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# Visualizing Changes in Cytosolic-Free $Ca^{2+}$ during the Response of Stomatal Guard Cells to Abscisic Acid

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**In this paper, we report the results of a detailed investigation into abscisic acid (ABA)–stimulated elevations of guard cell cytosolic-free $Ca^{2+}$ ($[Ca^{2+}]_{cyt}$). Fluorescence ratio photometry and ratio imaging techniques were used to investigate this phenomenon. Guard cells of open and closed (opened to 10 to 12 μm before treatment with ABA) stomata were microinjected with the fluorescent $Ca^{2+}$ indicator Indo-1. Resting $[Ca^{2+}]_{cyt}$ ranged from 50 to 350 nM. ABA (100 nM) stimulated an increase in $[Ca^{2+}]_{cyt}$ in 68 and 81% of guard cells microinjected in the open and closed configuration, respectively. All stomata were observed to close in response to ABA. Increases ranged from 100 to 750 nM above the resting concentration and were arbitrarily grouped into five "classes." ABA-stimulated increases in $[Ca^{2+}]_{cyt}$ were not uniformly distributed across the cytosol of guard cells. Rapid transient increases in $[Ca^{2+}]_{cyt}$ were also observed in the guard cells of stomata microinjected in the closed configuration. We concluded that the ABA-induced turgor loss in guard cells is a $Ca^{2+}$-dependent process.**

## INTRODUCTION

Stomatal guard cells constitute an attractive system for the study of plant cell signal transduction, responding to a wide variety of signals in a readily quantifiable manner. They are also tractable to a range of cell biological techniques. Accordingly, guard cells have been used as model systems for the molecular dissection of the indole-3-acetic acid (IAA) (Marten et al., 1991), abscisic acid (ABA) (Hetherington and Quatrano, 1991), and blue light (Assmann et al., 1985; Shimazaki et al., 1986) signal transduction pathways (Schroeder and Hedrich, 1989).

Many of the components that would be expected to be present in signal transduction pathways have been identified in stomatal guard cells, including ion channels that respond to plant growth regulators (Blatt, 1990; Schroeder and Hagiwara, 1990; Marten et al., 1991), stretch-activated ion channels (Cosgrove and Hedrich, 1991), and G-protein–regulated ion channels (Fairley-Grenot and Assmann, 1991). It is also known that guard cells are at least competent to respond to the intracellular second messengers inositol 1,4,5-trisphosphate (Blatt et al., 1990; Gilroy et al., 1990) and 1,2-diacylglycerol (Lee and Assmann, 1991).

Perhaps of greater importance, however, is the observation that the plant growth regulator ABA stimulates an increase in cytosolic-free $Ca^{2+}$ ($[Ca^{2+}]_{cyt}$) in stomatal guard cells (McAinsh et al., 1990). The significance of this demonstration lies in the fact that it provides direct support for the hypothesis that $Ca^{2+}$ acts as a second messenger in plant cells. Our initial report of an ABA-stimulated increase in $[Ca^{2+}]_{cyt}$ has subsequently been confirmed in both guard cells and a number of other cell types (Gehring et al., 1990; Schroeder and Hagiwara, 1990; Gilroy et al., 1991; Irving et al., 1992). In addition, it has been reported that ABA causes a rapid, small reduction in $[Ca^{2+}]_{cyt}$ in ABA-sensitive barley aleurone layer cells (Wang et al., 1991).

The nature of the ABA-stimulated increase in guard cell $[Ca^{2+}]_{cyt}$ has been observed to vary from cell to cell. McAinsh et al. (1990) reported sustained increases in $[Ca^{2+}]_{cyt}$ that peaked at approximately 1 μM, whereas Gilroy et al. (1991) observed a range of responses to ABA. Schroeder and Hagiwara (1990) have also demonstrated a rapid, transient increase in $[Ca^{2+}]_{cyt}$ in guard cell protoplasts of broad bean. Interestingly, a similar degree of variability is also encountered in certain animal cells (Tsien and Tsien, 1990).

The proportion of guard cells that fail to exhibit an ABA-stimulated increase in $[Ca^{2+}]_{cyt}$ but that respond to ABA by decreasing their turgor also varies between individual studies. McAinsh et al. (1990) observed that 80% of guard cells in detached epidermis of *Commelina communis* showed an increase in $[Ca^{2+}]_{cyt}$ in response to ABA, whereas Gilroy et al. (1991) detected a change in approximately 40% of guard cells

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tested using similar material and comparable photometric equipment. Recently, Irving et al. (1992) have reported that 70% of guard cells of orchid show an increase in $[Ca^{2+}]_{cyt}$ in response to ABA. In addition, in broad bean the percentage of guard cell protoplasts that exhibit an ABA-stimulated increase in $[Ca^{2+}]_{cyt}$ is equal to the proportion of stomata that close in response to ABA (approximately 37%) (Schroeder and Hagiwara, 1990).

It has been suggested that the presence of cells that reduce their turgor in response to ABA, but that do not appear to show an elevation in $[Ca^{2+}]_{cyt}$, may indicate the involvement of a $Ca^{2+}$-independent ABA signal transduction pathway in guard cells (Trewavas and Gilroy, 1991). To determine the validity of this suggestion, it is first necessary to establish that the failure to detect an increase in $[Ca^{2+}]_{cyt}$ reflects what occurs in vivo and does not simply reflect methodological variability. We have conducted a detailed study of both spatial and temporal changes in guard cell $[Ca^{2+}]_{cyt}$ in response to ABA to examine the variable nature of the response and to address this question. In addition, we have developed a novel microinjection protocol whereby guard cells of closed stomata can be loaded with the $Ca^{2+}$-sensitive indicator Indo-1 (Callaham and Hepler, 1991), induced to open with light, and subsequently treated with ABA. This ensures that only fully competent cells are used for measurements of $[Ca^{2+}]_{cyt}$.

Using this methodology, we have, in contrast to previous studies (Gilroy et al., 1991), successfully imaged the ABA-stimulated increase in guard cell $[Ca^{2+}]_{cyt}$. Schroeder and Hagiwara (1990) provide evidence that ABA stimulates an influx of $Ca^{2+}$ from the apoplast into the cytosol. The results of the present comprehensive ratio photometric and ratio imaging studies support this conclusion and suggest that $Ca^{2+}$ released from intracellular stores may also be involved. As in previous studies, the nature of the ABA-stimulated increases in $[Ca^{2+}]_{cyt}$ was found to be variable. In this paper, we draw attention to the fact that such variability is often found in animal cells. Furthermore, we suggest that, using the experimental conditions described, much of the failure to detect ABA-stimulated increases in $[Ca^{2+}]_{cyt}$ can be explained in terms of methodology. Consequently, in contrast to previous reports (Gilroy et al., 1991), we concluded that an increase in guard cell $[Ca^{2+}]_{cyt}$ is required during ABA-stimulated stomatal closure.

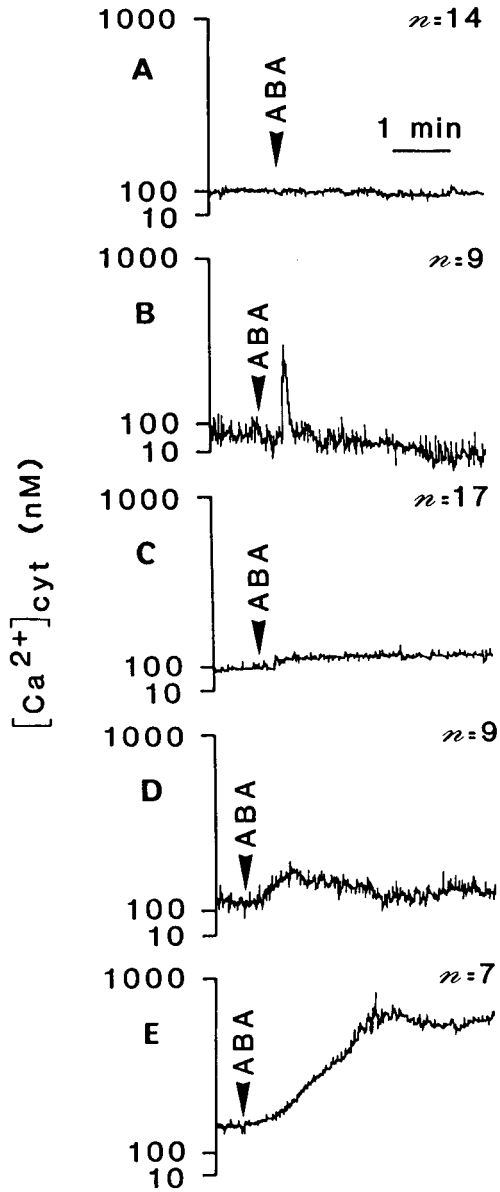


**Figure 1.** ABA-Stimulated Changes in Guard Cell $[Ca^{2+}]_{cyt}$.

Indo-1–loaded guard cells of Commelina were perfused with 100 nM ABA. Changes in $[Ca^{2+}]_{cyt}$ in response to ABA can be grouped into five "classes" on an arbitrary basis. All of these appear to be variations upon a common theme. Examples of each class of response are shown.

**(A)** Class 1: No response ($n$ = 14 cells).

**(B)** Class 2: Rapid, transient increases ($n$ = 9 cells).

**(C)** Class 3: Sustained increases, less than 250 nM above the resting concentration ($n$ = 17 cells).

**(D)** Class 4: Sustained increases, between 250 and 500 nM above the resting concentration ($n$ = 9 cells).

**(E)** Class 5: Sustained increases, greater than 500 nM above the resting concentration ($n$ = 7 cells).

## RESULTS

### ABA-Stimulated Increases in Guard Cell $[Ca^{2+}]_{cyt}$

We have developed a new protocol to load stomatal guard cells with Indo-1 while reducing the chances of obtaining artifactual measurements of $[Ca^{2+}]_{cyt}$, i.e., microinjection of Indo-1 into guard cells of closed stomata that are opened to 10 to 12 μm before use (see Discussion). This procedure was routinely

used to examine ABA-stimulated increases in guard cell $[Ca^{2+}]_{cyt}$ by ratio photometry.

Resting $[Ca^{2+}]_{cyt}$ ranged from 50 to 350 nM ($n$ = 56 cells) with membrane potentials ranging from −70 to −90 mV (50 mM external $K^+$). Treatment with 100 nM ABA was observed to cause an increase in $[Ca^{2+}]_{cyt}$ in 68% (16 of 24 cells) of guard cells of open stomata that were microinjected with Indo-1 and in 81% (26 of 32 cells) of guard cells of closed stomata that were microinjected with Indo-1 and then opened to 10 to 12 μm before treatment with ABA. All increases were initiated within 1 min following application of ABA and preceded any detectable alteration in the aperture of the stomatal pore by between 5 and 7 min. Stomatal closure was always observed following an increase in guard cell $[Ca^{2+}]_{cyt}$. The minority of guard cells that did not show an ABA-stimulated increase in $[Ca^{2+}]_{cyt}$ also closed in response to ABA.

The magnitude of ABA-stimulated increases in $[Ca^{2+}]_{cyt}$ ranged from 100 to 750 nM above the resting concentration. Similar types of increases were observed using both ratio photometric and ratio imaging techniques. As can be seen in Figure 1, for illustrative purposes, these can be grouped into five "classes" on an arbitrary basis, depending on the magnitude and pattern of the increases: (1) no response ($n$ = 14 cells); (2) a rapid, transient increase ($n$ = 9 cells); (3) a small (less than 250 nM above the resting concentration), sustained increase ($n$ = 17 cells); (4) a medium (between 250 and 500 nM above the resting concentration), sustained increase ($n$ = 9 cells); (5) a large (greater than 500 nM above the resting concentration), sustained increase ($n$ = 7 cells). These data correlate with the results of Schroeder and Hagiwara (1990), who have also reported variable responses to ABA, including rapid, transient increases in $[Ca^{2+}]_{cyt}$, slower, transient increases in $[Ca^{2+}]_{cyt}$ that remain at an elevated level, and cells that failed to exhibit any increases in $[Ca^{2+}]_{cyt}$ in response to ABA (McAinsh et al., 1990; Gilroy et al., 1991).

Rapid, transient increases in $[Ca^{2+}]_{cyt}$ (class 2) were only observed in guard cells of closed stomata that were microinjected with Indo-1 and then opened to 10 to 12 μm before treatment with ABA. This may be due to two factors. The use of guard cells microinjected in this manner may enable very early events in the response to ABA to be detected. This microinjection protocol may also preferentially select the least damaged cells and, therefore, those cells most likely to respond to ABA in a physiological manner. In cells microinjected in this manner, 22 of the 26 increases in $[Ca^{2+}]_{cyt}$ in response to ABA were measured using ratio photometry. This provides a higher degree of temporal resolution than ratio imaging, which only allows $[Ca^{2+}]_{cyt}$ to be monitored at discrete time intervals. If rapid, transient changes occur in between these intervals, they may not be detected. In microinjected guard cells of closed stomata that were opened to 10 to 12 μm before treatment with ABA, approximately 34% (nine of the 26 cells) of the ABA-stimulated increases in $[Ca^{2+}]_{cyt}$ exhibited a rapid, transient increase in $[Ca^{2+}]_{cyt}$ as part of the response. From Figure 2, it can be seen that these rapid, transient increases in $[Ca^{2+}]_{cyt}$ vary between guard cells.

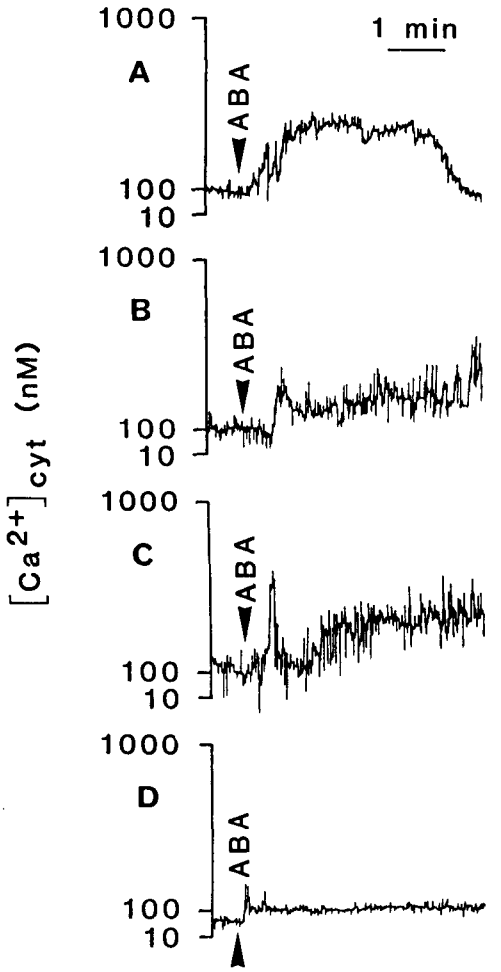


**Figure 2.** Rapid, Transient Increases in Guard Cell $[Ca^{2+}]_{cyt}$ in Response to 100 nM ABA.

Guard cells of closed stomata that were microinjected with Indo-1 into the cytosol and subsequently opened to 10 to 12 μm exhibit rapid, transient increases in $[Ca^{2+}]_{cyt}$ in response to 100 nM ABA (class 2, $n$ = 9). The range of responses, measured using fluorescence ratio photometry, are shown [**(A)** to **(D)**; also see Figures 1B and 3].

**(A)** Transient increase (up to 500 nM above the resting concentration) returning to resting.

**(B)** Rapid, transient increase followed by a sustained increase (both less than 500 nM above the resting concentration).

**(C)** Rapid, transient increase (greater than 500 nM above the resting concentration) followed by a sustained increase (less than 500 nM above the resting concentration).

**(D)** Rapid, transient increase followed by a sustained increase (both less than 250 nM above the resting concentration).

### Spatial Distributions of ABA-Stimulated Increases in Guard Cell $[Ca^{2+}]_{cyt}$ Are Uneven

In contrast to previous reports (Gilroy et al., 1991), we have used fluorescence ratio imaging to successfully visualize ABA-stimulated increases in $[Ca^{2+}]_{cyt}$. ABA-stimulated increases in

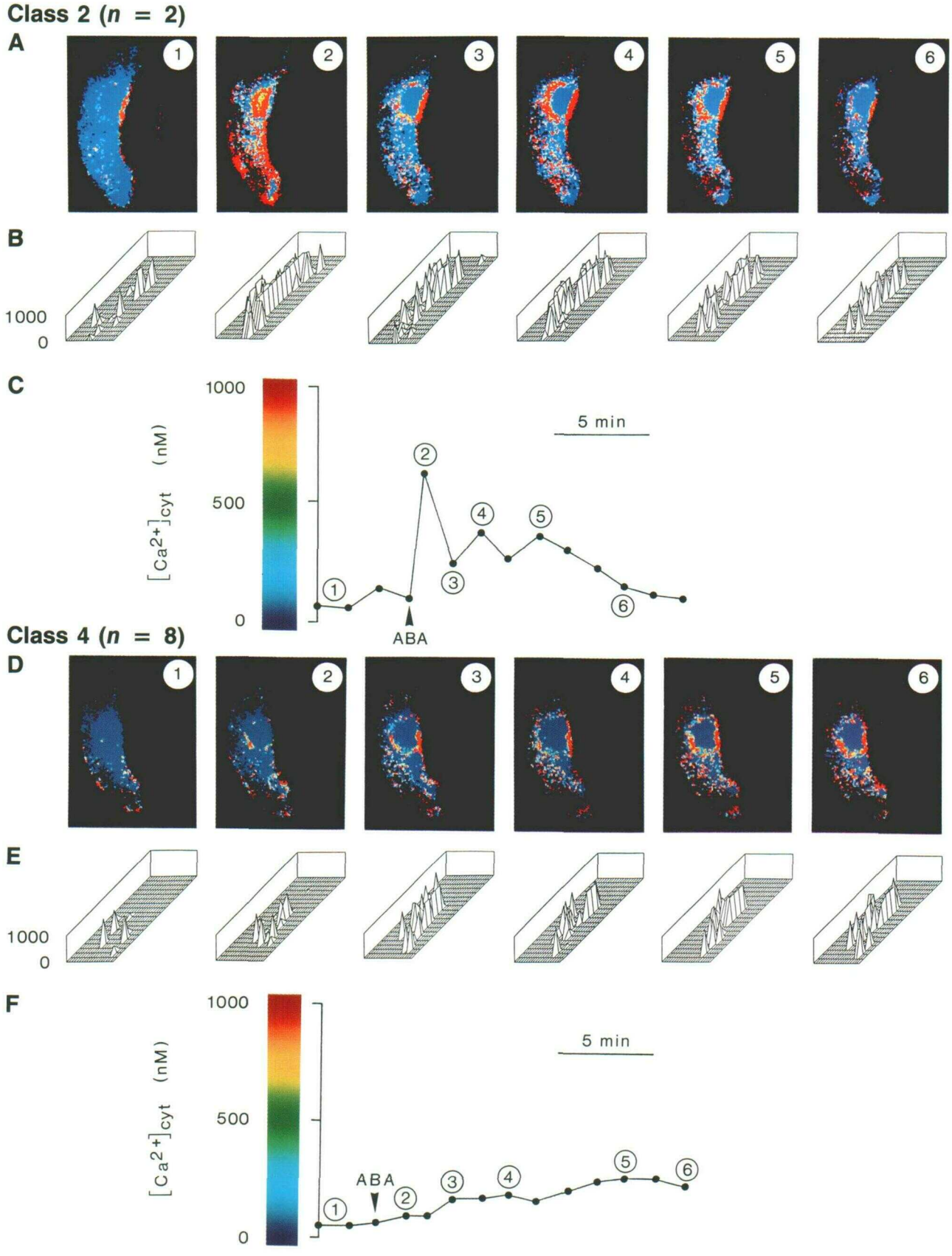


**Figure 3.** Fluorescence Ratio Imaging of ABA-Stimulated Increases in Guard Cell $[Ca^{2+}]_{cyt}$.

$[Ca^{2+}]_{cyt}$ were observed in 20 of the 28 guard cells analyzed in this manner. Using the arbitrary classification system described above, eight cells exhibited a class 1 response. The other responses fell into the following classes: class 2, $n$ = 2 cells; class 3, $n$ = 6 cells; class 4, $n$ = 8 cells; class 5, $n$ = 4 cells. Figure 3 illustrates examples of the results obtained using this technique. As with ratio photometric measurements, the nature of the response was variable. Using ratio imaging techniques, there is an inherently greater chance of observing this apparent variability, because $[Ca^{2+}]_{cyt}$ is determined at discrete time points following treatment with ABA (as opposed to being more or less monitored continuously during ratio photometry). Under these circumstances, it is possible that the first measurement made after the application of ABA forms only part of an upward or downward trend.

In contrast to ratio photometry, ratio imaging allows spatial heterogeneities in $[Ca^{2+}]_{cyt}$ to be detected. ABA-stimulated increases in $[Ca^{2+}]_{cyt}$ are not uniformly distributed across the cell (Figure 3). There is evidence to indicate that ABA may stimulate either $Ca^{2+}$ entry from the apoplast (Schroeder and Hagiwara, 1990) or $Ca^{2+}$ release from intracellular stores (Gilroy et al., 1991). The images presented in Figure 3 support both these possibilities. For example, Figure 3A suggests that an early response to ABA involves an increase in $[Ca^{2+}]_{cyt}$ at the periphery of the cell, possibly resulting from an influx of apoplastic $Ca^{2+}$. In contrast, Figure 3D suggests that ABA stimulates the release of $Ca^{2+}$ from internal stores. However, before it is possible to assess the full significance of these observations, it will be necessary to conduct further work.

## DISCUSSION

### Reducing the Occurrence of Artifactual Results

Stomatal guard cells do not readily take up the acetoxymethyl ester form of Indo-1, and neither are they compatible with the low pH loading method (Bush and Jones, 1987); therefore, Indo-1 must be microinjected into the cytosol using iontophoresis (McAinsh et al., 1990). In our attempts to identify whether there was a methodological basis for the apparent failure to detect an ABA-stimulated increase in $[Ca^{2+}]_{cyt}$ in all cells, we initially focused on the highly invasive and potentially disruptive process of microinjection. In previous studies (McAinsh et al., 1990; Gilroy et al., 1991), the turgid guard cells of open stomata have been used for microinjection. Subsequently, Indo-1–loaded cells have been treated with ABA, and the changes in $[Ca^{2+}]_{cyt}$ and stomatal aperture determined. Although stringent precautions were taken in these studies to ensure that guard cells remained viable following microinjection, it is quite possible that ABA-stimulated closure in the absence of an increase in $[Ca^{2+}]_{cyt}$ could simply reflect a damaged-induced reduction in guard cell turgor. Because an undamaged, nonmicroinjected partner (control) guard cell would also close under such conditions (i.e., in the presence of ABA), this constitutes an inherent weakness in the existing protocol.

To reduce the chances of including artifactual measurements in our results, we adopted a novel experimental protocol in which Indo-1 was microinjected into the cytosol of guard cells of closed stomata that were subsequently opened to an aperture of 10 to 12 μm before treatment with ABA. By monitoring the ability of cells to increase their turgor under conditions promoting stomatal opening (De Silva et al., 1985), together with the usual criteria for estimating viability (Blatt et al., 1990; McAinsh et al., 1990; Gilroy et al., 1991), we were able to ensure that only healthy guard cells were employed in further experimental manipulations. Accordingly, the chances of simply measuring damaged-induced closure and mistakenly ascribing it to the effects of ABA were reduced.

### Involvement of Second Messengers during ABA-Stimulated Reductions in Guard Cell Turgor

It is important to determine whether an increase in guard cell $[Ca^{2+}]_{cyt}$ is an essential requirement for the transduction of the ABA signal. To address this question, we have selected experimental conditions where the occurrence of artifactual results can be minimized. Under the conditions used in previous studies, it is likely that the number of cells exhibiting an ABA-stimulated increase in $[Ca^{2+}]_{cyt}$ has been underestimated. Using our new experimental protocol, more than 80% of guard cells were observed to show an increase in $[Ca^{2+}]_{cyt}$ in response to ABA.

In addition to $Ca^{2+}$, there may be other second messengers involved in coupling the ABA stimulus to the fluxes of anions and cations associated with stomatal closure (MacRobbie, 1992). There is evidence to suggest that pH changes occur during ABA stimulus–response coupling (Gehring et al., 1990; Irving et al., 1992). However, our data indicated that in the majority of guard cells an increase in $[Ca^{2+}]_{cyt}$ is at least a component of the response to ABA. There is a considerable body of evidence to support the role of $[Ca^{2+}]_{cyt}$ in the modulation of the activity of both the anion and cation channels that regulate stomatal turgor (MacRobbie, 1992). Therefore, the

**Figure 3.** (continued).

Guard cells of closed stomata were microinjected with Indo-1 into the cytosol and subsequently opened to 10 to 12 μm. Changes in $[Ca^{2+}]_{cyt}$ in response to 100 nM ABA were recorded by fluorescence ratio imaging, and the ratio images were color coded. Typical examples of responses are shown.

**(A)** and **(D)** Fluorescence ratio images of $[Ca^{2+}]_{cyt}$.

**(B)** and **(E)** Two-dimensional response surfaces showing $[Ca^{2+}]_{cyt}$.

**(C)** and **(F)** Changes in the mean $[Ca^{2+}]_{cyt}$ throughout the whole cell with time.

present observations fit closely the current theories on the regulation of ion channel activity during ABA-stimulated turgor loss in guard cells (Schroeder and Hedrich, 1989; Mansfield et al., 1990; Hetherington and Quatrano, 1991; MacRobbie, 1992). It is also important to recognize that without such an ABA-stimulated increase in $[Ca^{2+}]_{cyt}$, it is necessary to advocate a completely new system for the regulation of ion channel activity during stomatal closure, because on the basis of current evidence, alterations to pH alone are insufficient to explain stomatal closure observed in response to ABA.

### Failure to Detect Changes in Guard Cell $[Ca^{2+}]_{cyt}$ in Response to ABA

Stimulus–response coupling studies in animal cells indicate several additional experimental factors that may also contribute toward the failure to detect ABA-stimulated increases in guard cell $[Ca^{2+}]_{cyt}$. For example, in mammalian salivary acinar cells, activation of the $Ca^{2+}$-sensitive $K^+$ conductance in the basolateral membrane has been observed to occur even in the absence of any detectable increase in $[Ca^{2+}]_{cyt}$ using fluorescence ratio photometry. However, the increase in $K^+$ conductance is abolished if cells are loaded with the $Ca^{2+}$ chelator BAPTA, suggesting that increases in $[Ca^{2+}]_{cyt}$ occur despite the failure to detect a change in whole-cell $[Ca^{2+}]_{cyt}$ (Foskett et al., 1989). A similar phenomenon has been reported in mouse pancreatic acinar cells in which stimulation of the $Ca^{2+}$-dependent $Cl^-$ current occurs in the absence of any detectable increase in whole-cell $[Ca^{2+}]_{cyt}$ (Osipchuk et al., 1990). These events have been explained by the agonist inducing a highly localized increase in $[Ca^{2+}]_{cyt}$ that is not of sufficient magnitude to be detected (for full discussion, see Tsien and Tsien, 1990). Whether such an explanation has relevance to the guard cell must for the moment remain untested.

Other possible explanations for the failure to detect ABA-stimulated increases in $[Ca^{2+}]_{cyt}$ include variations in the concentration of the fluorescent $Ca^{2+}$ indicator loaded into the cytosol. This may effect changes in $[Ca^{2+}]_{cyt}$. It has been reported that iontophoretic microinjection of Indo-1 results in a cytosolic concentration of <10 μM and that this contributes little to the $Ca^{2+}$ buffering capacity of the cytosol (Gilroy et al., 1991). However, slight differences in the cytosolic concentration of the fluorescent $Ca^{2+}$ indicator may differentially buffer whole-cell changes in $[Ca^{2+}]_{cyt}$ while allowing localized changes to occur. Although it may be possible to image such localized changes under certain conditions, it is likely that the acquisition of high-resolution fluorescence images would require more of the fluorescent $Ca^{2+}$ indicator to be loaded into the cytosol, which could then buffer $[Ca^{2+}]_{cyt}$. In addition, it is possible that ratio photometry, which measures an average $[Ca^{2+}]_{cyt}$ across the whole cell, might not register these "buffered" increases as significant changes. This suggestion is supported by the observation that altering the $Ca^{2+}$ buffering capacity of the cytosol using $Ca^{2+}$ buffers has a marked effect on whole-cell $[Ca^{2+}]_{cyt}$, while still allowing the $Ca^{2+}$ concentration at the "mouth" of $Ca^{2+}$ channels to remain high (Alexandre and Lassalles, 1992). Overloading Fucus eggs with dextran-linked Fura-2 has also been observed to prevent the whole-cell $Ca^{2+}$ transient in the egg following fertilization without affecting the $Ca^{2+}$ fertilization current or partial activation of the egg as assessed by the production of the cell wall (S. K. Roberts, A. Taylor, and C. Brownlee, unpublished data).

The data reported in this paper do not exclude the possibility that other second messengers, such as inositol phospholipid derivatives (Hetherington and Drobak, 1992) and pH (Irving et al., 1992), operate in concert with $Ca^{2+}$ during the stimulus–response coupling of the ABA signal. However, on the basis of the present data, we suggest that guard cells respond to ABA by exhibiting an increase in $[Ca^{2+}]_{cyt}$ and that failure to detect this increase can be ascribed to methodological problems. Consequently, we find it unnecessary at this stage to invoke a $Ca^{2+}$-independent signaling pathway for ABA (Trewavas and Gilroy, 1991).

### Variability in ABA-Stimulated Increases in $[Ca^{2+}]_{cyt}$

Previous attempts to image ABA-stimulated changes in guard cell $[Ca^{2+}]_{cyt}$ have been unsuccessful (Gilroy et al., 1991). Our imaging studies indicate that increases in $[Ca^{2+}]_{cyt}$ in response to ABA are unevenly distributed across the cytosol of the guard cell. Similar heterogeneities have also been observed in animal cells (for a full discussion of the significance of such heterogeneities, see Tsien and Tsien, 1990). In guard cells, it is apparent that the distribution of $Ca^{2+}$ varies with time, with certain areas exhibiting highly localized increases in $[Ca^{2+}]_{cyt}$, whereas others remain comparatively unchanged (Figure 3). This indicates spatial and temporal localization of the ABA-stimulated increases in $[Ca^{2+}]_{cyt}$ that may reflect both the influx of $Ca^{2+}$ from the apoplast and the release of $Ca^{2+}$ from internal stores. However, to fully characterize the early ABA-stimulated changes in the spatial distribution of $[Ca^{2+}]_{cyt}$ in guard cells, it will be necessary to use rapid–real time confocal microscopy utilizing ratiometric $Ca^{2+}$ indicators. This technique, together with additional electrophysiological studies, should enable the question of the source of the ABA-stimulated increase in guard cell $[Ca^{2+}]_{cyt}$ to be resolved.

There is also considerable cell-to-cell variation in the ABA-stimulated increases in guard cell $[Ca^{2+}]_{cyt}$. This has been observed both in the current study and in previous work using ratio photometry (McAinsh et al., 1990; Schroeder and Hagiwara, 1990; Gilroy et al., 1991). The present data correlate well with the range of responses observed in these studies (McAinsh et al., 1990; Schroeder and Hagiwara, 1990; Gilroy et al., 1991). Variability of this nature has also been reported in experiments using animal cells. For example, a range of responses has been observed in single BC3H-1 muscle cells following treatment with phenylephrenine and histamine (Ambler et al., 1988). Individual cells within the population differed in the kinetics of the agonist-stimulated increase in $[Ca^{2+}]_{cyt}$, with some cells exhibiting oscillations in $[Ca^{2+}]_{cyt}$,

whereas this response was not detected in other cells from the same population. Interestingly, although all cells were observed to respond to phenylephrenine, between 5 and 26% failed to exhibit an increase in $[Ca^{2+}]_{cyt}$ following treatment with histamine. Kawanishi et al. (1989) have also reported heterogeneity in the response of hepatocytes to phenylephrenine and vasopressin, and the variability observed in the response of insulinoma cells to carbamylcholine has prompted Prentki et al. (1988) to suggest that each cell exhibits a unique $Ca^{2+}$ signal that they term the "$Ca^{2+}$ fingerprint."

Further work will be required before it is possible to assess accurately which components of this variability are due to subtle alterations in experimental conditions, such as agonist concentration and temperature, both of which are known to influence the form of the response in animal cells (Gray, 1988; Berridge, 1990), and what component reflects real changes in the response of individual cells to ABA.

## Synopsis

Our results correlate with those of previous studies (McAinsh et al., 1990; Schroeder and Hagiwara, 1990; Gilroy et al., 1991; Irving et al., 1992), demonstrating that ABA stimulates an increase in $[Ca^{2+}]_{cyt}$ in stomatal guard cells. An increase in $[Ca^{2+}]_{cyt}$, possibly associated with an increase in the cytosolic pH, is believed to be capable of regulating the activity of the ion channels associated with the ionic fluxes that occur during the ABA-stimulated reductions in guard cell turgor. This supports the hypothesis that $Ca^{2+}$ acts as a second messenger in plant cells.

We have imaged the ABA-stimulated increase in $[Ca^{2+}]_{cyt}$ and have demonstrated that such increases are heterogeneous both at the intracellular and intercellular level. In Commelina grown under the conditions described in this paper, we believe that the 20% of guard cells that fail to exhibit increases in $[Ca^{2+}]_{cyt}$ in response to ABA, but which show a decrease in their turgor, can be explained on the basis of methodology. This indicates that in stomatal guard cells an increase in $[Ca^{2+}]_{cyt}$ is a central requirement for the coupling of the ABA stimulus to its characteristic response.

In this present study, we have not attempted to address questions of seasonal variation, the "metabolic poise" of individual guard cells, or the role of other putative second messengers in the response to ABA (Trewavas and Gilroy, 1991). The possibility that these factors may assume importance under other as yet unspecified conditions awaits detailed analysis.

## METHODS

### Chemicals

ABA, Indo-1, and Br-A23187 were obtained from Calbiochem (Novabiochem Ltd., Nottingham, UK). Unless stated otherwise, all other chemicals were of BioChemika MicroSelect quality and obtained from Fluka (Glossop, Derbyshire, UK). All solutions were prepared in ultrahigh-quality water purified using an Elgastat UHQ II system (Elga Products Ltd., Berks, UK).

### Plant Material

*Commelina communis* plants were grown and maintained as described previously (McAinsh et al., 1991). All experiments were conducted during the middle of the photoperiod, between 10 AM and 6 PM, to minimize the effects of diurnal changes in stomatal responses. Immediately prior to each experiment, the epidermis was peeled carefully from the abaxial surface of the first fully expanded leaf of 4-week-old plants (Weyers and Travis, 1981) and floated on $CO_2$-free 10 mM Mes, 10 mM KCl, pH 6.15.

### Perfusion System

Epidermal strips of >2 cm in length were mounted, cuticle downward, on a long No. 1.5 coverslip (Chance Propper Ltd., Warley, UK) and secured around the edge with four smaller coverslips. These were attached to the bottom coverslip using low-melting point wax (Agar Scientific Ltd., Essex, UK), clamping the epidermis to the bottom coverslip, and creating a small open chamber, approximately 5 × 5 mm and one coverslip deep, over the center of the strip. This chamber was used to perfuse the central area of the strip continuously with $CO_2$-free medium at a rate of 6 mL/min. The small volume of the chamber allowed rapid changeover of the perfusion media. Measurements were made only on stomata in the center of the perfusion chamber to minimize possible edge effects. The perfusion medium was supplied along an insulated pipe from a reservoir where it was aerated with $CO_2$-free air and maintained at 25°C in a temperature-controlled water bath. There was no difference in temperature between the medium in the reservoir and that in the perfusion chamber.

### Microinjection

#### *Electrophysiology*

Guard cells were impaled with microelectrodes containing 10 mM Indo-1 in their tips. Microelectrodes (<0.25-μm tip diameter) were pulled from 0.68-mm filamented electrode glass (World Precision Instruments, Hastings, UK) using a Kopf 730 microelectrode puller (Clark Electromedical, Reading, UK). The membrane potential of the cell was recorded, and Indo-1 was microinjected iontophoretically using current pulses (1.0 nA negative pulses, 2 Hz, 200 msec duration) for up to 1 min (McAinsh et al., 1990) using an isolated stimulator and an intracellular amplifier (World Precision Instruments, Sarasota, FL). Following microinjection, the microelectrode was removed.

#### *Guard Cells of Open Stomata*

Epidermal strips were incubated under conditions promoting stomatal opening for 1 to 2 hr (McAinsh et al., 1991) to give a stomatal aperture of 12 to 15 μm before use. Subsequently, strips were mounted for microinjection, during which they were perfused with $CO_2$-free 10 mM Mes, 50 mM KCl, pH 6.15. Strips were discarded after 30 to 60 min

if microinjection was unsuccessful or if dark-induced stomatal closure was observed. Injected cells were allowed to recover for 15 min before use under continuous illumination (photon flux density of 100 μmol $m^{-2}$ $sec^{-1}$, 400 to 700 nm) from the microscope-transmitted light source (12 V tungsten lamp).

### *Guard Cells of Closed Stomata*

Freshly prepared epidermal strips in which the stomata were open to 0 to 2 μm were mounted for microinjection and were perfused with $CO_2$-free 10 mM Mes, 10 mM KCl, pH 6.15, at 25°C to prevent stomatal opening. Strips were discarded after 30 to 60 min if microinjection was unsuccessful. Injected cells were perfused with $CO_2$-free 10 mM Mes, 50 mM KCl, pH 6.15, under continuous illumination (photon flux density of 1000 μmol $m^{-2}$ $sec^{-1}$, 400 to 700 nm) from a Schott KL1500 halogen cold light source (Cologne, Germany) for 1 hr to promote stomatal opening, producing stomatal apertures of 10 to 12 μm. This had no effect on the temperature in the perfusion chamber.

## Fluorescence Ratio Analysis

### *Microscope System*

The perfusion system was mounted on the stage of a Nikon (Telford, UK) Diaphot inverted epifluorescence microscope. Epidermal strips were illuminated using either the microscope's transmitted light source (12 V tungsten lamp) for bright-field observations or excited with light from a 75-W xenon lamp (Osram, Germany) in conjunction with a 350-nm, 10-nm bandwidth interference filter (Nikon). The excitation light was reduced to 3% using quartz neutral density filters (Ealing Electro-Optics, Watford, UK). The field of excitation was limited to a small area of the specimen using a diaphragm. A Nikon CF Fluor DL 40×, oil immersion lens (aperture 1.30) and Panscan Xtra nonfluorescent immersion oil (Hughes and Hughes Ltd., Essex, UK) were used for all observations. Measurements were determined from the approximate midpoint of each cell. Fluorescent light emitted from the specimen was passed to the side camera port of the microscope via a Nikon 400-nm dichroic mirror and focused with a 10× projection lens (Nikon). The fluorescence emissions were then quantified using the appropriate combination of filters and detector system.

### *Photometric System*

Fluorescence ratio photometric measurements were made using a Cairn spectrophotometer system (Cairn Research Ltd., Kent, UK). The filter assembly, containing a dichroic mirror (DM 450) with 405- and 480-nm (10-nm bandwidth) interference filters, was attached to the side camera port of the microscope through a variable aperture PFX shutter and a rectangular diaphragm (Nikon). The diaphragm was adjusted to allow fluorescence to be monitored from the whole guard cell, allowing for slight changes in shape resulting from stomatal movements. The fluorescence at 405 and 480 nm was measured using a dual photomultiplier system (Cairn Research Ltd.) consisting of two 9924B photomultiplier tubes (Thorn EMI) and amplifiers, an analog-digital converter, and an 80386 computer (Cairn Research Ltd.). The autofluorescence of each guard cell was determined prior to microinjection with Indo-1. Autofluorescence subtraction and 405/480-nm emission ratio calculations were performed on-line during the experiment. Typically, each fluorescence measurement was recorded as the mean of 256 individual readings, to increase the signal-to-noise ratio and the ratio calculated every 0.5 sec. Ratios were converted into measurements of whole-cell $[Ca^{2+}]_{cyt}$ using a predetermined calibration curve (see below).

### *Imaging System*

Fluorescence ratio imaging was performed using a Magiscan image analysis system with MagiCal software (Joyce-Loebl Ltd., Tyne and Wear, UK). Alternate 405- and 480-nm images were acquired using a single pair of 10-nm bandwidth interference filters located in the emission filter wheel attached to the side camera port of the microscope. Images were recorded using either an ISIS-M or an Extended ISIS-M intensified CCD camera (Photonic Science, Kent, UK) connected to the filter wheel through a variable aperture PFX shutter system (Nikon). For both cameras, the optimum signal-to-noise ratio was achieved at camera intensifier and video gains of 50 to 70% of maximum. The camera output was sent to the image store of the Magiscan image analyzer, allowing sequences of up to 100, 256 × 256 pixel images to be recorded and stored on hard disc. Using a 40× lens, each pixel represented approximately 0.5 μm. The camera dark signal was subtracted from each image on-line. Typically, each image recorded was the mean of up to 50 individual frames. Averaged images took approximately 1.5 sec to acquire, with the step between filter positions taking 0.6 sec. During experiments, 405/450-nm ratio image data were acquired every 15 to 60 sec. Excitation of the specimen in between the acquisition of images was prevented using a manual shutter in front of the xenon light source. This minimized the exposure of the microinjected cell to the excitation light and reduced the photobleaching of the Indo-1.

Autofluorescence corrections were complicated by stomatal movements during experiments. This prohibited the direct subtraction of the initial image of the guard cell, prior to microinjection, from the subsequent images. Therefore, the autofluorescence image from a uniform area of an adjacent epidermal cell, exhibiting a similar level of autofluorescence to the cytosol of the guard cell, was used for autofluorescence corrections. The average resting $[Ca^{2+}]_{cyt}$ throughout the cell calculated using this technique was comparable to that measured using ratio photometry. Although this may underestimate the $[Ca^{2+}]_{cyt}$ in certain regions of the cytosol, ratio images calculated in the absence of autofluorescence correction typically underestimate $[Ca^{2+}]_{cyt}$ by 25% (Gilroy et al., 1991). Previously, an average autofluorescence that was calculated from a 12.96 $\mu m^2$ area of the cytoplasmic region of the guard cell has also been used for autofluorescence corrections across the whole area of the cell (Gilroy et al., 1991).

Following autofluorescence corrections, the 405-nm image was divided by the 480-nm image, on a pixel-by-pixel basis, to produce the ratio image. The $[Ca^{2+}]_{cyt}$ was color coded in ratio images according to a predetermined calibration curve (see below). Sequences of calibrated ratio images were used to calculate changes in the mean $[Ca^{2+}]_{cyt}$ throughout the cell with time using the MagiCal software.

### *Calibration*

In vitro calibration was carried out using 100-μL drops of calcium calibration buffers (World Precision Instruments) containing 100-μM Indo-1 (Tsien and Rink, 1980). For in vivo calibrations, Indo-1–loaded cells were treated with 10 μM Br-A23187 and equilibrated with calcium

calibration buffers for 10 min (Williams et al., 1985; Poenie et al., 1986). Similar results were obtained from both in vitro and in vivo calibrations, and in vitro calibrations were therefore routinely used (Brownlee and Pulsford, 1988; McAinsh et al., 1990; Gilroy et al., 1991).

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