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Elevation of Cytosolic Calcium Precedes Anoxic Gene Expression in Maize Suspension-Cultured Cells

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Based on pharmacological evidence, we previously proposed that intracellular Ca^{2+} mediates the perception of O_2 deprivation in maize seedlings. Herein, using fluorescence imaging and photometry of Ca^{2+} in maize suspension-cultured cells, the proposal was further investigated. Two complementary approaches were taken: (1) real time analysis of anoxia-induced changes in cytosolic Ca^{2+} concentration ($[Ca]_i$) and (2) experimental manipulation of $[Ca]_i$ and then assay of the resultant anoxia-specific responses. O_2 depletion caused an immediate increase in $[Ca]_i$, and this was reversible within a few seconds of reoxygenation. The $[Ca]_i$ elevation proceeded independent of extracellular Ca^{2+}. The kinetics of the Ca^{2+} response showed that it occurred much earlier than any detectable changes in gene expression. Ruthenium red blocked the anoxic $[Ca]_i$ elevation and also the induction of *adh1* (encoding alcohol dehydrogenase) and *sh1* (encoding sucrose synthase) mRNA. Ca^{2+}, when added along with ruthenium red, prevented the effects of the antagonist on the anoxic responses. Verapamil and bepridil failed to block the $[Ca]_i$ rise induced by anoxia and were equally ineffective on anoxic gene expression. Caffeine induced an elevation of $[Ca]_i$ as well as ADH activity under normoxia. The data provide direct evidence for $[Ca]_i$ elevation in maize cells as a result of anoxia-induced mobilization of Ca^{2+} from intracellular stores. Furthermore, any manipulation that modified the $[Ca]_i$ rise brought about a parallel change in the expression of two anoxia-inducible genes. Thus, these results corroborate our proposal that $[Ca]_i$ is a physiological transducer of anoxia signals in plants.

INTRODUCTION

Oxygen deprivation is a serious problem for plants growing in flooded soils. Maize responds to O_2 deficit by altered gene expression (Sachs, 1993) that ultimately leads to metabolic and morphological adjustments. There is also evidence that this response is rapid and very sensitive to changes in O_2 levels (Paul and Ferl, 1991; Andrews et al., 1993). However, the nature of the sensor or the early events that lead to the perception of O_2 availability and the consequent reprogramming of gene expression are yet to be investigated.

Cytosolic calcium concentration ($[Ca]_i$) has been implicated in the transduction of many external stimuli in plants (Johannes et al., 1991; Poovaiah and Reddy, 1993). Recently, we investigated the role of Ca^{2+} in anoxia signaling by testing the effects of various Ca^{2+} antagonists on a set of measurable responses to anoxia (Subbaiah et al., 1994). Ruthenium red (RR), which is proposed to block Ca^{2+} fluxes from organelles (Kreimer et al., 1985; Knight et al., 1992), repressed anaerobic gene expression as well as poststress survival of maize pre-emergent seedlings. These effects of RR were accompanied by a blockage of anoxia-elevated $^{45}Ca^{2+}$ influx, and in addition, the RR effects were prevented by externally added Ca^{2+}. However, Ca^{2+} chelators failed to influence either anoxic gene expression or survival, thus indicating that an influx of Ca^{2+} may not be required for the anaerobic response. Therefore, we proposed that RR may block the anoxia-induced mobilization of Ca^{2+} from an intracellular source, thereby leading to the loss of an essential link in anoxic signaling (Subbaiah et al., 1994). Although the data are highly suggestive of the involvement of cytosolic Ca^{2+} in the anaerobic response, RR inhibition of organellar Ca^{2+} fluxes in intact plant cells needs to be demonstrated. In this study, we undertook to clarify the participation of Ca^{2+} in the perception of anoxia by addressing the following questions: (1) Does anoxia lead to an elevation of $[Ca]_i$? (2) If there is an anoxic elevation of $[Ca]_i$, what is the source of Ca^{2+}? Is the $[Ca]_i$ rise due to an influx of extracellular Ca^{2+} ($[Ca]_e$) or a release from intracellular Ca^{2+} stores, or both? (3) What are the effects of Ca^{2+} channel blockers on $[Ca]_i$ during anoxia, and do these agents have a corresponding

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action on anaerobic gene expression? (4) Can elevation of $[Ca]_i$ under aerobic conditions trigger anoxia-specific genes without O_2 deprivation?

To answer these questions, we analyzed the dynamics and spatial distribution of $[Ca]_i$ in maize suspension-cultured cells that were subjected to anoxia and various Ca^{2+}-related pharmacological treatments (potential modulators of $[Ca]_i$) and correlated these spatial and temporal Ca^{2+} fluxes with changes in anoxic gene expression and survival. The maize cell line used in this work (P3377; Duncan et al., 1985) shows an inducibility of anaerobic genes (Paul and Ferl, 1991) similar to that of intact roots. Although single cells, particularly from in vitro culture, do not necessarily reflect the organization or the physiology of cells in intact plants, they offer certain advantages besides the technical feasibility of conducting photometry and imaging studies. The responses of single cells are more likely to involve primary signal sensing and transduction events without the involvement of the slower systemic effects. With individual cells, it is also possible to investigate the question of cell autonomy in the signaling process. Furthermore, the use of cultured cells makes it easy to alter and study the effect of various external agents. As was done in a previous study, we have considered anoxic survival, induction of alcohol dehydrogenase (ADH) activity, and increase in the steady state levels of *adh1* and *sh1* (sucrose synthase) mRNAs as a paradigm of the anaerobic response. These parameters represent the end products of gene regulation and form part of the whole-cell adaptive response.

RESULTS

Loading of Maize Cells with Fluo-3 AM to Measure $[Ca]_i$

Loading of the Ca^{2+} probe and its subcellular distribution are critical in Ca^{2+} measurement studies. In plant cells, the process of dye loading is complicated because of the presence of cell wall–bound hydrolases and the ability of plants to accumulate organic anions in the vacuole, thus becoming a major limitation for these studies (Darjania et al., 1993; Graziana et al., 1993; Ayling et al., 1994). Hence, our initial objective was to investigate the suitability of a maize cell line, P3377, for Ca^{2+} measurement by fluorescence. Considerations were dye uptake and retention in the cytoplasm for at least a few hours without sequestration into organelles. This cell line was chosen based on its inducible expression of anaerobic genes,

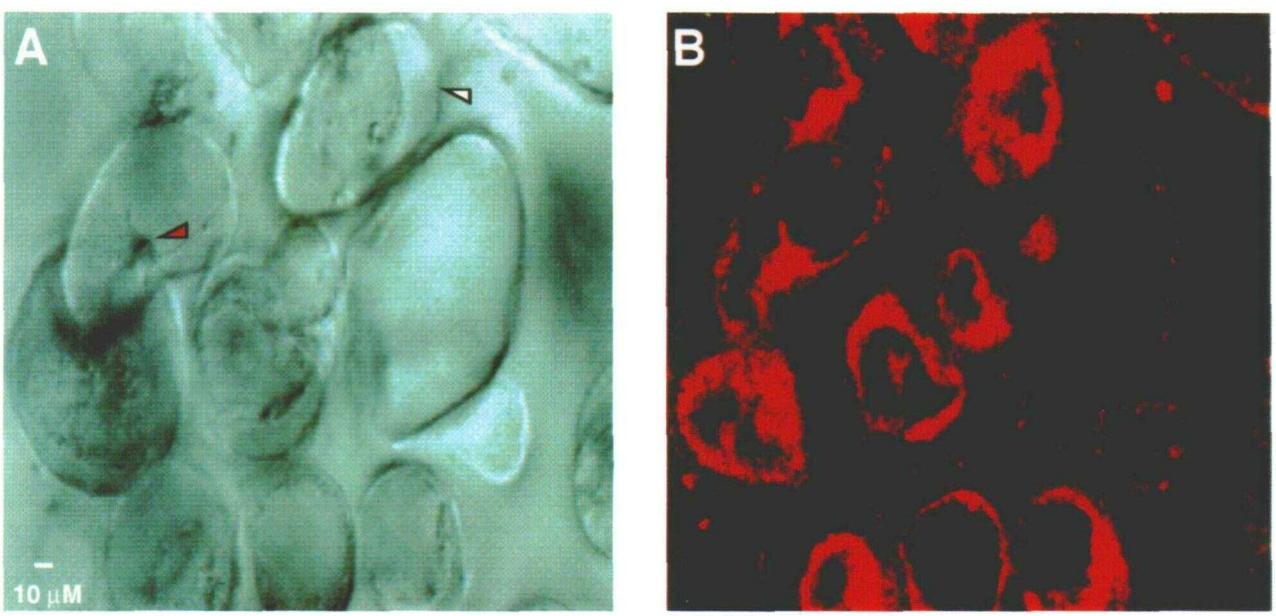


Figure 1. Localization of Fluo-3 AM in Maize P3377 Suspension Cells.

The cells were incubated in 5 μm Fluo-3 AM for 30 min in the dark and at room temperature before analyzing by laser confocal microscopy. The images were recorded 1 hr after incubation with Fluo-3 AM, using an on-line computer-controlled digital imaging system.
(A) Bright-field image. Cells were allowed to plasmolyze (by increasing the osmolarity of the medium) to distinguish the cell components, such as the cell wall (white arrowhead), cytoplasm, and the nucleus (red arrowhead), and to compare with the flourescence pattern shown in **(B)**.
(B) Distribution of fluorescence. The dye uptake by the cells started within 5 min of incubation, and fluorescence was distributed only in the nucleus and cytoplasm, with no discernible signal from the vacuole for at least 2 hr. Most of the experiments were conducted within this time period.
Bar = 10μm.

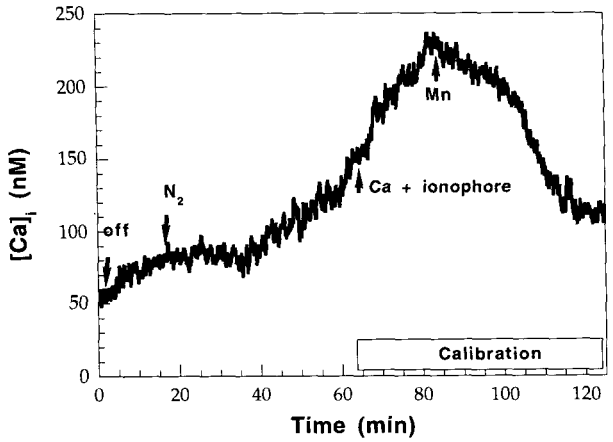


Figure 2. In Vivo Calibration of Fluorescence Intensity versus $[Ca]_i$.

Calibration was carried out as described in Methods. The additions are 1 mM $CaCl_2$, 10 μM A_{23187} (Ca + ionophore), and 5 mM $MnCl_2$ (Mn). $[Ca]_i$ values were calculated from the fluorescence intensities according to a formula described in Kao et al. (1989). In this experiment, anaerobic treatment was given both by stopping perfusion (off) followed by perfusion with N_2-saturated buffer.

which is equally critical in our studies. Fluorometric studies and microscopic observations indicated that dye (Fluo-3 AM) uptake started almost immediately after incubation (data not shown). This obviated the need for a more intrusive protocol, such as microinjection and its attendant problems (Ayling et al., 1994). Low concentrations of the dye (<10 μM) provided desirable dye distribution (cytoplasmic localization without punctate fluorescence), which was stable for at least 2 hr after transferring the cells into a dye-free medium. The cytoplasm and the nucleus, as seen in the bright-field image of Figure 1A, can be precisely overlaid on the fluorescence image shown in Figure 1B. There was only a minor amount of fluorescence in the cell walls compared to the cytoplasm (Figure 1B). Furthermore, the dye-loaded cells remained viable throughout the experiments, as determined by the continuation of saltatory movements of the organelles.

Because Fluo-3 AM shows only a quantitative difference in fluorescence without any spectral shift upon Ca^{2+} binding, it is crucial to calibrate the fluorescence intensity versus Ca^{2+} concentration in vivo. In vivo calibrations, involving clamping $[Ca]_i$ with an ionophore, are not always achievable (Malho et al., 1994). Although a few cells did not respond to Mn^{2+} quenching (Kao et al., 1989), calibration was possible in a majority of our experiments (e.g., see Figure 2). Thus, the cell line and the $[Ca]_i$ measurement protocols employed were appropriate for execution as well as interpretation of Ca^{2+} photometry and imaging experiments. From our measurements, the resting levels of $[Ca]_i$ were about 80 to 160 nM in this cell line.

Anoxia-Induced Changes in $[Ca]_i$ of Maize Cells

If the response to a signal is mediated by Ca^{2+}, the levels of $[Ca]_i$ should change when the signal is received (Hepler and Wayne, 1985), and these changes should precede or accompany the response (Jaffe, 1980). That is, changes in $[Ca]_i$ must occur within 1 to 2 min of exposure of the cells to anoxia, if they are to precede the observed changes in mRNA and protein synthesis following O_2 deprivation (Sachs, 1993). Anoxia was imposed in our experiments by turning off the perfusion system or perfusing with N_2-bubbled medium (Figure 2; also see Methods for details).

The resting levels of $[Ca]_i$ in the maize cells were ~80 to 160 nM. The cells responded to anoxia by a rapid (within a minute or two) elevation of $[Ca]_i$. The response was always reversible within a few seconds of reoxygenation. However, the anoxic elevation was not uniform in all the cells tested. This variability in the response is presented in Figure 3 and arbitrarily classified as follows: pattern I, majority (80%) of the

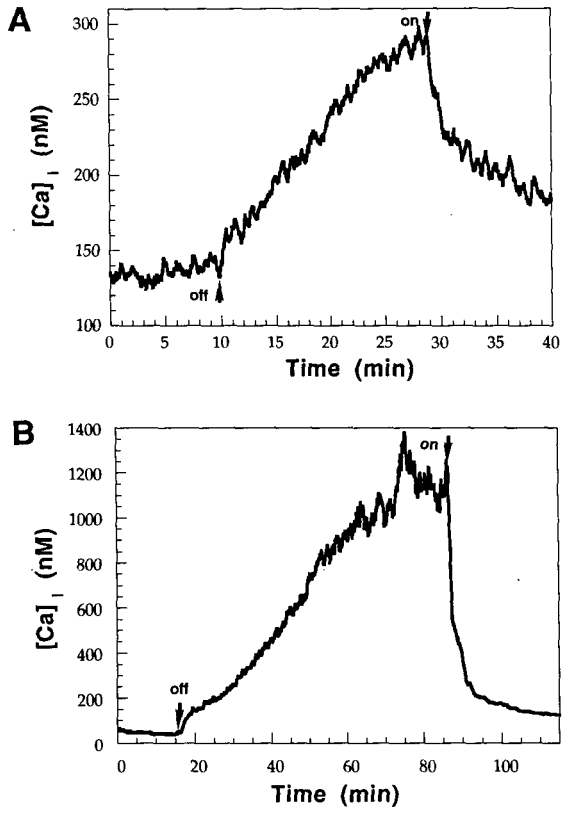


Figure 3. Anoxia-Induced Changes in $[Ca]_i$ of Maize Cells.

Measurement of $[Ca]_i$ changes by photometry was conducted. Anoxia was induced by shutting off the perfusion (off) and reoxygenation was by resuming circulation with an aerated buffer (on). The anoxic responses are grouped into two classes on an arbitrary basis.
(A) The majority of cells had this type of elevation.
(B) Only 20% of the cells tested showed this pattern.

cells tested showed an immediate but slow and steady rise in the signal, leading to a two- to threefold increase after ~20 min of anoxia (Figure 3A); pattern II, in 20% of the tested cells, the signal rose rapidly during the first minute followed by a slow elevation, leading to an eight- to 20-fold increase in $[Ca]_i$ (Figure 3B).

Maize cells did not show any biphasic increase in their $[Ca]_i$ in response to anoxia (even after prolonged anoxia for 1 hr; e.g., pattern II in Figure 3B); this is contrary to what has been observed in certain animal cells (Gasbarrini et al., 1992a). The increase in $[Ca]_i$ was always immediate without a lag phase and continued until the stress was released by aerobic perfusion (Figures 3A and 3B).

Changes in $[Ca]_i$ were also imaged and measured in maize cells to visualize the changes consequent to the imposition or withdrawal of anoxia and/or various pharmacological agents. Although imaging gave results similar to those of the photometric measurements, the technique was used to detect spatial heterogeneities in the cellular Ca^{2+} at discrete time points of various treatments.

The anoxic treatments in our experiments (both photometry and imaging) were generally given only for a short duration (10 to 15 min). This was to allow the cells to restore their resting Ca^{2+} quickly, as well as avoid problems such as starvation of cells for the nutrients. In an attempt to localize grossly the origin and propagation of Ca^{2+} signals, we prolonged anoxia

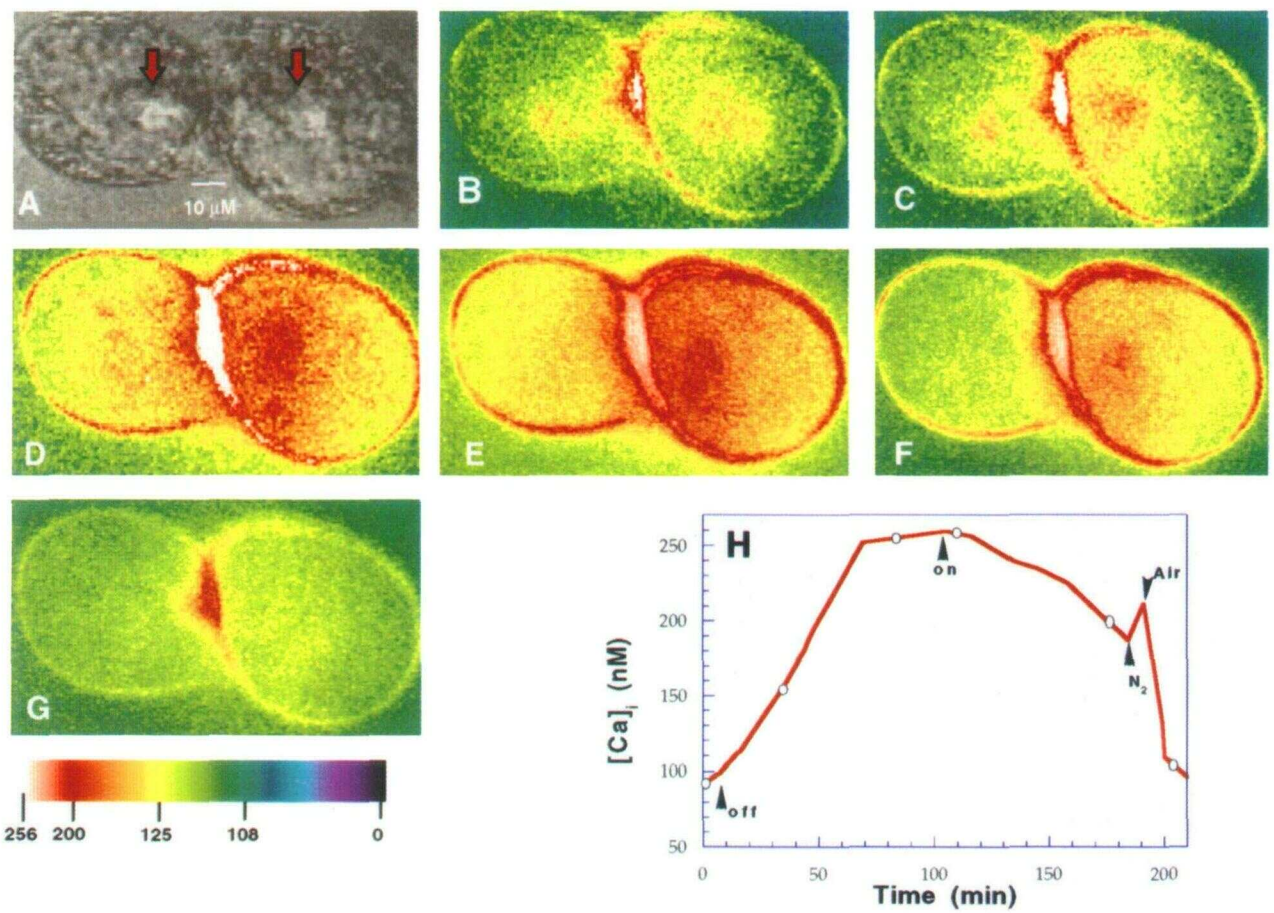


Figure 4. Fluorescence Imaging of Anoxia-Induced Changes in $[Ca]_i$ of Maize Cells.

The image capture and analysis were done as described in Methods. In this experiment, anoxia was prolonged for ~100 min. The color bar gives fluorescence intensity values. The calibrated $[Ca]_i$ values from a selected area of the cell are presented as a graph.

(A) Bright-field image. The red arrows mark the position of the nucleus. Bar = 10 μm.

(B) Perfusion on.

(C) and **(D)** Perfusion off. The rise in $[Ca]_i$ was initially observed around the nucleus where most of the cytoplasm was distributed.

(E) to **(G)** Perfusion on.

(H) Graphical presentation of $[Ca]_i$ changes averaged from the interior of the right cell. At 183 min, the perfusion buffer was bubbled with N_2, and after 7 min, aerobic perfusion (Air) was resumed. Circles on the graph indicate the time points when the images were extracted for the preparation of the montage.

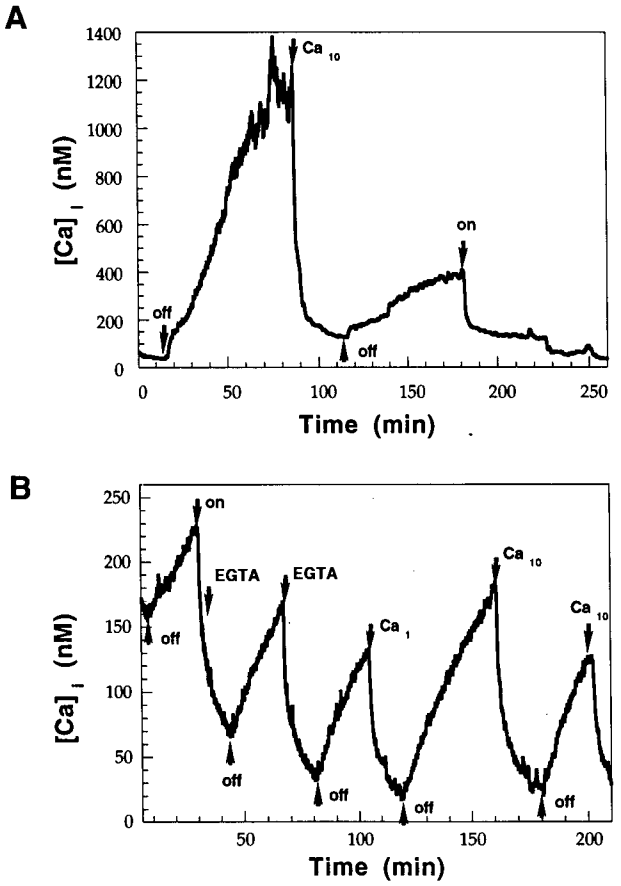


Figure 5. Anoxia-Induced Changes in $[Ca]_i$ Are Independent of Extracellular Ca^{2+}.

(A) Effect of extracellular Ca^{2+}. The cells were initially perfused without added Ca^{2+} in the perfusion buffer. At the end of the first perfusion off treatment, the cells were reperfused in 10 mM $CaCl_2$-supplemented buffer (Ca_{10}) before another anoxia treatment was given.

(B) Effect of EGTA perfusion. After one anoxia and reaeration (off–on) cycle, the effects of perfusion with 0.2 mM EGTA (higher concentrations of EGTA and/or 1,2-*bis*(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, of up to 5 mM, had the same effect) followed by 1 and 10 mM $CaCl_2$ (Ca_1 and Ca_{10}) on the $[Ca]_i$ changes induced by anoxia treatment (off) were tested.

for more than an hour in the imaging experiment shown in Figure 4. The increase in the Ca^{2+} signal (induced by O_2 deprivation) started as hot spots around the nucleus (red arrow in the bright-field image shown in Figure 4A), where most of the cytoplasm was localized in the cell, and subsequently spread throughout the cell (Figures 4C and 4D). The increase in the signal around the periphery of the cytoplasm was mild throughout the progress of anoxia. This is expected because the perfusion medium had 1 mM EGTA with no added Ca^{2+}. However, there was a substantial increase in the fluorescence at the periphery of the cell during late anoxia (Figures 4F and 4D). It continued to increase further, upon reoxygenation (i.e., reperfusion), when the interior cytoplasmic signal in contrast went down (Figure 4E). This suggested that Ca^{2+} efflux was stimulated in the cell (this was not due to dye efflux, because bubbling with N_2 reversed the decrease in the cytoplasmic signal; cf. $[Ca]_i$ graph in Figure 4H). This efflux possibly led to the restoration of the resting levels of $[Ca]_i$ (Figures 4F and 4G). The rate of $[Ca]_i$ decrease upon reoxygenation was slower in this experiment, probably due to prolonged anoxic treatment. This slow decrease (different from either of the patterns shown in Figure 3, although the anoxia-induced $[Ca]_i$ rise was similar to pattern I shown in Figure 3A) enabled us to localize the fluorescence changes and to interpret the mode of $[Ca]_i$ restoration (possibly by efflux) during reperfusion.

Does Elevation of $[Ca]_i$ Depend on Extracellular Ca^{2+}?

The perfusion buffer used in our experiments was essentially free of Ca^{2+} except for the Ca^{2+} (micromolar concentration, if any) carried through the deionized water. However, anoxia induced a 20-fold increase in $[Ca]_i$ even after prolonged perfusion (30 to 60 min) with this buffer (Figure 5A; see Methods for details), and in fact, perfusion with 10 mM $CaCl_2$ led to a $[Ca]_i$ increase of lower magnitude (threefold compared to >20-fold during the first spell of anoxia) in the subsequent period of anoxia (Figure 5A). The question of $[Ca]_e$ requirement for this response was further addressed by perfusing the cells with Ca^{2+} chelators prior to imposing anoxia. As shown in Figure 5B, EGTA perfusion did not prevent the anoxic $[Ca]_i$ rise even after two cycles of anoxia and reoxygenation, although the resting $[Ca]_i$ levels were lowered in the presence of EGTA. Subsequent perfusion with $CaCl_2$ stopped this fall but did not restore the resting $[Ca]_i$. Furthermore, $[Ca]_e$ (even at 10 mM) did not significantly alter either the pattern or the rate of anoxic $[Ca]_i$ elevation (3.6-fold increase with EGTA in comparison to 4.8-fold increase with $CaCl_2$ during ~20 min of anoxia; Figure 5B). These observations suggested that an influx of $[Ca]_e$ is not required for the anoxic $[Ca]_i$ rise in maize cells and that $[Ca]_e$ may only modulate the resting levels of the cation following a possible depletion of internal stores by anoxia.

Effect of RR Pretreatment or Perfusion on $[Ca]_i$ Changes

In our previous study, we reported that RR pretreatment prevented both anoxic gene expression and survival of maize seedlings. Combined with our observations on Ca^{2+} chelator–treated seedlings, we proposed that RR interferes with one of the intracellular Ca^{2+} stores that releases the cation during anoxia and that this RR-sensitive mobilization of Ca^{2+} is essential for the anoxic response (Subbaiah et al., 1994). This also implied that RR penetrates into the cell interior, which is crucial for its proposed intracellular effect.

We investigated the entry of RR into maize cells and its effects on $[Ca]_i$ during anoxia. Incubation of cells in 25 μM RR even for a few minutes resulted in the entry of the antagonist into the cell, and by ~3 hr, the nucleus and cytoplasm were stained (Figures 6A to 6C). Cells pretreated with RR (for 1 hr before the dye loading) steadily lost their $[Ca]_i$ even during aerobic incubation (note the low resting level of $[Ca]_i$ even at the beginning of the recording in Figure 7A) and more rapidly when perfusion was turned off (Figures 7A and 7D). An in vitro fluorometric study indicated that this decrease in $[Ca]_i$ signal was not due to interference by RR, the fluorescence properties of Fluo-3 AM, or the fluoroprobe's sensitivity to Ca^{2+} (data not shown). It follows that RR blocked the ability of the cells to respond to anoxia in terms of $[Ca]_i$ elevation. Untreated cells that showed anoxic elevation of $[Ca]_i$ also lost this ability after a few minutes of perfusion with 10 or 25 μM RR (Figures 7B and 7D), suggesting that RR penetration and its intracellular action were very rapid. The occurrence of $[Ca]_i$ depletion (in RR-treated cells) in the presence of EGTA (Figure 7D) confirmed that RR affected the intracellular fluxes of Ca^{2+}. This inhibition by RR perfusion was subsequently reversible (only the repression of 10 μM but not of 25 μM RR) by $CaCl_2$ treatment (Figures 7B, 7C, and 7D). Furthermore, preincubation of cells with RR (10 or 25 μM) in the presence of 5 mM $CaCl_2$ prevented the decrease of $[Ca]_i$, and the cells responded normally to anoxia (Figure 7C).

A rapid loss of the $[Ca]_i$ signal (>50% diminution in 5 to 10 min) was observed following perfusion of cells with 10 or 25 μM RR (Figure 8). RR appeared to induce a localized loss of Ca^{2+} from the cytoplasm (data not shown). Perfusion of cells even with very high doses of Ca^{2+} chelators (EGTA and 1,2-*bis*(2-aminophenoxy)ethane-*N*-*N*-*N*′-*N*′-tetraacetic acid, each at 5 mM) failed to prevent either anoxic elevation of $[Ca]_i$ or its abolition by RR (Figures 8C to 8F) in this experiment. This further corroborated the inference that both anoxia and RR acted on Ca^{2+} mobilization, because the $[Ca]_i$ changes

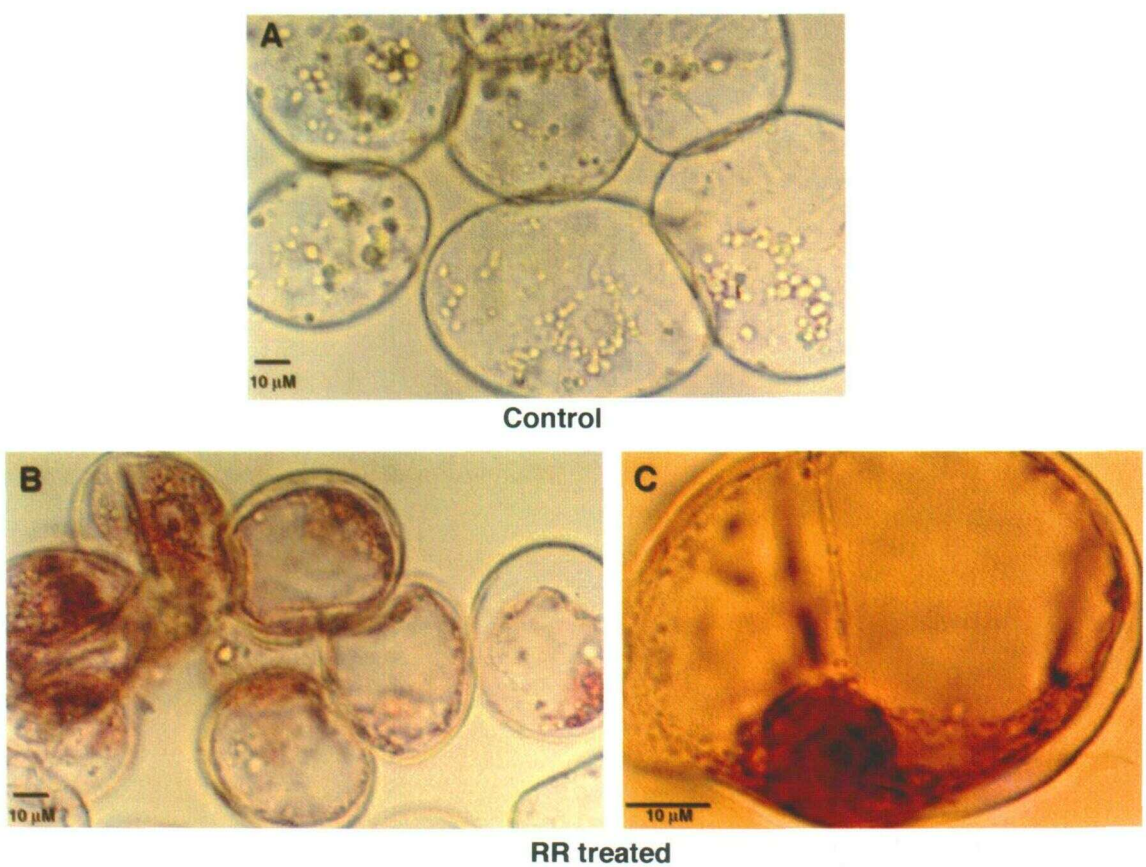


Figure 6. Entry of RR into Maize Cells.

(A) Cells incubated in the perfusion buffer without RR.

(B) Cells incubated with 25 μM RR in the perfusion buffer for 3 hr.

(C) One of the RR-treated cells is shown at higher magnification to indicate the preferential localization of RR in the cytoplasm and the nucleus in comparison to the cell wall and the vacuole. Bars = 10 μm.

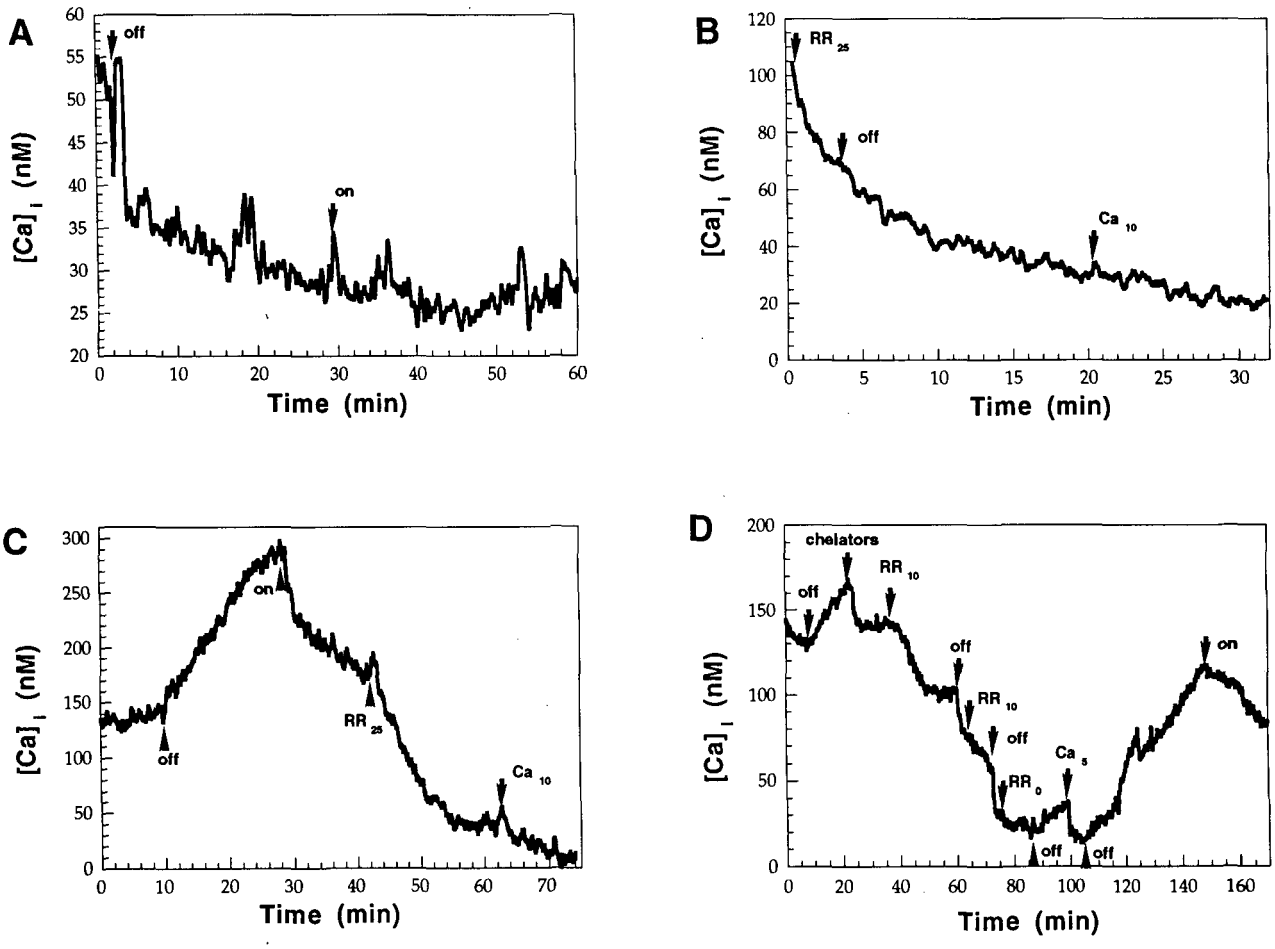


Figure 7. RR-Induced Changes in $[Ca]_i$ of Aerobic and Anoxic Maize Cells.

(A) and **(B)** RR decreases $[Ca]_i$ and blocks the anoxic elevation. Cells in **(A)** pretreated with RR (for 1 hr in 10 μM antagonist prior to Fluo-3 AM loading) or cells in **(B)** that received no pretreatment but were perfused with 25 μM antagonist (RR_{25}) for 3 min were given aerobic and anoxic treatments, and $[Ca]_i$ was measured. In **(B)**, after 17 min of anoxia (off), perfusion was resumed with 10 mM $CaCl_2$ (Ca_{10}).

(C) Ca^{2+} prevents RR suppression of anoxic response. Cells pretreated with RR (25 μM) along with 5 mM $CaCl_2$ (for 1 hr before fluoroprobe loading) show normal anoxic elevation of $[Ca]_i$. Subsequent perfusion of cells with RR (25 μM, RR_{25}) decreased the signal, and the cells failed to respond to the addition of $CaCl_2$ (Ca_{10}).

(D) Extracellular Ca^{2+} reverses the inhibition of 10 μM RR. The cells were perfused with 10 μM RR (RR_{10}) after one anoxia and reoxygenation cycle. Anoxia (off) was imposed intermittently during RR perfusion. The reversibility of the RR_{10} effect was tested by perfusion with a buffer lacking RR (RR_0) or containing 5 mM $CaCl_2$ (Ca_5).

caused by either of them were independent of Ca^{2+} influx from the extracellular medium.

Effect of Plasma Membrane Ca^{2+} Channel Blockers on $[Ca]_i$ of Maize Cells during Anoxia

In our earlier study, none of the plasma membrane Ca^{2+} channel blockers tested had an effect on the anoxic gene expression or survival of maize seedlings (Subbaiah et al., 1994). Two of these compounds, bepridil and verapamil, were tested for their effect on the anoxia-induced elevation of $[Ca]_i$ in maize cells. Either of the channel inhibitors given during preincubation (for 1 hr, prior to dye-loading) failed to block the elevation of $[Ca]_i$ by anoxia (Figure 9). Comparable results were also obtained when the channel blockers were given not as a pretreatment, but during perfusion (data not shown). The decrease in $[Ca]_i$ upon reoxygenation in the cells treated with bepridil or verapamil proceeded at a slower rate (20 to 30 min; Figure 9) than in the case of untreated cells, which quickly (within the first minute or two) restored their $[Ca]_i$ after reperfusion (Figures 3 and 5).

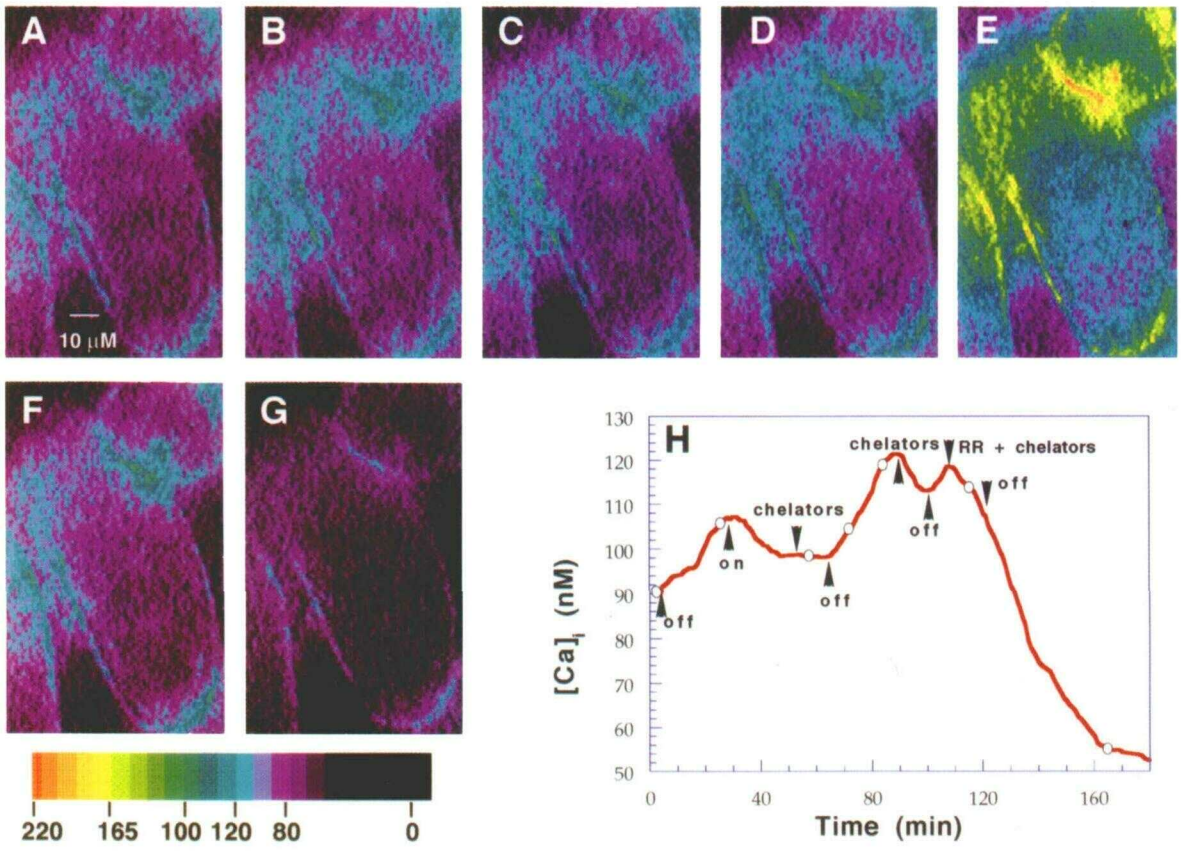
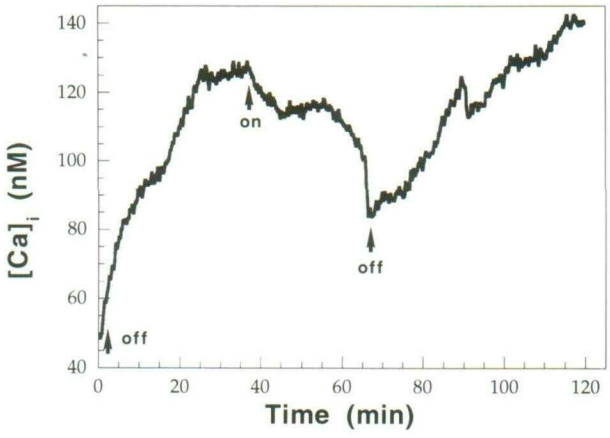


Figure 8. Fluorescence Imaging of RR-Induced Changes in $[Ca]_i$ of Aerobic and Anoxic Maize Cells.

The color bar gives fluorescence intensity values. The calibrated Ca^{2+} values from a selected area of the cell are presented as a graph.
(A) Perfusion on. Bar = 10 μm.
(B) Perfusion off.
(C) Perfusion on with Ca^{2+} chelators, 5 mM EGTA, and 5 mM 1,2-*bis*(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.
(D) and **(E)** Perfusion off.
(F) Perfusion on with 25 μM RR and the chelators.
(G) Perfusion off.
(H) Changes in $[Ca]_i$ from the entire cell during the experimental period. The time points at which images were extracted to prepare the montage are indicated as circles on the graph.



In Figure 10, after an anoxia–reoxygenation cycle in Ca^{2+}-free buffer, the cells were perfused with 50 μM bepridil, a Ca^{2+} channel inhibitor. Bepridil, probably by blocking plasma membrane Ca^{2+} channels, induced a further decrease in resting levels of $[Ca]_i$ (Figures 10D and 10I), similar to the consequence of EGTA perfusion (Figure 5B). In addition, it failed to block the rise in $[Ca]_i$ induced by O_2 deprivation (Figures 10E and 10I), similar to Ca^{2+} chelators. Furthermore, the severe depletion of $[Ca]_i$ caused by repetitive anoxia–

Figure 9. Ca^{2+} Photometry in Bepridil-Pretreated Cells.

Cells that were preincubated in 50 μM bepridil were used for the measurement of anoxia-induced changes in $[Ca]_i$. Cells pretreated with verapamil (50 μM) gave similar results.

reoxygenation cycles and treatment with bepridil led to a rapid influx of Ca^{2+} when the medium was supplemented with $CaCl_2$ (Figure 10F). This influx apparently allowed the cells to refill their Ca^{2+} stores, as evidenced by the return of $[Ca]_i$ to the resting levels. However, the extent of $[Ca]_i$ restoration or the subsequent response to anoxia was not uniform in all of the cells that were visible in the microscopic field (Figures 10F and 10G). Thus, the imaging technique not only depicted the spatial heterogeneities in the cytoplasm of an individual cell, but also presented the differential response of individual cells. This is a more representative estimation of the variability in response (than observed by photometry), because all

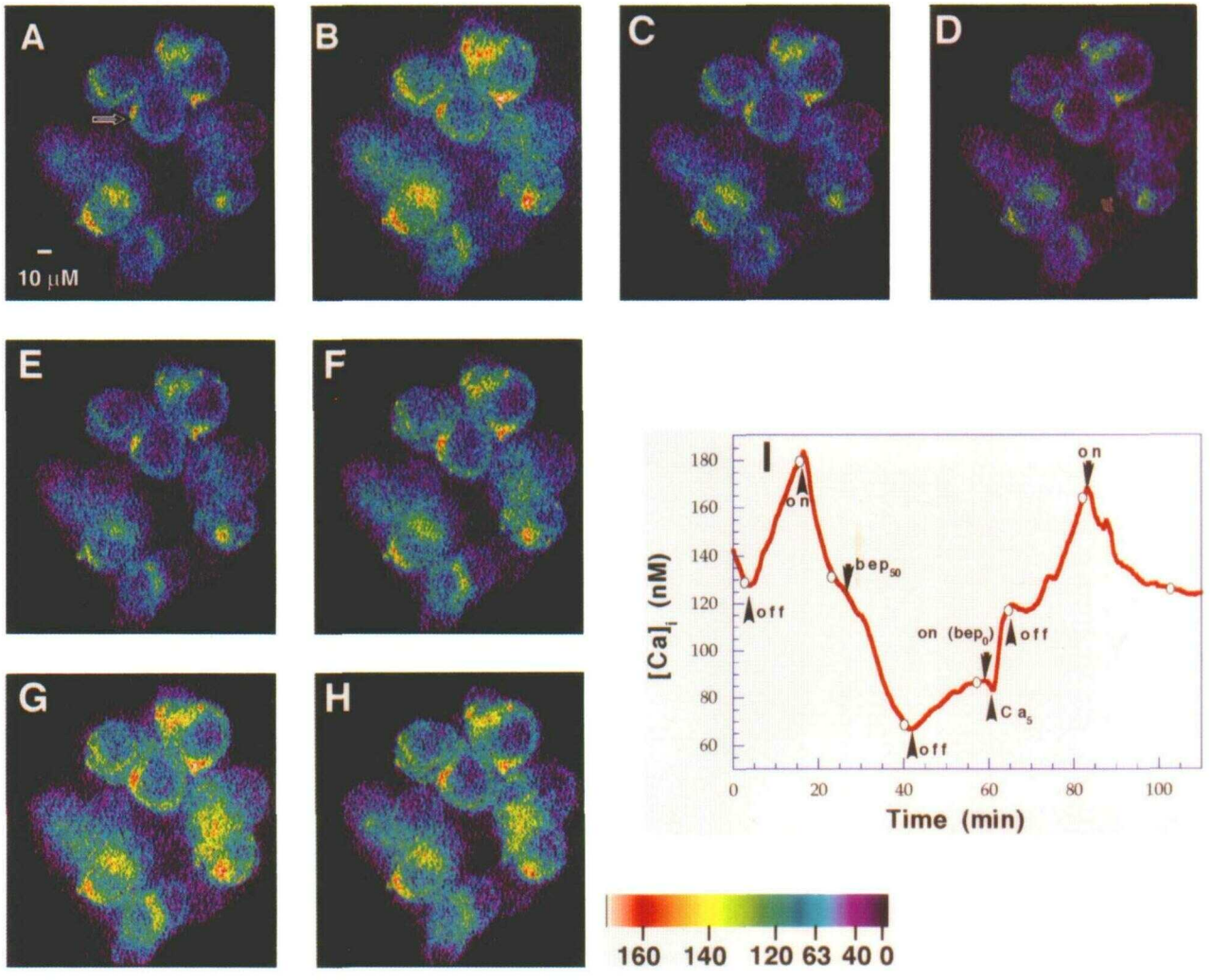


Figure 10. Fluorescence Imaging of $[Ca]_i$ in Maize Cells Perfused with Bepridil.

Micromolar concentrations of bepridil (bep) and millimolar concentrations of $CaCl_2$ (Ca) are given as subscripts in **(I)**. The color bar gives fluorescence intensity values. The calibrated $[Ca]_i$ values from a selected area of the cell are presented as a graph.

(A) Perfusion on. Bar = 10 μm.

(B) Perfusion off.

(C) Perfusion on.

(D) Perfusion continued with buffer containing 50 μM bepridil.

(E) Perfusion off.

(F) Perfusion on with 5 mM $CaCl_2$ but no bepridil in the medium.

(G) Perfusion off.

(H) Perfusion on.

(I) Quantification of the fluorescence and calculation of $[Ca]_i$ changes from one of the cells (indicated by an arrow in **[A]**). For the preparation of the montage, images were extracted from the time points indicated as circles on the graph.

the cells under comparison were present in the same microscopic field and were subjected to uniform experimental conditions (Figure 10).

Caffeine Stimulates an Elevation of $[Ca]_i$ in Maize Cells during Normoxia

Caffeine is known to stimulate Ca^{2+} release from intracellular stores in animal cells (Vites and Pappano, 1992; McNulty and Taylor, 1993). Reports on its effects in plants have been conflicting (Keifer et al., 1992; Roberts and Haigler, 1992; Robin et al., 1993). We studied the effect of caffeine on the $[Ca]_i$ of maize cells to evaluate the drug as a potential surrogate of anoxia in elevating $[Ca]_i$ as well as activating marker genes. As shown in Figure 11, caffeine (2.5 or 5 mM) elevated $[Ca]_i$ and withdrawing the compound restored $[Ca]_i$. The elevation occurred in a Ca^{2+}-free perfusion medium containing 1 mM EGTA and thus was independent of extracellular Ca^{2+}, similar to the anoxic increase. Caffeine-treated cells responded subsequently to anoxia with a rise in $[Ca]_i$ similar to untreated controls, showing that anoxia and caffeine acted either on different Ca^{2+} stores or a common store with a large capacity.

The aerobic $[Ca]_i$ elevation caused by caffeine also started as hot spots in the cytoplasm, much like the effect of O_2 deprivation (Figure 12B). However, the caffeine-stimulated Ca^{2+} mobilization was much less sensitive to RR than the anoxia-elevated $[Ca]_i$. The depletion of $[Ca]_i$ after caffeine treatment required prolonged perfusion (>30 min) with a greater concentration of RR (50 μM; Figures 12D and 12F). This, combined with the observation that anoxia stimulated a significant elevation of $[Ca]_i$ in caffeine-treated cells (Figure 12C), indicates that caffeine and O_2 deprivation were probably mobilizing the cation from different intracellular Ca^{2+} stores. Caffeine is known to deplete Ca^{2+} rapidly from the endoplasmic reticulum in animal cells (Bassani et al., 1993; Janczewski and Lakatta, 1993).

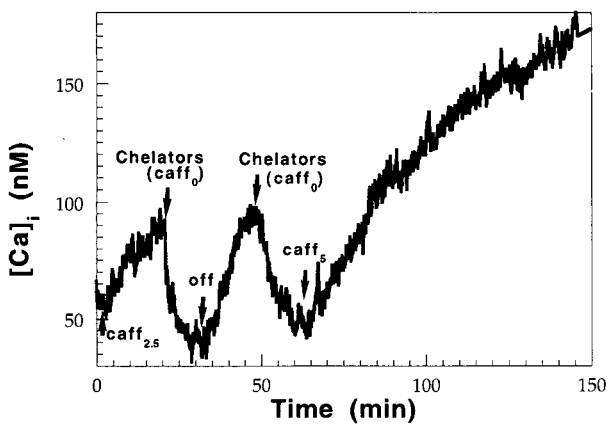


Figure 11. Effect of Caffeine on $[Ca]_i$ of Maize Suspension Cells.

The perfusion buffer was nominally free of Ca^{2+} because the buffer contained 1 mM EGTA. Millimolar concentrations of caffeine (caff) are given as subscripts.

Anoxic Tolerance of Maize Suspension-Cultured Cells

We measured the tolerance of the P3377 cells to anaerobiosis in terms of their ability to reduce triphenyltetrazolium chloride (TTC; Monroy et al., 1993). This assay was found to correspond well quantitatively with the capacity of the cells to divide (as revealed by plating efficiency experiments) in many cell lines (Towill and Mazur, 1975). The ability to reduce TTC was completely retained by the cells even after 24 hr of complete anoxia. After 4 days of continuous anoxia, however, TTC reduction declined by 50%. From the TTC assay, the P3377 cells appeared to be more tolerant to anoxia than the maize (cv B73) seedlings (Figure 13). Although we have not correlated this assay with the actual plating efficiency of this cell line, it appeared that TTC reduction overestimated the actual viability based on the relative sensitivity of maize seedlings to anoxia. Nonetheless, the decrease in the rate of TTC reduction by the cells with the lengthening of anoxia (Figure 13) indicated that the assay served as a relative estimate of anoxia tolerance.

Effect of Pharmacological Compounds on Anaerobic Survival and Gene Expression in Maize Cells

The Ca^{2+} antagonists that were employed on seedlings in our previous study (Subbaiah et al., 1994) were tested for their effects on the anoxia tolerance and induction of ADH activity in maize cells also (Table 1). None of the agents had any effect on the aerobic survival of the cells as measured by the ability to reduce TTC. All of them, except for RR, also failed to affect anoxia tolerance, ADH activity (Table 1), and levels of *adh1* and *sh1* mRNA (data not shown).

RR-treated cells showed 50% survival after 24 hr of anaerobic treatment. Simultaneous addition of 5 mM $CaCl_2$ counteracted the repressive effect of RR on cell survival. RR apparently had a less severe effect on the post-anoxic survival of cells compared to its complete repression of anoxic survival observed on the B73 seedlings (Subbaiah et al., 1994). This further confirmed that the TTC reduction assay did not precisely quantify (but instead overestimated) the survival of the cells. However, the assay served as a rapid tool (but only as an index of tolerance) to evaluate the relative effects of Ca^{2+} antagonists, as indicated by the microscopic appearance of the cells from different treatments (data not shown). RR completely abolished the anoxic induction of ADH activity (Table 1) and repressed the levels of *adh1* and *sh1* mRNA (Figure 14). The addition of Ca^{2+} along with RR partly prevented the repression of ADH activity (Table 1).

Caffeine caused induction of ADH activity in maize cells under aerobic conditions (Table 1) to the same magnitude as in anoxically treated cells. At the same time, it did not affect the

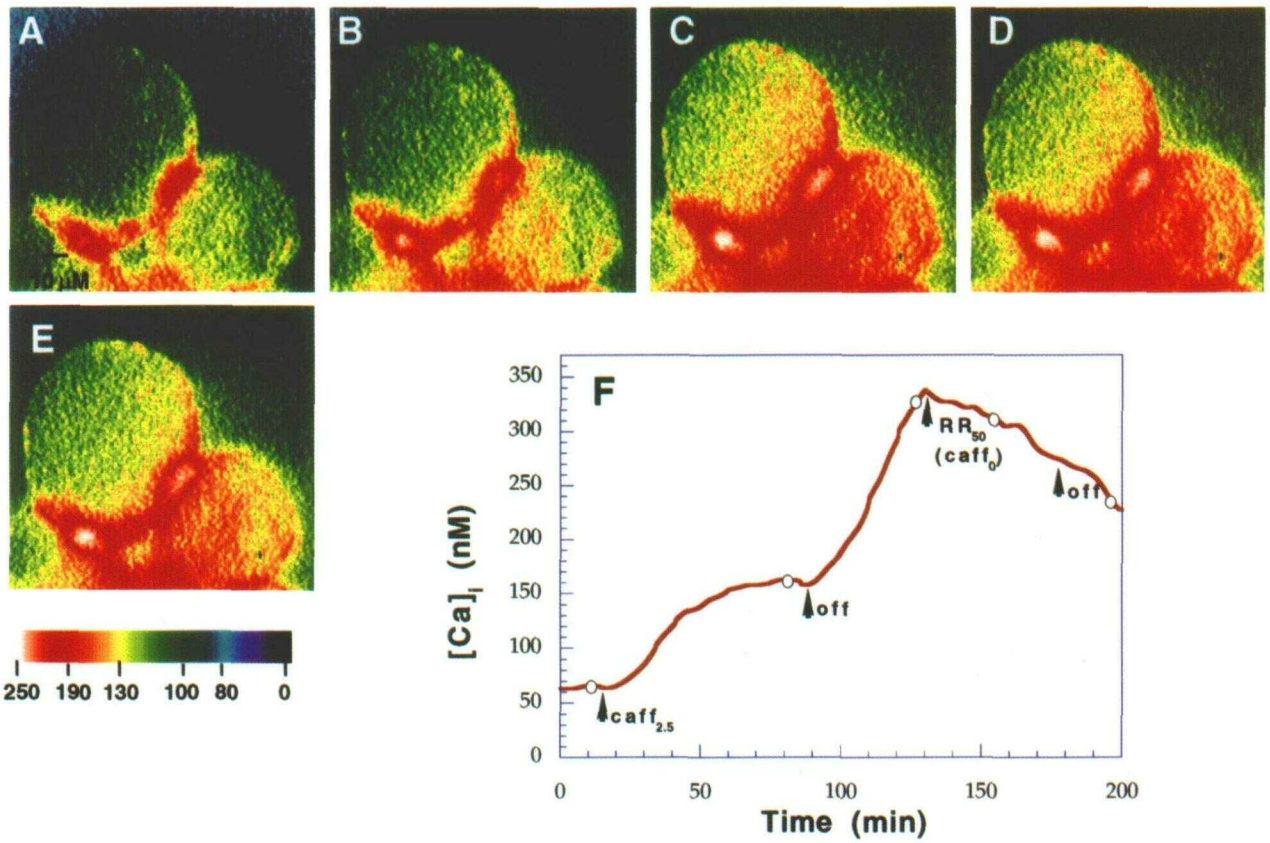


Figure 12. Fluorescence Imaging of Caffeine-Induced Rise in $[Ca]_i$ of Aerobic Maize Cells.

The millimolar concentrations of caffeine (caff) and micromolar concentrations of RR are given as subscripts in **(F).** The color bar gives fluorescence intensity values. The calibrated $[Ca]_i$ values from a selected area of the cell are presented as a graph.

(A) Perfusion on. Bar = 10 μm.

(B) Perfusion with 2.5 mM caffeine.

(C) Perfusion off.

(D) Perfusion with 50 μM RR.

(E) Perfusion off.

(F) Graphical presentation of $[Ca]_i$ changes from the entire top cell. Images used in the preparation of the montage were from the time points indicated as circles on the graph.

survival of the cells under these conditions. However, caffeine-treated cells were less tolerant to anoxia than untreated controls. The drug-treated cells also showed poor anaerobic induction of ADH activity (data not shown). This was possibly due to toxicity from Ca^{2+} overloading of the cytosol by both caffeine and anoxia, which independently mobilized Ca^{2+} from different intracellular stores.

DISCUSSION

Anoxia-induced cytosolic Ca^{2+} fluxes have been well studied in animal systems (e.g., Gasbarrini et al., 1992a), and the cellular injury from anoxia has been attributed to Ca^{2+} overloading (Gasbarrini et al., 1992b). However, there have been few investigations of the involvement of Ca^{2+} as a possible intracellular messenger in sensing O_2 availability (Aldashev et al., 1991). In plants, there has been only circumstantial evidence for the occurrence/participation of $[Ca]_i$ fluxes in response to O_2 deprivation (such as the rapid depolarization of the plasma membrane following anoxia [Buwalda et al., 1988] and the in vitro Ca^{2+} binding ability of a transacting factor of *adh* [Lu et al., 1994]). We have initiated studies on the role of Ca^{2+} in O_2-sensing in plants and recently presented pharmacological evidence for the participation of this cation as a transducer of anoxia signals in maize seedlings (Subbaiah et al., 1994). Here, we measured and imaged $[Ca]_i$ in maize

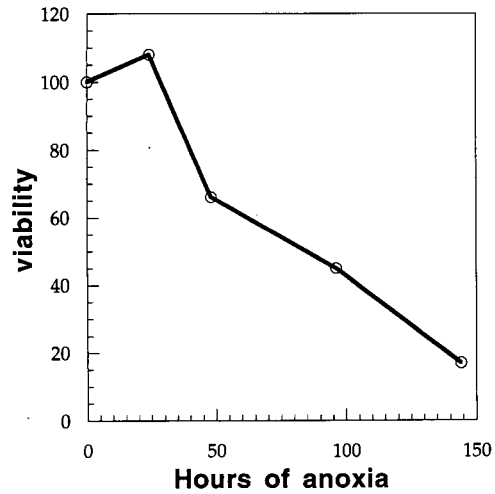


Figure 13. Effect of Progressive Anoxia on the Relative Viability of Maize Cells.

Viability was estimated as the ability to reduce TTC (Towill and Mazur, 1975) after the indicated hours of anaerobic incubation. The values are the percentages of aerobic cells. The aerobic cells reduced TTC at a rate of 0.15 per mg $A_{485\ nm}$ dry weight per hr.

suspension-cultured cells during O_2 deprivation to assess further the role of Ca^{2+} in triggering gene expression changes during anoxia.

Optical fluorescent probes have been successfully used for measurement of Ca^{2+} in animal systems. However, their application to plant cells is technically demanding (Graziana et al., 1993). Our requirement for a suitable maize cell line was further constrained by a need for inducibility of anoxic response. The only maize cell line, among those tested so far, that shows inducibility of anoxic genes is P3377 (other cell lines are constitutive in their expression of anaerobic genes; Paul and Ferl, 1991). To our advantage, this cell line has also been found to be amenable to measuring $[Ca]_i$ in terms of rapid uptake of dye at low concentrations, localization of dye in the cytoplasm, and feasibility of calibrating fluorescence intensity to Ca^{2+} concentration. Thus, the cell line P3377 served as a good model system for our studies on anoxia signaling. The resting level of $[Ca]_i$ in maize cells on average was 100 nM (ranged between 80 and 160 nM) based on both photometric and imaging experiments.

Anoxia Rapidly Elevates $[Ca]_i$ by Mobilizing Ca^{2+} from Internal Stores of Maize Cells

Our photometric and imaging studies showed that an elevation of $[Ca]_i$ occurred within 1 to 2 min after O_2 deprivation in a majority of cells tested. The increase was variable and ranged from three- to 20-fold. We lack a direct assay to determine the threshold value of $[Ca]_i$ that may bring about changes in gene expression of maize cells; however, an increase as small as 2.5- to 3.0-fold has been shown to be physiologically significant in other Ca^{2+}-mediated signal transduction pathways (Gilroy et al., 1991; McAinsh et al., 1992; Malho et al., 1994).

Most of the anoxia-induced rise in $[Ca]_i$ appeared to come from intracellular stores, even in the presence of extracellular Ca^{2+} (Figures 5A and 5B). $[Ca]_e$ may sustain the response by restoring the resting levels of $[Ca]_i$. Rat hepatocytes, under O_2 deprivation, show a distinct biphasic elevation of $[Ca]_i$ involving both mobilization from internal stores (peak I, occurring within the first 10 min of anoxia) and an influx occurring 30 min later (peak II; Gasbarrini et al., 1992a). The mobilization induces only an approximate threefold elevation, with the major increase (15-fold above resting $[Ca]_i$) being from influx (Gasbarrini et al., 1992a). On the contrary, even the largest anoxia-induced increase in $[Ca]_i$ in maize cells (~20-fold) occurred as a single peak without any dual component and was independent of extracellular Ca^{2+} (Figure 5A). In hepatocytes, abolition of the peak II (influx) by perfusion with either Ca^{2+} chelators or fructose alleviates anoxic injury, showing that the influx is in fact inhibitory for anoxic survival (Gasbarrini et al., 1992a, 1992b) in accordance with the effect of Ca^{2+} chelators on maize seedlings (Subbaiah et al., 1994) or cells (Table 1).

Our imaging analysis showed that the $[Ca]_i$ rise started as discrete spots around the nucleus. However, these studies did not reveal the precise organelle of Ca^{2+} release in the cell. Given the profound effect that anoxia is known to have on mitochondria, this could be a primary source for the $[Ca]_i$ elevation during anoxia. In fact, mitochondria of intact rat hepatocytes (Aw et al., 1987) or isolated from rat liver (Nishida et al., 1989) lose their matrix Ca^{2+} immediately after O_2 deprivation. On the other hand, the vacuole could be a source of anoxic elevation of $[Ca]_i$ in maize cells, because this is a major intracellular store of Ca^{2+} in plants. We also do not know

Table 1. Effect of Pharmacological Agents on Viability and ADH Activity in Maize Cells Incubated Anaerobically for 24 hr

Treatment	TTC Reduction (% of Control)	ADH Activity (% of Control)
RR, 25 μM	50	5
$CaCl_2$, 5 mM	110	90
$CaCl_2$ + RR	95	70
Caffeine, 2.5 mM (aerobic)	105	300
Bepridil, 50 μM	100	90
Verapamil, 100 μM	80	120
Lanthanum, 1 mM	92	75
EGTA, 5 mM	119	125

Values are averages of two experiments. After 24 hr of anoxia, the untreated cells (control) maintained a viability of 100%, but the ADH activity increased from 53 units per min per $A_{280\ nm}$ (in aerobic cells) to 269 units per min per $A_{280\ nm}$.

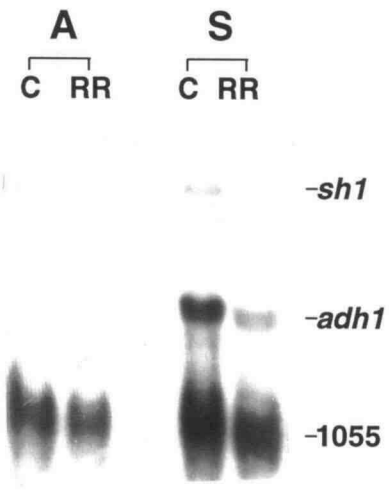


Figure 14. Effect of RR (25 μM) on the Steady State Levels of *adh1* and *sh1* mRNA in Cells Treated Anaerobically for 12 hr.

C, control; RR, ruthenium red treated; A, aerobic; S, anoxic.
A cDNA from maize root tissue, 1055, whose transcript levels are unaffected by anoxia (Sachs, 1991), is used to show loading variations.

the route of Ca^{2+} influx that may occur during or following anoxia. $^{45}Ca^{2+}$ flux experiments with plasma membrane channel blockers on maize seedlings revealed that plasma membrane Ca^{2+} channels may not be involved in the influx (Subbaiah et al., 1994). This is in accordance with the observations in animal systems; for example, in rat hepatocytes, where plasma membrane Ca^{2+} channel blockers fail to prevent anoxia-induced $[Ca]_i$ elevation or release of lactate dehydrogenase, although extracellular Ca^{2+} promotes these processes (Gasbarrini et al., 1992a). By analogy with animal cells, this influx may be mediated by a H^+/Ca^{2+} exchanger at the plasma membrane. This proton–Ca^{2+} exchange may also be important for restoring the cytosolic pH, because a rapid acidosis of cytoplasm occurs during hypoxia or anoxia (Roberts et al., 1984; also see following discussion).

O_2 Deprivation–Induced Changes in Gene Expression Are Preceded by $[Ca]_i$ Elevation

Repression of ongoing protein synthesis occurs almost immediately after O_2 deprivation but requires absolute anoxia (Sachs et al., 1980). However, transcriptional activation (as indicated by protein–DNA interactions and subsequent mRNA accumulation) of anoxia-specific genes can occur even under mild hypoxia but takes at least 1 hr of O_2 deprivation (Paul and Ferl, 1991). In our anoxia induction procedures (either "perfusion off" or "N_2 perfusion"), the system took at least a few minutes to become completely anoxic and the $[Ca]_i$ elevation occurred within this hypoxic period, well before any changes in gene expression are known to take place. Thus, $[Ca]_i$ elevation may be one of the earliest cellular responses that precede reprogramming of gene expression in response to O_2 deprivation.

The earliest change in response to anoxia reported in the literature is cytosolic acidification, occurring 2 min after O_2 deprivation (Roberts et al., 1984). $[Ca]_i$ elevation appears either to precede or to accompany this pH change (Figure 5A). It is not clear whether there is a causal relationship between these two anoxia-induced events. In a preliminary study using a pH-sensitive fluorescent probe, we observed that a treatment that abolished Ca^{2+} fluxes in maize cells (i.e., RR perfusion; Figures 7A to 7D) also prevented the occurrence of cytosolic pH changes during O_2 deficiency (C.C. Subbaiah, D.S. Bush, and M.M. Sachs, unpublished data). In an analogous study, Gibbon and Kropf (1994) observed that La^{3+}, which reduced $[Ca]_i$, also eliminated the pH gradient (both associated with tip growth) in *Pelvetia* rhizoidal cells. The authors reasoned that the pH gradient may be dependent on Ca^{2+} homeostasis. Although the relationship between the two events is not clear in maize cells, they represent the earliest responses to anoxia and may operate coordinately in regulating anoxic gene expression.

Experimental Manipulation of $[Ca]_i$ Is Accompanied by Changes in Anoxia Responses in Maize Cells

Because $[Ca]_i$ elevation preceded anoxic gene activation and thus may be involved in transducing low O_2 signals, we tested whether pharmacological manipulations of $[Ca]_i$ would be accompanied by alterations in anoxic gene expression and survival. Based on the photometric and imaging studies, these agents belonged to three categories: (1) RR, which blocked anoxia-induced $[Ca]_i$ fluxes, (2) Ca^{2+} chelators and plasma membrane channel inhibitors, which failed to affect these Ca^{2+} fluxes, and (3) caffeine, which mimicked anoxia in causing $[Ca]_i$ elevation. The effects of these agents on anoxic response of cells (Table 1; Figure 14) or of the seedlings reported earlier (Subbaiah et al., 1994) closely corresponded with their effects on $[Ca]_i$. RR abolished anoxia-induced $[Ca]_i$ elevation (e.g., Figure 7), $^{45}Ca^{2+}$ influx (Subbaiah et al., 1994), and anoxic gene expression as well as survival (Table 1 and Figure 14; Subbaiah et al., 1994). Plasma membrane Ca^{2+} channel blockers or Ca^{2+} chelators affected neither anoxic Ca^{2+} fluxes (Figures 9, 10, and 5B; Subbaiah et al., 1994) nor any of the other responses, both in seedlings (Subbaiah et al., 1994) and cultured cells (Table 1). In addition, caffeine, which induced an aerobic elevation of $[Ca]_i$ (Figures 11 and 12), also caused the induction of ADH activity under normoxia without affecting the survival of the cells (Table 1). Furthermore, the amplitude of $[Ca]_i$ changes (two to three times the resting level) induced by caffeine (Figures 11 and 12) was apparently enough in maize cells to bring about changes in gene expression.

The efflux of mitochondrial Ca^{2+} induced by anoxia or respiratory uncouplers is known to be blocked by RR in animal cells (Bernardi et al., 1984; Riley and Pfeiffer, 1986; Gunter and Pfeiffer, 1990). Very little is known of the effect of RR on plant mitochondrial Ca^{2+} fluxes, and our imaging experiments did not resolve the site of action for RR in the cell. However, suppression of anoxic $[Ca]_i$ elevation by RR in maize cells implicates mitochondria as both a source of Ca^{2+} mobilization and the target of RR action under O_2 deprivation in plants.

Where Is O_2 Deprivation Sensed in the Cell?

In conclusion, a rise in $[Ca]_i$ appeared to be the primary response to O_2 deprivation in maize cells, and any experimental manipulation that inhibited or promoted this elevation had a corresponding effect on the expression of two anaerobic genes as well as on anoxic tolerance, in both individual cells and whole plants. Taken together, these results strongly implicate $[Ca]_i$ as an essential player in the transduction of anoxia signals in plants. Furthermore, it is important to investigate where O_2 levels are sensed in the cell and what leads to $[Ca]_i$ elevation. Because O_2 is more diffusive than any potential signal molecule that has to traverse the cellular membranes, anoxia may be first sensed at the mitochondrial electron transport chain, where O_2 can no longer be available as an electron acceptor. However, the roles of plasma membrane redox systems and associated second messengers also need to be examined. We expect that detecting the origin of the Ca^{2+} signal may reveal the nature of the primary sensor, because $[Ca]_i$ rise appears to be an essential link in the chain of events that lead to the whole plant response to anoxia.

METHODS

Chemicals

Reagents of tissue culture grade, pharmacological agents, and most of the biochemicals were obtained from Sigma. Ruthenium red (RR; 98%) was from K & K Laboratories, Division of ICN Biomedicals (Costa Mesa, CA). Fluo-3 AM was purchased from Molecular Probes (Eugene, OR), and A_{23187} was from Boehringer Mannheim. All solutions were prepared in ultra-high quality water purified using the Milli Q-UF Plus system (Millipore, Bedford, MA).

Cell Culture

The maize cell line P3377 was grown and maintained in AMCF-ARM medium (Duncan et al., 1985) in disposable tissue culture flasks (Corning Corp., Corning, NY) on a rotary shaker at 100 rpm at 26 ± 2°C in the dark. Subculturing was done once in 4 days.

Dye Loading

The cells were incubated in 5 μM Fluo-3 AM, a Ca^{2+}-sensitive fluorescent dye (cell-permeant form; 0.1 M methanolic stock diluted in the perfusion medium: 2% sucrose, 3% mannitol, inorganic macronutrients except Ca^{2+}, pH 5.8) for 15 min. Higher concentrations (30 to 50 μM) of the dye were used with some batches of cells that had stronger autofluorescence. However, at these concentrations the dye was sometimes sequestered, as indicated by a continuous increase in the fluorescence. Therefore, we limited the dye concentration to below 10 μM but increased the incubation period to 30 min. This protocol gave a desirable dye distribution (Figure 1).

Perfusion System

Cells incubated in Fluo-3 AM (in some cases after preincubation with pharmacological agents for 1 hr) were loaded in a metallic perfusion plate containing a drop of low-melting agarose (1% solution in perfusion medium) maintained at 40°C. The perfusion plate was immediately cooled to room temperature and sealed air-tight using vacuum grease, a cover slip, a metallic clamp, and screws. The plate was immediately connected (using Intramedic polyethylene tubing, i.d. 0.58 mm; Clay Adams, Parsippany, NJ) to a reservoir containing perfusion medium bubbled vigorously with air. All of these operations were performed in less than 5 min. Circulation with the aerated medium (at a flow rate of 0.3 mL/min) was continued for at least 30 min to equilibrate the cells and wash off extraneous dye or pharmacological agents before starting the measurements. The small volume of the chamber in the perfusion plate (2 × 7 mm with 1-mm depth) allowed rapid equilibration with changes (addition of chemicals and alteration of pH) made to the perfusion medium.

We imposed anoxia in most of our experiments by shutting down the perfusion. O_2 deprivation was induced in a few experiments also by perfusing with N_2-saturated buffer (N_2 perfusion). Although the results were comparable, the cells took a longer time to respond to N_2 perfusion (Figure 2), probably due to leakage of air through the perfusion tubing. Therefore, turning off perfusion was the preferred method of imposing anoxia in our experiments. However, two trivial explanations are possible in interpreting the results from turning off perfusion: (1) accumulation of any leaked dye in the extracellular medium, leading to an artifactual increase in signal and (2) starvation of cells from nutrients during longer periods of "perfusion off" treatments. We ruled out these possibilities by (1) restricting the measurement area to a small region in the cytoplasm while excluding any extracellular space and (2) giving N_2 perfusion following "perfusion off" treatment to observe the effect of perfusion (without O_2) on the stability of fluorescence (Figure 2). These controls indicated that dye leakage and starvation did not interfere with our measurements.

Fluorescence Measurements

Microscopy

The perfusion plate was mounted on the stage of a Nikon (Nikon Corp., Tokyo, Japan) Diaphot inverted epifluorescence microscope. The cells were excited using light from a xenon lamp passed through a 490 ± 10-nm band-pass filter. A 490-nm dichoric mirror mounted on the side port of the microscope allowed us to view the specimen in the bright field and to mark its position on a video monitor during fluorescence measurements. The objectives routinely used were LWD Fluor (Modulation Optics, Greenvale, NY) 40× and 60× with numerical apertures of 0.7 to 1.3.

For monitoring the cellular distribution of Fluo-3 AM (Figure 1), a confocal laser-scanning microscope (Axioplan; Carl-Zeiss, Oberkochen, Germany), located at the Beckman Institute Optical Visualization Facility on the University of Illinois campus, was used. The dye-loaded

cells were mounted on a microscope slide (without the perfusion system), and optical sections were obtained both in transmission and confocal modes.

Photometry

The cells were selected (generally from the middle of the plate) using bright-field illumination based on their apparent healthiness (unplasmolyzed and showing cytoplasmic streaming) and fluorescence (restricted to the cytoplasm). The area of measurement was controlled using a standard Nikon variable aperture and PFX shutter system placed on the emission port of the microscope. The dye was excited at 490 ± 10 nm, and the emission was measured at 535 nm. A photomultiplier selected for extremely low dark current was used to detect light at the emission wavelength. The photomultiplier signals were passed to a photon counter (Hamamatsu, Japan) and analyzed with a Dell computer (Dell Computer Corp., Austin, TX) using a Flopro software program (D.S. Bush, unpublished data). An automatic filter changer (Lamda 10; Sutter Instruments, Novato, CA) was used for excitation of the sample.

Imaging System

An intensified CCD video camera replaced the photomultiplier system previously described. Data acquisition and image analysis were performed using the Metafluor program (Universal Imaging, Westchester, PA). The fluorescence intensity was mapped into color pixels with values from 0 to 256. Consequently, the video color images represent only the fluorescence values and not actual Ca^{2+} values due to unequal thickness of the cell as well as the possibility of unequal distribution of the dye in the cell. The Ca^{2+} values calibrated from fluorescence measured in a selected area of the cell are presented separately in each imaging experiment.

Calibration

At the end of an experiment (photometric or imaging), in vivo calibration of fluorescence intensity versus Ca^{2+} concentration was performed according to Kao et al. (1989) with a few modifications. The cells were perfused with perfusion buffer, pH 7.5, containing 1 mM $CaCl_2$ and the ionophore A_{23187} at 10 μM. After $[Ca]_i$ reached a saturation equilibrium, the fluorescence was quenched with 5 mM $MnCl_2$. The ionophore was used to speed up the Mn^{2+} equilibration. The quenched fluorescence value was taken as 100 nM Ca^{2+}. For accurate estimation of autofluorescence, the cells were finally perfused in some experiments with Triton X-100, which released all of the intracellular dye and Ca^{2+}. The values from calibrations need to be viewed with caution and considered as mostly approximate rather than absolute (Read et al., 1992).

Anaerobic Incubation and TTC Reduction Assay

Cells were incubated either aerobically (normal growth conditions) or in an Anaerobic System (Model No. 1025; Forma Scientific, Marietta, OH) containing 90% N_2 and 10% H_2 at 28°C. Cell viability was estimated in terms of triphenyltetrazolium chloride (TTC) reduction according to Towill and Mazur (1975) without further modifications.

Soluble Protein Extraction and Alcohol Dehydrogenase Assay

Cells from aerobic and anaerobic (24 hr) treatments were filtered on sterile Miracloth (Calbiochem, LaJolla, CA). They were washed and ground in ice-cold extraction buffer (50 mM Tris-Cl, pH 6.8, 15% glycerol, 20 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride) using a chilled mortar and pestle with the aid of acid-washed sand. The extracts were spun at 5000*g* for 5 min. The supernatant was clarified and concentrated with dry Sephadex G-50 gel (Pharmacia, Piscataway, NJ) and used for the alcohol dehydrogenase (ADH) assay according to Russell et al. (1990). The activity of each sample was normalized against its absorbance at 280 nm (Russell et al., 1990).

RNA Extraction and Gel Blot Analysis

Total RNA from aerobic or anaerobic cells was prepared by ultracentrifugation over a CsCl cushion according to Cone et al. (1986). RNA gel blot analysis and probes used were as described previously (Subbaiah et al., 1994).

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Names are necessary to report factually on available data; however, the U.S. Department of Agriculture neither guarantees nor warrants the standard of the product, and the use of the name by the Department implies no approval of the product to the exclusion of others that may also be suitable.

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