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Involvement of Intracellular Calcium in Anaerobic Gene Expression and Survival of Maize Seedlings[1]

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Ca-mediated processes are known to be involved in transducing many developmental, hormonal, and environmental cues in plant cells. In this study, the role of Ca in the perception of anoxic stress signals by maize (*Zea mays* L. cv B73) roots was assessed by studying the effect of various Ca antagonists on the induction of alcohol dehydrogenase (ADH) and sucrose synthase mRNA as well as ADH activity under anoxia. The effect of these compounds on the poststress recovery of the seedlings was also monitored. Ruthenium red (RR), an inhibitor of organellar Ca fluxes, repressed the anoxic activation of the *alcohol dehydrogenase1* and *shrunken1* genes as measured by their transcript levels as well as ADH activity. Furthermore, RR-treated seedlings could not recover even after only 2 h of flooding, in contrast to untreated B73 seedlings that survived 72 h of submergence. Ca, when supplied along with RR, allowed normal anoxic gene expression and also prevented the RR-imposed death of the seedlings from short-term anoxia. Ca (^{45}Ca) fluxes were measured in maize roots to elucidate the mode of action of RR. RR abolished anoxia-stimulated ^{45}Ca influx into maize roots but did not affect aerobic Ca^{2+} uptake, unlike a few other antagonists that blocked both the aerobic and anoxic fluxes. However, Ca uptake across the plasma membrane was not necessary for the adaptive response to anoxia, since chelation of extracellular Ca by ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid or 1,2-bis(2-aminophenoxy)ethane *N,N,N',N'*-tetraacetic acid did not affect the induction of ADH activity or poststress survival of flooded seedlings. The data suggest that RR may act on one of the intracellular stores of Ca and the Ca mobilized from this source is a physiological transducer of anoxic stress signals in maize roots.

Oxygen deprivation is the primary stress factor in flooded soils. Maize (*Zea mays* L.) roots respond to anoxia by synthesizing a unique set of polypeptides (Sachs et al., 1980). The genes coding for these polypeptides (e.g. ADH) are rapidly induced even under mild hypoxia (Wignarajah and Greenway, 1976; Paul and Ferl, 1991; Andrews et al., 1993) probably as an adaptive mechanism. Such a response also implicates a fast and precise oxygen-sensing system operating in plant cells. However, there is little information concerning how plants sense the changes in external oxygen concentration and how this information is translated into a physiological response.

Transient changes in cytosolic Ca^{2+} have been implicated in many signal transduction pathways, including tip growth in root hairs and pollen tubes, morphogenesis, phytohormone responses, tropic movements, stomatal closure, and environmental stresses (see reviews by Hepler and Wayne, 1985; Poovaiah and Reddy, 1993). Ca fluxes across the plasma membrane, ER, vacuole, and mitochondria are proposed to modulate cytosolic Ca^{2+} levels precisely within the physiological range (Tester, 1990). Ca antagonists as well as Ca^{2+} channel blockers have been successfully used to elucidate the role of Ca in stimulus-response coupling in animal systems and more recently in plants as well (Saunders and Hepler, 1983; Reddy et al., 1988; Knight et al., 1992; Schiefelbein et al., 1992). In the absence of appropriate mutants or genetic variants, these chemical agents facilitate experimenting with intact plants. However, their penetration into plant cells, appropriate concentrations, specificity, and reversibility of their effects are important considerations when using these pharmacological agents.

In this study, we show the impairment of anoxic gene expression and poststress recovery of maize roots by a specific Ca^{2+} antagonist and prevention of the antagonist's action by Ca. We propose that Ca is involved in communicating low oxygen signals in intact maize roots.

MATERIALS AND METHODS

Plant Material

Caryopses of inbred maize (*Zea mays* L.) line B73 were germinated and grown aseptically at 28°C and 90% RH in the dark for 5 d. The seedlings used had primary roots of 5 to 7 cm and preemergent shoots.

Chemicals

RR (98%) was purchased from K & K Laboratories (Division of ICN Biomedicals). The other Ca^{2+} antagonists were from Sigma or Calbiochem. All chemicals used in the study were analytical or Ultrapure grade.

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Abbreviations: ADH, alcohol dehydrogenase; *adh1*, *alcohol dehydrogenase1* gene; BAPTA, 1,2-bis(2-aminophenoxy)ethane *N,N,N',N'*-tetraacetic acid; RR, ruthenium red; *sh1*, *shrunken1* gene; SS, sucrose synthase.

Seedling Treatments

Ca antagonists were dissolved in either water (lanthanum chloride, verapamil, methoxy verapamil, diltiazem, bepridil, and RR), methanol (TMB-8, final concentration of methanol not exceeding 0.1%), or DMSO (nifedipine and thapsigargin; final concentration of DMSO not exceeding 0.2% [v/v] in water) and used in the concentrations given in Table I. Ten to 20 seedlings were incubated aerobically on wet Kimwipes with or without the above compounds. After 1 h, one-half of the seedlings from each treatment was submerged in flooding buffer (5 mM Tris-Cl [pH 7.5], Augmentin [Beecham; 250 mg of amoxicillin plus 125 mg of potassium clavulanate/L]) alone or with the corresponding compound for different periods. The other half remained under aerobic incubation.

Soluble Protein Extraction and ADH Assay

Roots from aerobic and submerged seedlings (generally a 16-h treatment) were sliced and ground in ice-cold extraction buffer (50 mM Tris-Cl [pH 6.8], 15% glycerol, 20 mM 2-mercaptoethanol, and 1 mM PMSF) in 1.5-mL microfuge tubes, with a Pellet Pestle (Kontes) and acid-washed sand. The extracts were spun at 6000 rpm for 5 min, and the supernatant was used for the ADH assay according to the method of Russell et al. (1990). The activity of each sample was normalized against A_{280} (Russell et al., 1990).

RNA Extraction and Northern Analysis

Seedlings from both aerobic and 2-h submergence treatments were blotted and immediately frozen in liquid N_2. Total RNA was isolated from root tissue according to the method of Russell et al. (1990). RNA was electrophoresed on formaldehyde-agarose gels and blotted onto a nylon membrane by capillary action. A single hybridization (Church and Gilbert, 1984) was done using three cDNA clones radioactively labeled by random priming. Clones used in this study were pZmL793 (maize ADH cDNA corresponding to the *adh1* gene [Dennis et al., 1984]), psh (a 700-bp genomic clone corresponding to the maize *sh1* gene, which encodes SS, encompassing the last two exons [Russell et al., 1990]), and pZmL 1055 (a maize cDNA clone whose transcript level does not change during anoxia [Sachs, 1991]). The *adh1* and *sh1* genes respond within 2 h of flooding, unlike another anoxic gene of unknown function that shows delayed induction (Sachs, 1991). ADH and SS also have different transcript sizes, allowing them to be probed concurrently. Hence, these two clones were chosen as markers of anoxic gene activation.

Although rRNA bands were used to equalize loading, clone 1055 (no. 55, hereafter) was selected as an internal control to normalize the hybridization data for the following reasons: (a) This clone hybridizes to a constitutive mRNA that is unusually stable and remains steady even up to 72 h (the anaerobic survival limit of B73) of anoxia (Sachs, 1991). Any nonspecific effects (toxic and those unrelated to anoxia) of the pharmacological agents used are expected to reflect in the transcription and/or stability of no. 55 transcript. rRNA, being a structural RNA, may be less affected by these chemicals. (b) ADH and SS transcripts banded close to the 18S and 28S rRNA bands, respectively. Hence, using rRNA levels for normalization of hybridization data necessitated stripping the blot to reprobe with rDNA. The distinct size of the no. 55 transcript allowed probing with all three clones in a single hybridization reaction and also comparing the signals (after quantification) more precisely from a single autoradiograph. Thus, no. 55 proved to be a better internal control than rRNA. Furthermore, using these three probes together did not affect any of their hybridization properties.

Autoradiograms with exposures that were within the linear range of measurement (lower than that used for photography) were densitometrically scanned. ADH and SS signals were measured relative to the no. 55 signal in each lane. Furthermore, the corresponding aerobic values were subtracted from each treatment, and the difference was expressed as relative intensity.

Table I. *Effect of Ca^{2+} antagonists on anoxia-induced ADH activity and poststress recovery of maize seedlings*

Antagonist	Concentration Range	ADH Activity[a]	Poststress Recovery[a]
	nM		
Lanthanum	0.10–1.00	90	70–100
Verapamil	0.05–0.25	95	100
Methoxy verapamil	0.01–0.10	87	100
Diltiazem	0.02–0.10	100	90
Bepridil	0.02–0.10	84	80
RR	0.01–0.10	<10	0–20
Nifedipine	0.01–0.05	50–70	100
Thapsigargin	0.10	120	Not tested

[a] Percentage of control values.

Measurement of Ca Flux

Ca exchange into the roots was measured by incubating whole plants in 0.01 μCi ^{45}Ca/mL of flooding buffer with or without the Ca antagonists for 2 h at 28°C in the dark, both under aerobic and submerged conditions. The plants were then washed in 1 mM Na EDTA (pH 8.0) for 10 min and for another 10 min in 2 mM $LaCl_3$ to remove the cell-wall-bound Ca. The apical 2-cm fragment of the root (since there were no allometric changes in the root during the treatments) was used to determine the ^{45}Ca levels by liquid scintillation counting. Ten seedlings were used for each treatment.

Since the EDTA and lanthanum washes do not completely displace cell-wall-bound ^{45}Ca, seedlings that were pretreated overnight with 2% Triton X-100 were used in the assays to determine the cell-wall-sequestered ^{45}Ca. Alternatively, seedlings after ^{45}Ca uptake, as well as the washes, were incubated in the detergent for the same period, and the residual ^{45}Ca (in the cell wall) was determined.

Flood Tolerance Tests

Following aerobic or submergence treatment, seedlings were transferred to a sand bench in the greenhouse. A 10-d period was allowed for seedling establishment, after which the plants were uprooted and scored for root and shoot growth.

Effect of Ca Chelators on Anoxic Survival and ADH Activity

To test the effect of extracellular Ca deprivation, maize seedlings were pretreated and submerged in the presence of 1 or 5 mM EGTA or BAPTA. The postanoxic survival and ADH activity were assayed as described earlier.

RESULTS

Screening for Ca^{2+} Antagonists that Affect the Flooding Response

We developed a simple physiological test to identify Ca channel blockers that may possibly interfere with the early molecular response to anoxia. We tested various pharmacological agents for their effect on the induction of ADH activity as well as postsubmergence survival after 24 h of flooding (Table I). ADH activity is induced severalfold in maize seedlings during flooding and is essential for their anoxic survival (Schwartz, 1969). Since the enzyme can also be rapidly assayed, it served as a biochemical marker of the adaptive response. Preemergent seedlings of B73, the cultivar used in this study, can survive 3 d of submergence (Lemke-Keyes and Sachs, 1989), and the extent of survival after 1 d of flooding was chosen as the other criterion for screening. Of the chemicals tested and selected for those without any adverse effect on the aerobic growth of the seedlings, only nifedipine and RR affected ADH activity. However, nifedipine, like most other antagonists, did not affect the poststress recovery of the seedlings, whereas RR impaired the survival of anoxic seedlings (Table I). Some agents such as A_{23187}, millimolar levels of lanthanum, and cadmium affected anoxic survival and induction of ADH activity, but they also impaired the aerobic growth or survival of seedlings. TMB-8, a known inhibitor of Ca release from ER, also selectively blocked anaerobic survival and induction of ADH activity. However, methanol, the solvent used for dissolving TMB-8, had toxic effects on the anoxic seedlings and made it difficult to distinguish the specific effects of TMB-8. In addition, the compound itself was effective at or above 100 μM (data not shown).

Effect of Antagonists on the Induction of Anaerobic Genes

A few of these compounds were further tested for their effect on the steady-state level of transcripts for two anaerobically inducible genes, namely *adh1* and *sh1*, at the end of 2 or 16 h of flooding. Only nifedipine and RR decreased the levels of the two mRNAs in submerged seedlings. Nifedipine, however, was effective only after 16 h of anoxia (data not shown). At 2 h of submergence, RR was found to be the only Ca^{2+} antagonist that affected the early response to anoxia in maize (Fig. 1). Even at the lowest concentration used (10 μM), RR repressed ADH and SS mRNA to one-third of the levels induced in the untreated (control) maize root tissue. At 50 μM, RR almost completely abolished the induction of these messages (Fig. 1). The RR levels used here are in the same range as were shown to be effective for the inhibition of Ca fluxes in other systems (Chanson, 1991; Currie et al., 1992; Fletcher et al., 1993; Wilkinson and Duncan, 1993). In tobacco seedlings, RR at 50 μM prevented the wind-induced cytosolic Ca increase (Knight et al., 1992).

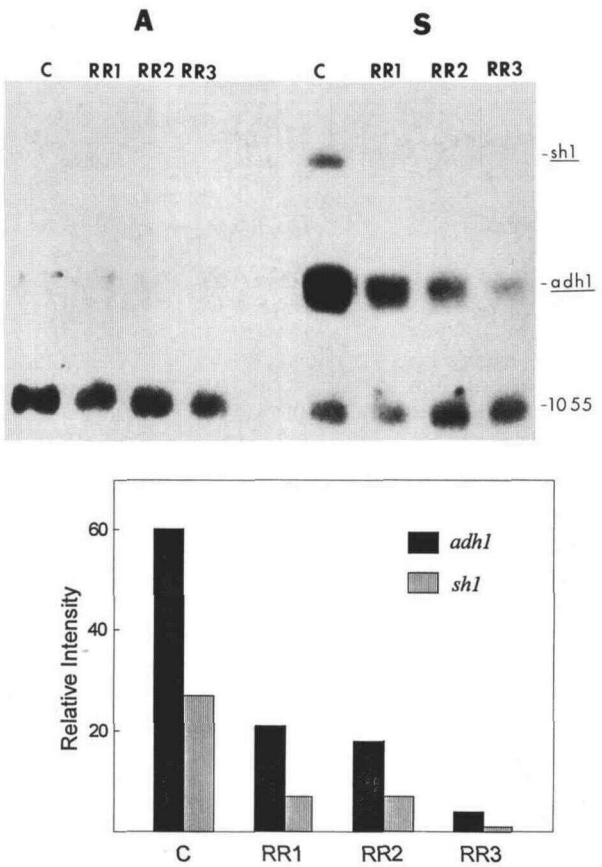

Figure 1. Effect of RR on the expression of *adh1* and *sh1* after 2 h of submergence in maize seedlings. Four to 5-d dark-grown maize seedlings were pretreated for 1 h in 0 μM (C), 10 μM (RR1), 25 μM (RR2), or 50 μM (RR3) RR and later either submerged for 2 h (S) or maintained aerobically (A). Total RNA (10 μg) from each treatment was used to determine the levels of ADH (*adh1*), SS (*sh1*), and no. 55 mRNAs by northern analysis. The levels of ADH and SS transcripts were densitometrically quantified and normalized to the level of no. 55 in each sample. The corresponding aerobic values were subtracted from these, and the differences were expressed as the relative intensities. The names of the bars and lanes correspond.

The transcript for no. 55 was unaffected by RR even at the highest concentration used, both in aerobic and anoxic conditions (Fig. 1), suggesting that the Ca^{2+} antagonist was not indiscriminately inhibitory to transcription and/or stability of the transcripts under anoxia.

Effect of Ca Antagonists on ^{45}Ca Uptake by Maize Roots

Maize roots took up more Ca under anoxia than during aerobic incubation (Table II). We used detergent-incubated (either before or after the ^{45}Ca uptake, see "Materials and Methods" for details) seedlings to determine the residual ^{45}Ca in the cell wall that was unavailable to EDTA and lanthanum washes. The Triton X-100-treated roots had only about 10% of ^{45}Ca taken up by those from untreated seedlings after the EDTA and lanthanum washes. Neither RR nor anoxia influ-

Table II. *Exchange of ^{45}Ca by maize roots during a 2-h incubation*

The experimental details are given in "Materials and Methods." The values are means ± SD of three replicates.

Treatment	Exchange	
	Aerobic	Anoxic
	ng of Ca	
Control	0.64 ± 0.14	3.60 ± 0.74
RR		
10 μM	0.60 ± 0.05	3.11 ± 0.12
25 μM	0.50 ± 0.12	0.64 ± 0.03
50 μM	0.51 ± 0.03	0.55 ± 0.01
Bepridil, 50 μM	0.46 ± 0.07	3.05 ± 0.79
Verapamil, 100 μM	0.54 ± 0.03	3.44 ± 0.62
0.1% DMSO	0.84 ± 0.14	3.28 ± 0.38
Nifedipine, 50 μM	0.27 ± 0.04	2.64 ± 0.13

enced this cell-wall component of ^{45}Ca flux. Thus, our assay accurately reflected Ca fluxes into the symplast. The anoxia-stimulated Ca influx was consistent at 5-fold of aerobic uptake. RR had little effect on the Ca flux into well-aerated maize roots but decreased the anoxia-induced influx (Table II). The uptake was inversely related to RR concentration, and the anoxia stimulation was almost completely abolished at 25 μM (Table II). Other antagonists, e.g. nifedipine, verapamil, and bepridil, also affected Ca uptake. However, their inhibition was mild (except for the effect of nifedipine on the aerobic uptake), and the anoxia-induced Ca^{2+} flux was not reversed by any other antagonist (Table II).

Ca Prevention of RR Effect on Anoxic Gene Expression

Since RR is shown to act on Ca^{2+} transport (Table II), prevention or reversibility of its effect by Ca would support the premise that RR effects on anaerobic gene expression and survival are due to a specific blockage of Ca^{2+} fluxes. We chose 25 μM RR in this set of experiments because this concentration was nearly optimal for the repression of ADH and SS mRNA as well as Ca^{2+} uptake, and it was devoid of any visible secondary effects. $CaCl_2$ (2 mM), when supplied concurrently with RR, prevented the effect of the antagonist (Fig. 2). A 9-fold increase of ADH mRNA level was effected in Ca plus RR-treated seedlings relative to the level in plants treated with RR alone. The changes (either inhibition by RR or recovery with Ca) in the SS messenger levels were not as dramatic in this experiment.

Ca was also tested for its effect on RR inhibition of ADH activity (Table III). Although distinct induction of ADH mRNA is achieved within the first 2 h of anoxia, a comparable increase in the enzyme activity can be detected only after 8 to 10 h of submergence (Russell et al., 1990; Andrews et al., 1993). Hence, flooding was continued for up to 16 h in this set of experiments. RR at 25 μM almost completely blocked induction of ADH activity after 16 h of flooding, and this inhibition was prevented by Ca (Table III). This suggested that the effects of RR and Ca were not transient. Addition of Ca to RR-treated seedlings 1 h after the onset of submergence failed to reverse the repression of ADH activity (Table IV), suggesting that the presence of Ca very early is critical for

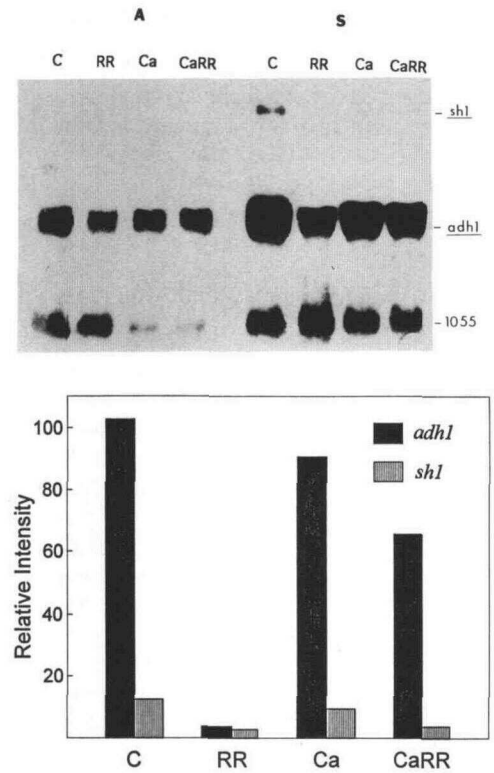

Figure 2. Ca prevents RR repression of anoxic gene expression in maize seedlings. Dark-grown maize seedlings, pretreated for 1 h with none (C), 25 μM RR (RR), 2 mM $CaCl_2$ (Ca), or both (CaRR), were used for flooding and northern analysis as described in Figure 1.

the prevention of RR action on the anaerobic response. However, the inhibition of ADH activity by RR at 10 μM was partly reversed by subsequent incubation in $CaCl_2$ (Table IV).

Addition of RR or Ca to the soluble extract did not affect ADH activity, showing that their in vivo effects are not caused by any direct effect on the enzyme itself. Also, Ca apparently did not block the uptake of RR by the roots as evidenced by the staining of roots. In fact, at lower concentrations of RR (2–5 μM), roots were more intensely colored in the presence of $CaCl_2$ than without, ruling out any extracellular interaction between RR and Ca^{2+} being responsible for the observed antagonism.

Table III. *Effect of RR and Ca on the induction of ADH activity in the root tissue of maize seedlings submerged for 16 h*

Each value is the mean ± SD of three separate experiments.

Treatment	ADH Activity	
	Aerobic	Anoxic (16 h)
	enzyme units min^{-1} A_{280}^{-1}	
Control	105 ± 7	216 ± 19
RR (25 μM)	75 ± 14	82 ± 9
Ca (2 mM)	78 ± 13	226 ± 19
RR (25 μM) + Ca (2 mM)	93 ± 7	216 ± 22

Table IV. *Ca reversibility of RR inhibition on the induction of ADH activity in maize roots*

After pretreatment (1 h) and submergence (1 h) in the presence of RR, the seedlings were transferred to flooding buffer with or without 5 mM $CaCl_2$ (RR to Ca or RR to H_2O, respectively) and submerged for an additional 15 h. The RR and control seedlings were submerged for 16 h in the flooding buffer with or without RR, respectively. Each value is an average of two experiments.

Treatment	ADH Activity		Percentage of Control[a]
	Aerobic	Anoxic (16 h)	
	Enzyme units min^{-1} A_{280}^{-1}		
Control	78	162	100
RR (10 μM)	70	82	9
RR to H_2O	73	71	0
RR to Ca	72	135	75
Control	104	210	100
RR (25 μM)	75	85	10
RR to H_2O	80	107	25
RR to Ca	80	115	35

[a] The aerobic enzyme activity in each treatment is subtracted from the corresponding anoxic ADH activity before computing the percentages.

Effect of RR and Ca on Seedling Recovery from Short-Term Anoxia

Seedlings treated with RR (25 μM) failed to recover after as few as 2 h of anoxia. At the same time, the compound did not affect the survival and growth of their aerobic counterparts (Fig. 3). Even a mild hypoxia caused by an excess of incubation medium during "aerobic" treatment affected the growth and root survival of RR-treated seedlings (data not shown). Aerobic incubation of poststressed control seedlings in RR also did not affect their survival and growth, indicating that the effect of this antagonist was specific to anoxia. During flooding, roots of the RR-treated seedlings apparently died, as evidenced by their decay soon after planting (Fig. 3). We noted a temporary recovery of shoots in some RR-treated seedlings after 2 h of anoxia (the preemergent shoots were apparently impenetrable to the drug as denoted by the non-acquisition of red color when submerged), but subsequently these plants also died. More recently, we observed that even 10 μM RR was toxic to roots after 1 h of submergence (data not shown). In contrast, untreated B73 seedlings show more than 60% survival even after 72 h of anoxia (data not shown; Lemke-Keyes and Sachs, 1989). Even the ADH nulls of maize are known to survive at least 6 h of flooding (Schwartz, 1969; Lemke-Keyes and Sachs, 1989).

The prevention of RR action on anaerobic gene expression by Ca was also manifested in the postanoxic recovery of the seedlings (Fig. 3). Ca addition along with RR prevented the seedlings from the anoxic death, and the effect was linear up to 5 mM $CaCl_2$ (data not shown). The concurrent presence of Ca with 25 μM RR was again found to be critical during the first 1 or 2 h of anoxia. Subsequent addition of Ca^{2+} did reverse the repression of 10 μM RR on seedling survival (data not shown).

Effect of EGTA and BAPTA on Anoxic Survival and ADH Activity

Since RR prevented anoxia-induced Ca influx into maize roots (Table II) and addition of $CaCl_2$ prevented RR repression of the anaerobic response (Figs. 2 and 3 and Tables III and IV), it was inferred that a rapid influx of Ca across the plasma

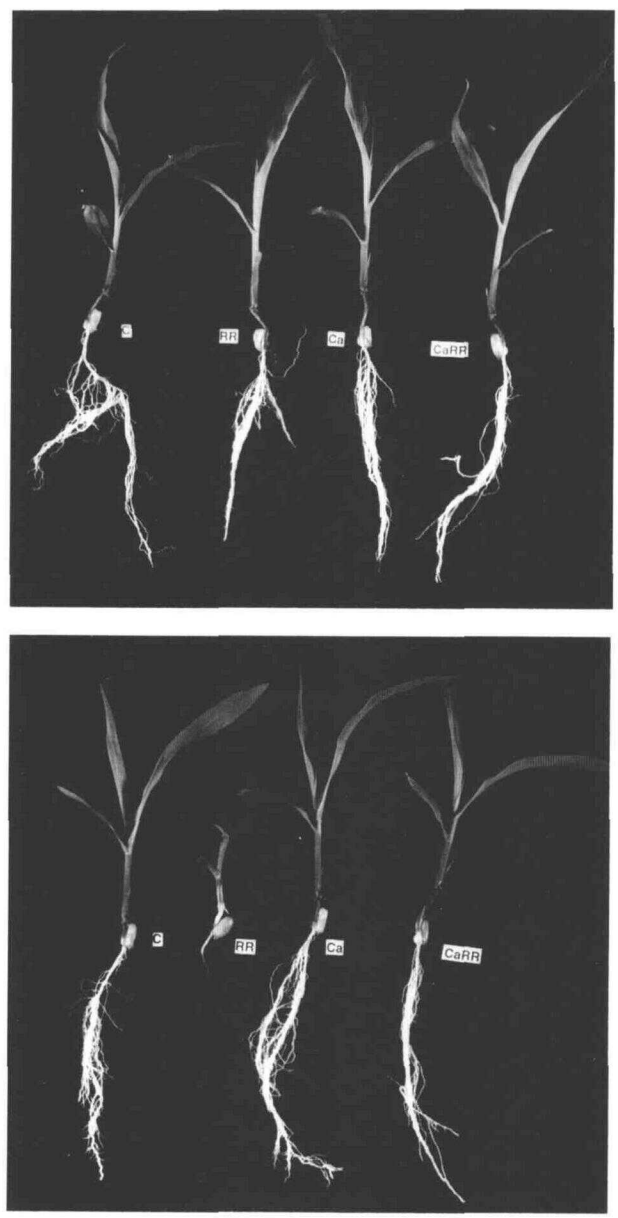

Figure 3. Effect of RR and $CaCl_2$ on the poststress recovery of maize seedlings submerged for 2 h. Maize seedlings were pretreated with none (C), 25 μM RR (RR), 2 mM $CaCl_2$ (Ca), or both $CaCl_2$ and RR (CaRR) for 1 h, and one-half of the plants from each treatment was subjected to 2 h of flooding. Subsequently, the seedlings were planted in the sand bench and allowed to establish. After 10 d, the plants were uprooted, and the ones with the mean root and shoot lengths from each treatment were photographed. The top panel shows "aerobic" seedings and the bottom panel "anoxically" treated seedings.

membrane may be essential for the expression of anoxic genes and seedling survival. We tested this possibility by pretreating and submerging the seedlings in the presence of the Ca chelator EGTA or BAPTA. The poststress recovery after 2 h of flooding and ADH activity (after 16 h of anoxia) were monitored (Tables V and VI). Neither chelator affected the induction of ADH activity (Table V) or the recovery of the submerged seedlings (Table VI). In fact, deprivation of the external Ca even for 3 h affected the aerobic growth of the seedlings (e.g. treatment with 5 mM BAPTA) but mildly stimulated their root growth during recovery after anoxia (Table VI).

DISCUSSION

Ca is implicated in the perception of many environmental stimuli such as mechanical stress (Jones and Mitchell, 1989), salinity (Lynch et al., 1988), wind, and cold shock (Knight et al., 1992) by plants. However, there has been no information concerning the role of Ca^{2+} in the anaerobic stress response of plants. In animal cells, hypoxic or anoxic elevation of cytosolic Ca has been very well documented (Shen and Jennings, 1972; Allshire et al., 1988; Gasbarrini et al., 1992a, 1992b; Miyata et al., 1992). Despite this, the role of Ca in anoxic gene activation has not been investigated even in animal systems, except for some circumstantial evidence (Aldashev et al., 1991; Gasbarrini et al., 1992a, 1992b).

Our observation that RR-treated maize seedlings could not tolerate even a very brief period of submergence (or even mild hypoxia without submergence) suggests that RR may be interfering with a step critical for the survival of the plants during oxygen deprivation. This interference was associated with the repression of ADH activity (Table III) and other genes important for anaerobic metabolism (Fig. 1). However, RR effects on submerged seedlings cannot be explained by mere inhibition of ADH activity, since ADH nulls can survive at least 6 h of anoxia. Induction of anoxic genes (those involved in glycolytic and sugar-phosphate metabolism) is no doubt part of the adaptive response to anoxia. However, several events are expected to precede gene activation, and RR may be impairing one of these early steps rather than directly affecting gene expression. The inhibitory effect of RR on Ca transport is well documented (Moore, 1971; Ferrari et al., 1982; Chanson, 1991; Wilkinson and Duncan, 1993).

Table V. *Effect of EGTA and BAPTA on the induction of ADH activity after 16 h anoxia in maize roots*

Each value is the mean ± SD of three replicates.

Treatment	ADH Activity	
	Aerobic	Anoxic
	units min^{-1} A_{280}^{-1}	
Control	77 ± 4	165 ± 5
EGTA		
1 mM	83 ± 2	194 ± 15
5 mM	54 ± 3	197 ± 3
BAPTA		
1 mM	108 ± 6	170 ± 11
5 mM	25 ± 2	122 ± 35

Table VI. *Effect of EGTA and BAPTA on postanoxic growth of maize seedlings*

The values are means ± SE of 10 seedlings.

Treatment	Root		Shoot	
	Aerobic	Anoxic	Aerobic	Anoxic
	mg fresh wt/seedling			
Control	245 ± 43	193 ± 31	573 ± 60	575 ± 24
EGTA				
1 mM	311 ± 59	131 ± 42	531 ± 13	464 ± 41
5 mM	289 ± 41	340 ± 38	438 ± 46	607 ± 42
BAPTA				
1 mM	267 ± 35	317 ± 33	408 ± 81	515 ± 17
5 mM	170 ± 20	234 ± 34	267 ± 38	418 ± 32

In the present study, it was also shown that the anoxia-stimulated Ca^{2+} influx was abolished by RR (Table II; also by TMB-8, another inhibitor of intracellular Ca release, data not shown), whereas an exogenous supply of Ca prevented RR-induced impairment of anoxic survival (Fig. 3). Furthermore, the critical presence of Ca^{2+} very early and concurrently with RR for the preventive effect implicates Ca as a probable mediator of an early cellular response to anoxia. Our recent studies with maize suspension-cultured cells show that hypoxia rapidly elevates cytosolic Ca^{2+}, and this elevation is abolished by RR (C.C. Subbaiah, D.S. Bush, and M.M. Sachs, unpublished data). In addition to the anoxia-stimulated Ca^{2+} exchange through the apoplast demonstrated here (Table II), we postulate that a release of Ca from internal stores also is responsible for this elevation (also see below). Such a scenario is consistent with our cytosolic Ca measurements by photometry and imaging in the presence of RR and Ca chelators (C.C. Subbaiah, D.S. Bush, and M.M. Sachs, unpublished data).

Although the exact source of Ca release in anoxic maize root cells remains to be determined, the response is shown to be independent of extracellular Ca. The ineffectiveness of well-known plasma membrane Ca^{2+} channel blockers on the anoxic responses, including elevated Ca influx (Tables I and II), and its specific repression by RR (and probably TMB-8) indicate that the Ca might be mobilized from intracellular sources during anoxia. Our Ca chelators experiment also showed that any trans-plasma membrane Ca influx is not required for anoxia tolerance (Tables V and VI), since these polycarboxylic compounds effectively sequester apoplastic Ca^{2+} but do not reach the intracellular stores (McAinsh et al., 1991; Himpens et al., 1992; Tsien, 1992).

A biphasic cytosolic elevation of Ca involving mobilization from internal stores (peak I) followed by an influx (peak II) was shown to be induced by anoxia in hepatocytes (Gasbarrini et al., 1992a). Treatments that alleviated anoxic injury (either removal of extracellular Ca^{2+} by chelators or perfusion with Fru) abolished peak II (influx) with no effect or an increase in peak I, showing that the influx was not essential if not inhibitory for anoxic survival (Gasbarrini et al., 1992a, 1992b) in accordance with our results from the chelator experiment (Tables V and VI). Furthermore, RR at 10 μM inhibited anoxic survival (data not shown) and gene expres-

sion (Fig. 1 and Table IV) with little effect on extracellular ^{45}Ca influx (Table II). RR at this concentration can completely block the anoxic elevation of cytosolic Ca in maize suspension cells independently of extracellular Ca (C.C. Subbaiah, D.S. Bush, and M.M. Sachs, unpublished data). It therefore follows that RR may be blocking Ca^{2+} mobilization from an internal source that is critical for survival during submergence.

In contrast, the inhibition of anoxia-stimulated Ca^{2+} flux into roots by greater levels of RR (Table II) and prevention of the RR effect by extracellularly added Ca^{2+} (Figs. 2 and 3 and Table III) suggest that this RR-sensitive intracellular Ca^{2+} pool was rapidly exchanging (probably by capacitative influx due to emptying of the internal store or by a Ca release-activated Ca current, a process shown to be sensitive to RR, as reviewed by Putney [1993]) with the extracellular Ca.

Other explanations are also possible, e.g. RR might be blocking Ca transport through the plasma membrane independently and in addition to the organellar fluxes. RR is known to be ineffective on plasma membrane Ca channels (Currie et al., 1992, and refs. therein). However, micromolar levels of RR partially inhibited ^{45}Ca exchange by sorghum roots (Wilkinson and Duncan, 1993). In maize roots, RR inhibition was limited to anoxia-stimulated Ca uptake. This, we interpret, was due to RR blocking an internal store and, consequently, the capacitative influx rather than directly acting on the influx. The inability of RR at 10 μM to block anoxia-stimulated Ca^{2+} influx and the Ca reversibility of its effects indicate that RR at this concentration may not sufficiently block Ca mobilization from (and thus emptying of) intracellular stores and the coupled influx. Given the profound effect that anoxia might have on mitochondria and the well-known interference of RR with their normal Ca^{2+} metabolism (Moore, 1971; Ferrari et al., 1982), this organelle could be a primary source for the cytosolic Ca^{2+} elevation and thereby the site of RR action during anoxia. In fact, mitochondria of intact rat hepatocytes (Aw et al., 1987) or isolated from rat liver (Nishida et al., 1989) lost their matrix Ca immediately after oxygen deprivation.

The prevention or reversal of RR effect by added Ca and the dependence on concentration as well as timing of Ca addition for this antagonism may also suggest that Ca and RR compete for the same intracellular site very early during anoxia. RR binds with similar or greater affinity than Ca to several Ca-binding proteins (Charuk et al., 1990). Such proteins could be important targets of RR, e.g. its possible inhibition of Ca binding to calmodulin and the dependent reactions (Sasaki et al., 1992).

In animal cells also, verapamil, nifedipine, or diltiazem (plasma membrane Ca channel blockers) failed to prevent anoxia-induced cytosolic Ca^{2+} elevation or lactic dehydrogenase release, although extracellular Ca^{2+} promoted these processes (Gasbarrini et al., 1992a, 1992b). However, Ca appears to act differently in animal (at least on myocytes) and plant cells under anoxia. Extracellular Ca per se (in the absence of RR) does not seem to have any effect on the anaerobic response in plants. Furthermore, as inferred from our results, Ca mobilization from an internal store into the cytosol could be a necessary early step in the adaptive response of plants to submergence.

In contrast, under prolonged anoxia, Ca loading (by electrophoretic exchange from the cytosol) into mitochondria was reported to cause irreversible injury leading to death of cardiac myocytes (Shen and Jennings, 1972; Börgers and Piper, 1986). Furthermore, RR was shown to protect the myocytes from this injury (Ferrari et al., 1982; Park et al., 1990), contrary to our observations in maize.

The antagonism of the anoxic response by only RR (probably also by TMB-8) is not surprising in the wake of previous reports. For example, Knight et al. (1992) showed that each of the different abiotic stimuli that elevated cytosolic Ca in *Nicotiana plumbaginifolia* seedlings responded to only one or a specific set of Ca channel blockers. RR selectively blocked the wind-induced Ca increase but not the other blockers. Different Ca modulators were also reported to regulate specific time-dependent responses in germinating aplanospores of *Vaucheria* (Oliveira, 1992). Of many used, only RR blocked germination at late stage II irrespective of the time of its application.

A variety of effects of Ca antagonists have been presented as evidence for the participation of Ca in plant responses. For example, Reddy et al. (1988) showed that various Ca antagonists inhibited the auxin-induced elongation of pea epicotyls, but none had any effect on auxin-responsive mRNA induction. In pea stems, TMB-8 inhibited auxin-induced elongation and cell-wall synthesis, but only the inhibition of cell-wall synthesis was reversible by the addition of Ca (Brummel and Maclachlan, 1989). To our knowledge, the present work is the first report in which the effect of a Ca antagonist (i.e. RR) at the whole-plant level (Fig. 3) has been correlated with changes in anoxia-specific gene expression (Fig. 1 and Table III). These changes, caused by micromolar levels of RR, were associated with a decreased exchange of Ca (Table II) and were effectively prevented by a supply of Ca (Figs. 2 and 3 and Tables III and IV). In addition, our study also excluded the requirement of extracellular Ca for the tolerance to anoxia (Tables V and VI). Thus, the data lead us to propose that in maize roots intracellular Ca participates in the signaling of anoxia. Our results from the measurements of the intracellular Ca changes in corn cells during anoxia provide further experimental evidence for this proposal and will be presented in a separate communication (C.C. Subbaiah, D.S. Bush, and M.M. Sachs, unpublished data).

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