in cytosolic Ca\(^{2+}\) and ADH gene expression. The ability of OA to block GA-induced changes in cytosolic Ca\(^{2+}\) but not those induced by hypoxia further indicates that changes in Ca\(^{2+}\) induced by these two stimuli occur through modulation of different Ca\(^{2+}\) transport proteins. This is consistent with previous studies showing that intracellular stores of Ca\(^{2+}\) are the primary source of Ca\(^{2+}\) changes induced by hypoxia (Subbaiah et al., 1994), whereas in aleurone, extracellular Ca\(^{2+}\) is required for GA-induced changes in cytosolic Ca\(^{2+}\) (Gilroy and Jones, 1992; Bush, 1996). Our data confirm that the transduction pathways for hypoxia and GA are quite separate, even though both involve changes in cytosolic Ca\(^{2+}\).

ABA, like hypoxia, blocks the GA response in aleurone cells. Unlike hypoxia, however, ABA reduces cytosolic Ca\(^{2+}\) and induces a separate set of genes whose function in aleurone is poorly understood. The mechanism by which ABA blocks GA responses is not known. Although OA and ABA had similar effects in blocking GA responses, our data indicate that it is unlikely that ABA acts by inactivating the same OA-sensitive phosphatase that functions in the GA response, because OA also inhibits the ABA response (Figure 5). The importance of protein phosphatases in ABA responses has been elegantly demonstrated through the cloning of the ABA-insensitive locus, ABI (Leung et al., 1994; Meyer et al., 1994). ABI encodes a protein that belongs to the PP2C class of Ca\(^{2+}\)-dependent and OA-insensitive protein phosphatases. The effects of OA on reducing PHA\(\beta\)1 mRNA are therefore not expected to be the result of inhibition of ABI. Of course, an effect of OA on ABI cannot be eliminated until more is known about the characteristics and function of the ABI protein in aleurone cells. The likelihood that OA is acting on different phosphatases in the ABA and GA response pathways is further indicated by the incomplete inhibition of the ABA response at concentrations of OA that completely inhibited the GA response (Table 2).

We have summarized our findings in a working hypothesis regarding how transduction pathways for GA and hypoxia may function in aleurone cells (Figure 8). An essential feature of this hypothesis is that these two pathways can be distinguished by their sensitivity to OA. The ability of OA to block Ca\(^{2+}\) changes, gene expression, and accelerated cell death that are normally induced by GA indicates that an OA-sensitive protein phosphatase is an early part of the GA transduction pathway in aleurone cells. After GA perception, we propose that receptor binding leads to activation of an OA-sensitive protein phosphatase that alters cytosolic Ca\(^{2+}\) through regulation of a Ca\(^{2+}\) transport protein at the plasma membrane, thereby permitting influx of extracellular Ca\(^{2+}\) that is required for GA-induced increases in cytosolic Ca\(^{2+}\) (Gilroy and Jones, 1992; Bush, 1996). The connection between Ca\(^{2+}\) changes and gene expression is not well understood; however, Ca\(^{2+}\) changes by themselves are not sufficient to induce the GA response (Bush, 1996). We have therefore proposed a branch in the transduction pathway involving another, unidentified cellular event. If this model is correct, then we conclude that protein phosphatases are important before the branch point that separates Ca\(^{2+}\) changes and gene expression. Our model would also require that ABA acts before the branch point that separates Ca\(^{2+}\) changes from gene expression. We have not specifically included the action of ABA in our model, but our data indicate that ABA action includes a different set of protein phosphatases from those involved in GA action. Although both ABA and OA effectively block GA responses, the molecular basis for their action is clearly different because OA does not induce other ABA responses and, indeed, inhibits ABA-induced gene expression.

Our working hypothesis (Figure 8) emphasizes the difference in the signal transduction pathways for GA and hypoxia. Although both hypoxia and GA lead to increases in cytosolic Ca\(^{2+}\), the response to hypoxia is not affected by OA. Moreover, the receptor for hypoxia has not been localized to the plasma membrane, and hypoxia-induced Ca\(^{2+}\) changes are hypothesized to occur, as in maize cells, from mobilization of

![Figure 8. A Working Hypothesis of Signal Transduction Pathways of GA and Hypoxia in Wheat Aleurone Cells.](image)
intracellular stores, perhaps from mitochondria or vacuoles (Subbaiah et al., 1994). Our hypothesis predicts that these Ca\(^{2+}\) changes are involved in the regulation of genes, such as ADH, and hemoglobin (Taylor et al., 1994) that promote the survival of cells under unfavorable conditions. There is now considerable evidence that changes in Ca\(^{2+}\) by themselves are sufficient to induce ADH expression in other plant cells. Caffeine, for example, which has been shown to elevate cytosolic Ca\(^{2+}\) in maize suspension cells, also induces ADH gene expression in the absence of hypoxia (Subbaiah et al., 1994).

The signal transduction pathways for GA, ABA, and hypoxia are distinct, but they must interact at one or more points. Because OA inhibits ABA-induced gene expression and has no effect on hypoxia-induced gene expression, it seems unlikely that OA-sensitive phosphatases are a key point of intersection for the three transduction pathways. However, our data and those of others indicate that changes in cytosolic Ca\(^{2+}\) may be a point of intersection. GA and ABA induce opposite changes in cytosolic Ca\(^{2+}\), and it has recently been reported that manipulation of cytosolic Ca\(^{2+}\) levels modifies the ability of ABA to inhibit the GA response (S. Gilroy, personal communication). Although both GA and hypoxia increase levels of cytosolic Ca\(^{2+}\), the spatiotemporal properties of the Ca\(^{2+}\) changes that are induced by hypoxia are quite distinct from those induced by GA. The most current theory regarding Ca\(^{2+}\) signaling, the spatiotemporal model (Berridge, 1993), suggests that the differences in the properties of the Ca\(^{2+}\) change may account for the different responses. The existence of multiple Ca\(^{2+}\)-based transduction pathways in aleurone cells clearly provides an opportunity to test the spatiotemporal model of Ca\(^{2+}\) signaling in plant cells and to understand how multiple signals are integrated to modulate cell function.

**METHODS**

**Plant Material**

Aleurone layers were obtained from deembryonated wheat (Triticum aestivum cv Inia 66-R, 1986 harvest) grains that had been surface sterilized and allowed to imbibe in sterile water at room temperature for 48 hr. Aleurone layers were isolated from the imbibed grain by removing the starchy endosperm under sterile conditions. Isolated aleurone layers were incubated in a medium that contained CaCl\(_2\) (5 mM), gibberelglin (0 or 5 mM), acetic acid (ABA; 0 or 50 mM), and, where indicated, pharmacological agents. Hypoxia was induced in aleurone layers by incubating them in O\(_2\)-free solutions. Cell viability was determined using the vital dye methylene blue (Bush et al., 1986).

**Enzyme Assays**

Amylase activity in aleurone incubation medium was measured using the starch-iodine procedure (Jones and Varner, 1967). Total alcohol dehydrogenase (ADH) activity in cell extracts was assayed spectrophotometrically by measuring the production of NADH in the presence of ethanol, as described by Irish and Schwartz (1987). Cellular extracts were obtained by grinding four to seven aleurone layers in 1 mL of an extraction buffer that contained Tris-HCl (25 mM, pH 8) and \(\beta\)-mercaptoethanol (5 mM). The homogenization was done in an ice bath for 5 min or until no pieces of the layers could be seen. Cellular debris was removed from the homogenate by centrifugation at 16,000g for 15 min at 4°C; the supernatant was used in enzyme assays. Protein in cell extracts was determined according to the method of Bradford (1976), using BSA as the standard. ADH activity was visualized after electrophoresis of cellular extracts containing 20 \(\mu\)g of protein in 75% (w/v) polyacrylamide gels, as described by Russell et al. (1990).

**RNA Gel Blot Analysis**

Total RNA from aleurone layers was isolated and analyzed using modifications of standard procedures (Sambrook et al., 1989). Wheat or barley aleurone layers (1.5 g) were ground in liquid nitrogen to a fine powder, added to a buffer containing guanidine thiocyanate (5 M), sodium citrate (25 mM), sarcosyl (0.5% [w/v]), EDTA (2 mM), polyvinylpyrrolidone (1.5% [w/v]), and \(\beta\)-mercaptoethanol (1.5% [w/v]), and then further ground with a motorized tissue homogenizer (Polytron, Lucerne, Switzerland) for 2 min. The sample was centrifuged at 8000g for 20 min, the pellet was discarded, and 0.3 g/mL of CsCl was added to the supernatant, which was then centrifuged at 126,000g for 24 hr at 20°C in a 13-mL tube containing 3 mL of a CsCl cushion (5.7 M CsCl and 0.1 M EDTA). The supernatant from the 126,000g spin was discarded, and the RNA pellets below the CsCl cushion were dissolved in 400 \(\mu\)L of solution containing urea (7 M) and sarcosyl (2% [w/v]). The RNA was extracted with chloroform-i-butanol (4:1), precipitated, pelleted, washed, dried under vacuum, and stored at -80°C until use. The yield of isolated RNA was, typically, 1 \(\mu\)g per layer with A\(_{260}\)A\(_{280}\) ratios of 1.9 to 2.0. For RNA gel blot analysis, 20 \(\mu\)g of total RNA was denatured in a buffer containing formamide (2.2 M) and formamide (50% [w/v]) at 65°C for 15 min and chilled on ice. The samples were electrophoresed in an agarose gel (1.2% [w/v]) and transferred to a nylon membrane; the membrane was dried in a vacuum oven at 80°C for 2 hr before hybridization.

Levels of amylase and PHAV1 mRNA on the nylon membranes were determined by hybridization to radiolabeled DNA from plasmids containing either 1-28, for hybridization to high-pl forms of \(\alpha\)-amylase (Deikman and Jones, 1985) (obtained from Dr. J. Deikman, Pennsylvania State University, College Park, PA), or PHAV1, for hybridization with an ABA-inducible mRNA (Hong et al., 1988) (obtained from Dr. T-H. D. Ho, Washington University, St. Louis, MO). The plasmids were isolated and radiolabeled using standard methods (Sambrook et al., 1989). After prehybridization, the nylon membrane was then hybridized to the radiolabeled probes at 42°C for 17 hr, washed, and exposed to X-ray film.

Levels of ADH2 and ADH3 mRNA were determined by hybridization to a 40-bp oligonucleotide designed to recognize specifically ADH2 and ADH3 but not ADH1 RNA. The oligonucleotide 5'-ACAAAGGGTGGCTCTGCTCTGCAGTGGCGACAGCTCC-3' was designed from the published sequence for a wheat cDNA that was 98% homologous to ADH3 from barley and 92% homologous to ADH2 from barley (Mitchell et al., 1989). The oligonucleotide was not homologous to any ADH1 identified in any organism. The oligonucleotide was 3' end labeled and hybridized as described for PHAV1 and 1-28. For quantitative comparisons, RNA levels on the nylon membrane were checked by staining with methylene blue (Sambrook et al., 1989) and by measuring actin RNA levels. Actin levels were determined by hybridization with a 632-bp cDNA fragment of rat neuronal actin (Nudel et al., 1983).
which is 70 to 84% homologous to plant actins from a variety of sources, including cereal grains. Hybridization was performed as described for PHAV1 and 1-28.

Cytosolic Calcium

Cytosolic Ca\textsuperscript{2+} in single aleurone cells was measured using the Ca\textsuperscript{2+}-sensitive fluorescent dye fluo-3 (Molecular Probes, Eugene, OR). Aleurone layers were prepared and loaded with fluo-3, as described by Bush (1996). Fluo-3 fluorescence was measured photometrically in living cells that were perfused with the same incubation solutions described above. Hypoxia was induced by stopping the flow of oxygenated solution past the layer. Fluorescence levels were calibrated in terms of Ca\textsuperscript{2+} concentrations by saturating the dye with Mn\textsuperscript{2+} in the presence of the calcium ionophore A23187 (Bush, 1996).

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