

# Stimulus-Induced Oscillations in Guard Cell Cytosolic Free Calcium

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**Ca<sup>2+</sup> is implicated as a second messenger in the response of stomata to a range of stimuli. However, the mechanism by which stimulus-induced increases in guard cell cytosolic free Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) are transduced into different physiological responses remains to be explained. Oscillations in [Ca<sup>2+</sup>]<sub>i</sub> may provide one way in which this can occur. We used photometric and imaging techniques to examine this hypothesis in guard cells of *Commelina communis*. External Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>e</sub>), which causes an increase in [Ca<sup>2+</sup>]<sub>i</sub>, was used as a closing stimulus. The total increase in [Ca<sup>2+</sup>]<sub>i</sub> was directly related to the concentration of [Ca<sup>2+</sup>]<sub>e</sub>, both of which correlated closely with the degree of stomatal closure. Increases were oscillatory in nature, with the pattern of the oscillations dependent on the concentration of [Ca<sup>2+</sup>]<sub>e</sub>. At 0.1 mM, [Ca<sup>2+</sup>]<sub>e</sub> induced symmetrical oscillations. In contrast, 1.0 mM [Ca<sup>2+</sup>]<sub>e</sub> induced asymmetric oscillations. Oscillations were stimulus dependent and modulated by changing [Ca<sup>2+</sup>]<sub>e</sub>. Experiments using Ca<sup>2+</sup> channel blockers and Mn<sup>2+</sup>-quenching studies suggested a role for Ca<sup>2+</sup> influx during the oscillatory behavior without excluding the possible involvement of Ca<sup>2+</sup> release from intracellular stores. These data suggest a mechanism for encoding the information required to distinguish between a number of different Ca<sup>2+</sup>-mobilizing stimuli in guard cells, using stimulus-specific patterns of oscillations in [Ca<sup>2+</sup>]<sub>i</sub>.**

## INTRODUCTION

Strong evidence supports the role of Ca<sup>2+</sup>-based signal transduction pathways in stimulus–response coupling in plants (Bush, 1993; Gilroy et al., 1993; Poovaiah and Reddy, 1993; Webb et al., 1995). Increases in cytosolic free Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) have been observed in several cell types in response to a number of stimuli, including plant hormones, mechanical stimuli, high and low temperatures, salt stress, exposure to oxidative agents, red light, fungal elicitors, self-incompatibility factors, and electrical fields (for reviews, see Bush, 1993; Gilroy et al., 1993; Read et al., 1993; Webb et al., 1995). In addition, many components of Ca<sup>2+</sup>-based second messenger systems found in animals have also been identified in plant cells. These include primary effectors, such as Ca<sup>2+</sup>-dependent protein kinases and protein phosphatases (Bush, 1993; Gilroy et al., 1993; Poovaiah and Reddy, 1993; Webb et al., 1995).

Although considerable progress has been made in elucidating the transduction elements involved in plant cell signal transduction, little is known about the manner in which stimulus specificity is encoded in the Ca<sup>2+</sup> signal or the encryption process responsible for describing the strength of a stimulus. Several mechanisms may contribute to these processes. First, additional factors may act in parallel with Ca<sup>2+</sup> or modify the action of changes in [Ca<sup>2+</sup>]<sub>i</sub>. For example, both abscisic acid, which promotes stomatal closure (Mittelheuser and van

Steveninck, 1969), and auxin, which promotes stomatal opening (Pemadasa, 1982), induce an increase in [Ca<sup>2+</sup>]<sub>i</sub> in stomatal guard cells (Irving et al., 1992). However, whereas abscisic acid induces an increase in guard cell cytosolic pH, auxin induces a decrease in cytosolic pH (Irving et al., 1992). It has been suggested that the opposite changes in cytosolic pH are responsible for the differential response of stomata to these two plant hormones (MacRobbie, 1992; Assmann, 1993; Blatt and Thiel, 1993). Second, spatial heterogeneities in increases in [Ca<sup>2+</sup>]<sub>i</sub> may differentially activate discretely localized Ca<sup>2+</sup>-mediated signaling pathways (Tsien and Tsien, 1990). Third, oscillations in [Ca<sup>2+</sup>]<sub>i</sub> may increase the amount of information encoded by the changes in [Ca<sup>2+</sup>]<sub>i</sub> (Berridge and Galione, 1988; Berridge et al., 1988; Fewtrell, 1993).

To date, there has been little evidence of oscillations in [Ca<sup>2+</sup>]<sub>i</sub> in plants (Read et al., 1993). However, stimulus-induced oscillations in [Ca<sup>2+</sup>]<sub>i</sub> have been extensively studied in animal cells (for example, see Berridge and Galione, 1988; Berridge et al., 1988; Berridge, 1990, 1993; Tsien and Tsien, 1990; Meyer and Stryer, 1991; Fewtrell, 1993). A number of models have been proposed to account for the generation and maintenance of oscillations in [Ca<sup>2+</sup>]<sub>i</sub> in animals, and their possible physiological significance has been widely discussed (Berridge and Galione, 1988; Berridge et al., 1988; Berridge, 1990, 1993; Eichwald and Kaiser, 1993; Fewtrell, 1993). The pattern of oscillations observed is dependent on the cell type and varies depending on the strength and nature of the stimulus

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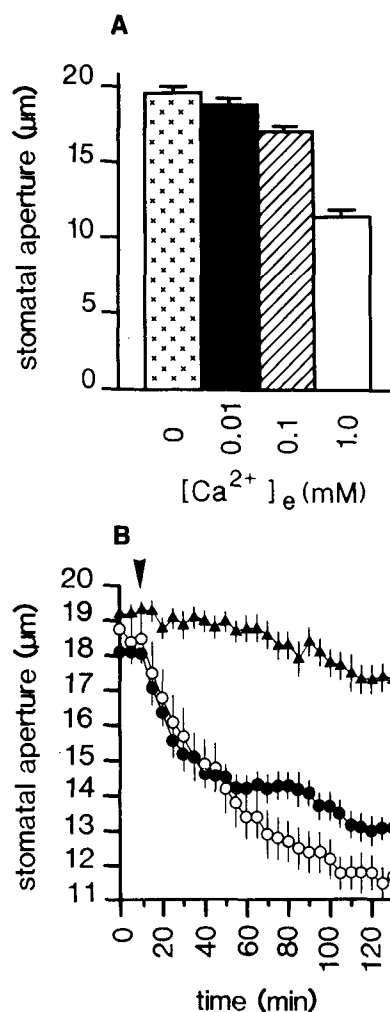
(Berridge et al., 1988; Meyer and Stryer, 1991; Fewtrell, 1993). Therefore, it has been suggested that the potential exists for encoding signaling information in the pattern of oscillations in  $[Ca^{2+}]_i$  (Berridge and Galione, 1988; Berridge et al., 1988; Fewtrell, 1993), providing a mechanism for introducing a high level of stimulus specificity into stimulus-induced increases in  $[Ca^{2+}]_i$ .

Stomatal guard cells constitute an attractive system in which to investigate intracellular signaling mechanisms in plants (MacRobbie, 1992; Assmann, 1993).  $Ca^{2+}$  has been shown to act as a second messenger in the response of stomata to both opening and closing stimuli (McAinsh et al., 1990, 1992; Schroeder and Hagiwara, 1990; Gilroy et al., 1991; Irving et al., 1992; Allan et al., 1994).  $Ca^{2+}$ -regulated ion channels, which act as primary effectors in the signal transduction cascade linking extracellular stimuli to alterations in guard cell turgor and hence stomatal aperture, have also been identified (MacRobbie, 1992; Assmann, 1993). In this study, we employed fluorescence photometric and imaging techniques (McCormack and Cobbold, 1991) to investigate the regulation of cellular  $Ca^{2+}$  homeostasis and possible mechanisms of encoding stimulus-specific information in the  $Ca^{2+}$  signal in plant cells by using the response of stomatal guard cells to external  $Ca^{2+}$  ( $[Ca^{2+}]_e$ ), a closing stimulus (Mansfield et al., 1990; MacRobbie, 1992; Assmann, 1993), as a model.

## RESULTS

### Response of Stomata to $[Ca^{2+}]_e$

Figure 1 shows that  $[Ca^{2+}]_e$  has a marked effect on stomatal aperture in *Commelina communis*, causing stomatal closure. The degree of closure is directly related to the concentration of  $[Ca^{2+}]_e$ , with the amount of stomatal closure increasing with the amount of  $[Ca^{2+}]_e$ . In the presence of 2 mM EGTA, reducing  $[Ca^{2+}]_e$  to vanishingly low levels, apertures were  $19.7 \pm 0.3 \mu\text{m}$  (Figure 1A). Incubation of open stomata for 1 hr with 0.01 mM  $[Ca^{2+}]_e$  had no significant ( $P > 0.05$ ) effect on apertures. In contrast, a 1-hr incubation in 0.1 or 1.0 mM  $[Ca^{2+}]_e$  resulted in significant ( $P < 0.05$ ) stomatal closure (giving final apertures of  $17.2 \pm 0.2$  and  $11.5 \pm 0.3 \mu\text{m}$ , respectively). Further examination of this relationship revealed that stomatal responses to  $[Ca^{2+}]_e$  were biphasic (Figure 1B). During the first phase, the initial rates of closure (determined in the first 30 min of the response) were the same for both 0.1 and 1.0 mM  $[Ca^{2+}]_e$  ( $15.9 \mu\text{m hr}^{-1}$ ). During the second phase of the response, the rate of closure induced by 1.0 mM  $[Ca^{2+}]_e$  decreased to  $\sim 3.0 \mu\text{m hr}^{-1}$ . However, there was a lag of  $\sim 30$  min when no change in stomatal aperture occurred, before the new rate of closure induced by 0.1 mM  $[Ca^{2+}]_e$  was established. The rates of closure induced by 0.1 and 1.0 mM  $[Ca^{2+}]_e$  were then the same. This resulted in the differences in stomatal aperture observed after 1 hr. There was no significant ( $P > 0.05$ ) effect of 0.01 mM  $[Ca^{2+}]_e$  on stomatal aperture until 80 min



**Figure 1.** Stomatal Responses to  $[Ca^{2+}]_e$ .

(A) Stomatal apertures following the incubation of stomata open to 18 to 20  $\mu\text{m}$  for 1 hr in the presence of 2 mM EGTA (bar at left), reducing  $[Ca^{2+}]_e$  to vanishingly low levels, or 0.01 (solid bar), 0.1 (hatched bar), or 1.0 mM (open bar)  $CaCl_2$ . Values are the means of 120 measurements ( $\pm$ SE).

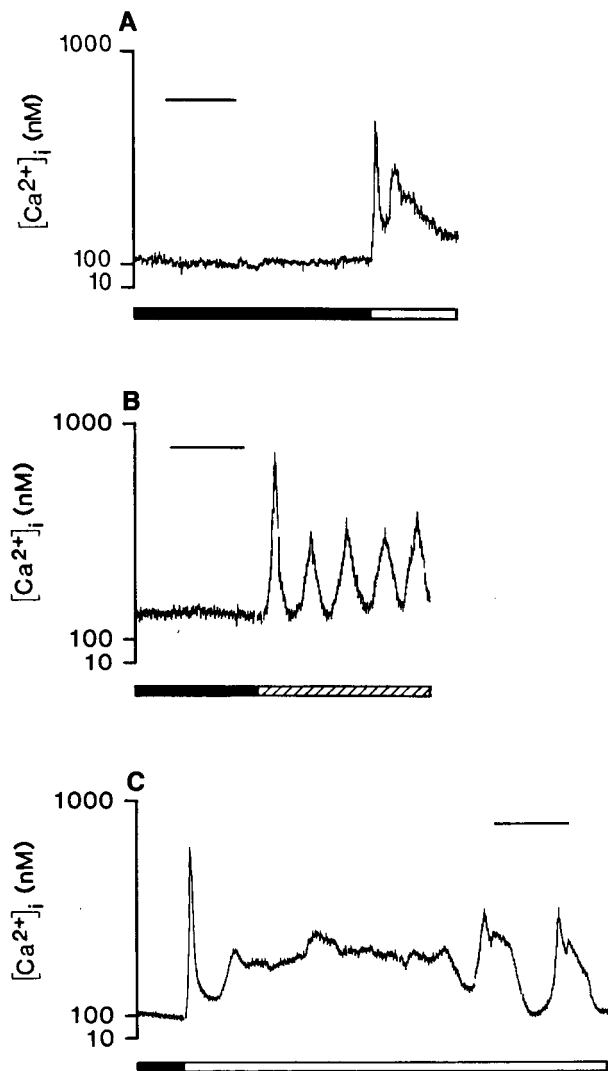
(B) Rates of stomatal closure in response to the addition (arrowhead) of 0.01 ( $\blacktriangle$ ), 0.1 ( $\bullet$ ), or 1.0 mM ( $\circ$ )  $CaCl_2$ . Values are the means of 15 measurements ( $\pm$ SE).

into the response; at this time, there was no difference between the final rates of closure induced by the three concentrations of  $[Ca^{2+}]_e$ . A two-stage response has also been reported during dark-induced stomatal closure (Mansfield and Meidner, 1966).

### Oscillations in $[Ca^{2+}]_i$ in Stomatal Guard Cells

The effects of  $[Ca^{2+}]_e$  on guard cell  $[Ca^{2+}]_i$  are shown in Figure 2. When guard cells were perfused with 0.01 mM  $[Ca^{2+}]_e$

( $n = 6$  cells), there was no change in  $[\text{Ca}^{2+}]_i$  (Figure 2A), although these cells were still capable of regulating  $[\text{Ca}^{2+}]_i$  under conditions of elevated  $[\text{Ca}^{2+}]_e$ , showing that they were undamaged. Consequently, the addition of 1.0 mM  $\text{CaCl}_2$  was



**Figure 2.** Effects of  $[\text{Ca}^{2+}]_e$  on Guard Cell  $[\text{Ca}^{2+}]_i$ .

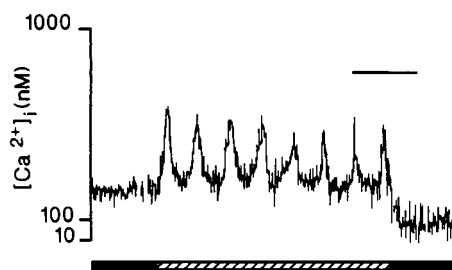
Fura-2-loaded guard cells of stomata open to 6 to 10  $\mu\text{m}$  were perfused with 0.01 (solid bars), 0.1 (hatched bar), or 1.0 mM  $[\text{Ca}^{2+}]_e$ , and  $[\text{Ca}^{2+}]_i$  was monitored by ratio photometry. Representative responses to the three concentrations of  $[\text{Ca}^{2+}]_e$  are shown in (A) to (C).

(A) No apparent effect on  $[\text{Ca}^{2+}]_i$  by 0.01 mM  $[\text{Ca}^{2+}]_e$ . The ability of cells to regulate  $[\text{Ca}^{2+}]_i$  was assessed by the addition of 1.0 mM  $\text{CaCl}_2$  ( $n = 6$  cells).

(B) Symmetrical oscillations in  $[\text{Ca}^{2+}]_i$  induced by 0.1 mM  $[\text{Ca}^{2+}]_e$  ( $n = 77$  cells).

(C) Asymmetrical oscillations in  $[\text{Ca}^{2+}]_i$  induced by 1.0 mM  $[\text{Ca}^{2+}]_e$  ( $n = 34$  cells).

Bars = 10 min.

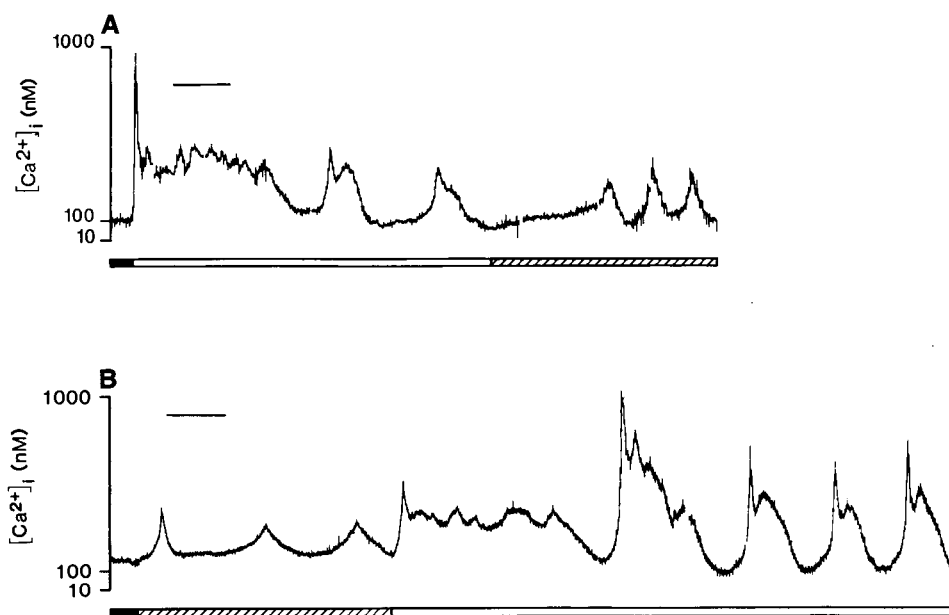


**Figure 3.** Abolition of Oscillations in  $[\text{Ca}^{2+}]_i$ .

Guard cells of closed stomata were microinjected with fura-2 into the cytosol, and the stoma opened to 6 to 10  $\mu\text{m}$ . Resting  $[\text{Ca}^{2+}]_i$  was determined during perfusion with 0.01 mM  $[\text{Ca}^{2+}]_e$  (solid bars). Guard cells were subsequently perfused with 0.1 mM  $[\text{Ca}^{2+}]_e$  (hatched bar) and then returned to 0.01 mM  $[\text{Ca}^{2+}]_e$ . Changes in  $[\text{Ca}^{2+}]_i$  were monitored by ratio photometry. The response shown is representative of measurements made with 10 cells. Bar = 10 min.

used as a positive control under these conditions and provided an additional indicator of cell viability (Gilroy et al., 1991; McAinsh et al., 1992). The resting  $[\text{Ca}^{2+}]_i$  during perfusion with 0.01 mM  $[\text{Ca}^{2+}]_e$  was determined at the start of each experiment. Resting  $[\text{Ca}^{2+}]_i$  remained at a constant level in unstimulated cells, with values ranging from 90 to 250 nM (Figures 2 to 5). These values are similar to those reported previously for guard cells (Gilroy et al., 1990, 1991; McAinsh et al., 1990, 1992; Schroeder and Hagiwara, 1990; Irving et al., 1992; Allan et al., 1994). When  $[\text{Ca}^{2+}]_e$  was increased to 0.1 mM (Figure 2B) and 1.0 mM (Figure 2C), there was a transitory rise in  $[\text{Ca}^{2+}]_i$ , which subsequently entered into a series of oscillations. Once established, the amplitude and period of the oscillations in a given cell remained constant, in the presence of the stimulus, for up to 3 hr. All cells exhibiting a regulated increase in  $[\text{Ca}^{2+}]_i$  in response to 0.1 or 1.0 mM  $[\text{Ca}^{2+}]_e$  displayed this oscillatory behavior ( $n = 102$  cells). This was not a consequence of changes in the concentration of external chloride ions rather than  $[\text{Ca}^{2+}]_e$ , because the addition of 2 mM chloride ions, by increasing the external concentration of KCl by 2 mM, had no effect on  $[\text{Ca}^{2+}]_i$  (data not shown).

The pattern of oscillations was dependent on the concentration of  $[\text{Ca}^{2+}]_e$ . Typically, 0.1 mM  $[\text{Ca}^{2+}]_e$  ( $n = 77$  cells) induced symmetrical oscillations with an amplitude of 300 to 560 nM and a period of 5 to 17 min (mean period of  $8.3 \pm 0.8$  min; Figure 2B), whereas 1.0 mM  $[\text{Ca}^{2+}]_e$  ( $n = 34$  cells) induced oscillations that were asymmetrical in character with an amplitude of 400 to 850 nM and a period of 10 to 16 min (mean period of  $13.6 \pm 0.6$  min; Figure 2C). The major oscillations characteristic of 1.0 mM  $[\text{Ca}^{2+}]_e$  were preceded by a series of minor oscillations around an elevated basal level of  $[\text{Ca}^{2+}]_i$ . These may represent an adjustment phase during which the cell was reestablishing intracellular  $\text{Ca}^{2+}$  homeostasis. The total increase in  $[\text{Ca}^{2+}]_i$  integrated over the period of the oscillations (Berridge and Prince, 1972) was 3.5 times greater when cells were perfused with 1.0 mM  $[\text{Ca}^{2+}]_e$  as



**Figure 4.** Modulation of Oscillations in  $[Ca^{2+}]_i$ .

Fura-2-loaded guard cells of open stomata (6 to 10  $\mu\text{m}$ ) were perfused with either 0.1 (hatched bars) or 1.0 mM (open bars)  $[Ca^{2+}]_e$ . After the characteristic oscillations in  $[Ca^{2+}]_i$  were established, the concentration of  $[Ca^{2+}]_e$  was changed (within the range that induced oscillatory behavior). Resting  $[Ca^{2+}]_i$  was determined at the start of each experiment during perfusion with 0.01 mM  $[Ca^{2+}]_e$  (solid bars). Representative responses are shown in (A) and (B).

(A) The effect of decreasing  $[Ca^{2+}]_e$  from 1.0 to 0.1 mM ( $n = 6$  cells).

(B) The effect of increasing  $[Ca^{2+}]_e$  from 0.1 to 1.0 mM ( $n = 9$  cells).

Bars = 10 min.

compared with oscillations induced by 0.1 mM  $[Ca^{2+}]_e$  ( $4991 \pm 781$  nM min and  $1418 \pm 260$  nM min, respectively).

#### Stimulus Dependence of Oscillations in $[Ca^{2+}]_i$

The continued presence of either 0.1 or 1.0 mM  $[Ca^{2+}]_e$  as a stimulus was required to maintain the oscillations in  $[Ca^{2+}]_i$ . Figure 3 shows that when guard cells exhibiting oscillations in response to 0.1 mM  $[Ca^{2+}]_e$  were returned to 0.01 mM  $[Ca^{2+}]_e$ , the oscillations were immediately abolished and replaced by the previous or a lower resting level of  $[Ca^{2+}]_i$  ( $n = 10$  cells). This also occurred in cells perfused with 1.0 mM  $[Ca^{2+}]_e$  and returned to 0.01 mM  $[Ca^{2+}]_e$  (data not shown). The lower resting level of  $[Ca^{2+}]_i$  observed in  $\sim 50\%$  of cells may represent a temporary adjustment phase before the reestablishment of the intracellular  $Ca^{2+}$  homeostasis.

#### Modulation of the Oscillatory Behavior

Figure 4 shows that changing  $[Ca^{2+}]_e$  within the concentration range that induced oscillations resulted in an alteration in the established oscillatory pattern. When guard cells exhibiting characteristic oscillations in response to 1.0 mM

$[Ca^{2+}]_e$  were perfused with 0.1 mM  $[Ca^{2+}]_e$  ( $n = 6$  cells), there was a change in the pattern of oscillations as the oscillations typical of the lower concentration were established (Figure 4A). This was reflected by a decrease in the ratio of the total change in  $[Ca^{2+}]_i$  integrated during the rising phase to the total change in  $[Ca^{2+}]_i$  integrated during the falling phase (from between 1.3 and 1.4 to  $\sim 1.0$ ), indicating a switch from asymmetrical to symmetrical oscillations. In addition, there was a lag phase before a new oscillatory pattern was established. This lag may reflect the cell adjusting to the new level of  $[Ca^{2+}]_e$ . In contrast, when  $[Ca^{2+}]_e$  was increased from 0.1 to 1.0 mM ( $n = 9$  cells), there was an increase in the ratio from  $\sim 1.0$  to between 1.4 and 1.7. This reflected an immediate switch from the symmetrical oscillations induced by 0.1 mM  $[Ca^{2+}]_e$  to the oscillatory behavior, which was characterized by asymmetrical oscillations, typical of that induced by 1.0 mM  $[Ca^{2+}]_e$  (Figure 4B).

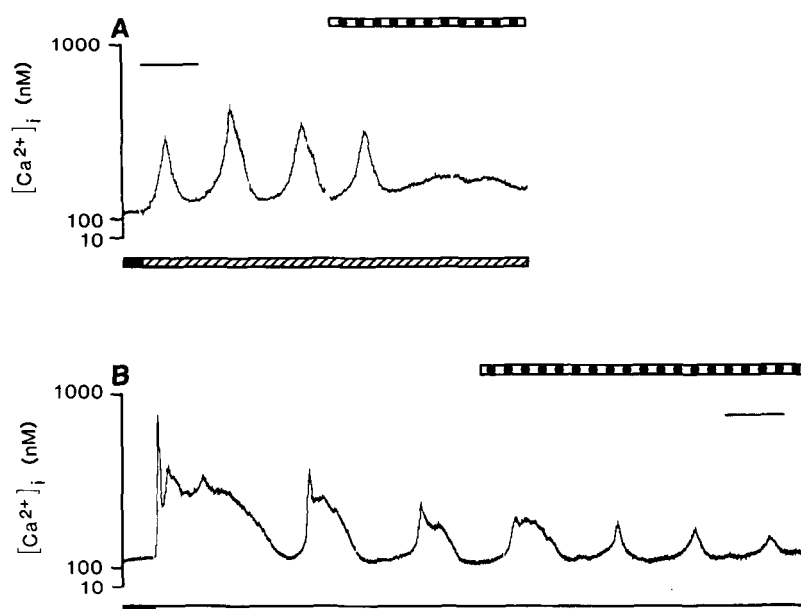
#### $Ca^{2+}$ Influx across the Plasma Membrane Contributes to the Rising Phase of Oscillations in $[Ca^{2+}]_i$

We have used two approaches to assess the contribution made by  $Ca^{2+}$  influx to the observed oscillatory behavior:  $Ca^{2+}$  channel blockers, which are known to inhibit  $Ca^{2+}$  entry into

plant cells (Hetherington et al., 1992) and have been previously shown to be active in stomatal guard cells (MacRobbie, 1989; McAinsh et al., 1991), and the Mn<sup>2+</sup>-quenching technique (Sage et al., 1989; Kass et al., 1990; Fasolato et al., 1993).

Figure 5 shows that the Ca<sup>2+</sup> channel blocker verapamil caused a marked change in the oscillatory behavior induced by [Ca<sup>2+</sup>]<sub>e</sub>. When cells exhibiting oscillations in response to 0.1 mM [Ca<sup>2+</sup>]<sub>e</sub> were perfused with 0.1 mM [Ca<sup>2+</sup>]<sub>e</sub> containing 0.01 mM verapamil (*n* = 10 cells), there was a reduction in the amplitude of the oscillations leading to the abolition of the oscillatory behavior (Figure 5A). A similar effect on 0.1 mM [Ca<sup>2+</sup>]<sub>e</sub>-induced oscillations was observed using 0.01 mM bepridil (data not shown). In contrast, 0.01 mM verapamil caused an alteration in the pattern of oscillations induced by 1.0 mM [Ca<sup>2+</sup>]<sub>e</sub> (*n* = 5 cells). Oscillations were converted from the characteristic asymmetrical pattern to symmetrical oscillations (Figure 5B). At both concentrations of [Ca<sup>2+</sup>]<sub>e</sub>, there was a lag of one oscillation before these changes became apparent. Increasing the verapamil concentration from 0.01 to 0.1 mM had no further effect on the pattern of oscillations (data not shown). Unfortunately, it was not possible to use La<sup>3+</sup>, which has been shown to inhibit <sup>45</sup>Ca<sup>2+</sup> influx into guard cells (MacRobbie, 1989), in these studies due to interference with the Ca<sup>2+</sup>-dependent fluorescence of fura-2 (data not shown).

Mn<sup>2+</sup> has been widely used as a tracer to study Ca<sup>2+</sup> influx across the plasma membrane in animal cells (Sage et al., 1989; Kass et al., 1990; Fasolato et al., 1993). This technique relies on two well-established experimental observations: (1) Mn<sup>2+</sup> competes with Ca<sup>2+</sup> for passage through Ca<sup>2+</sup>-permeable channels (Sage et al., 1989; Luckhoff and Clapham, 1992); and (2) Mn<sup>2+</sup> has a very high affinity for fura-2 (dissociation constant of 2 nM), so that when Mn<sup>2+</sup> binds to fura-2, it quenches the fluorescence of the dye at all excitation wavelengths (Kwan and Putney, 1990). Changes in both the Ca<sup>2+</sup>-independent fluorescence (used to follow changes in fluorescence due to quenching by Mn<sup>2+</sup>) and the Ca<sup>2+</sup>-dependent fluorescence (used to calculate [Ca<sup>2+</sup>]<sub>i</sub>) can be monitored simultaneously. This allows changes in [Ca<sup>2+</sup>]<sub>i</sub> to be related to the entry of Mn<sup>2+</sup>, and by inference, the Ca<sup>2+</sup> influx across the plasma membrane, into cells. Recently, the Mn<sup>2+</sup> permeability of Ca<sup>2+</sup>-selective channels has been clearly demonstrated in plants (Piñeros and Tester, 1995). This suggests that Mn<sup>2+</sup> entry can also be used to trace Ca<sup>2+</sup> influx into plant cells through Ca<sup>2+</sup>-permeable channels by following the reduction in the Ca<sup>2+</sup>-independent fluorescence of fura-2-loaded cells in the presence of external Mn<sup>2+</sup>. However, the Mn<sup>2+</sup> permeability of Ca<sup>2+</sup>-permeable channels still remains to be demonstrated in guard cells, and therefore, this



**Figure 5.** Effects of Verapamil on [Ca<sup>2+</sup>]<sub>e</sub>-Induced Oscillations in [Ca<sup>2+</sup>]<sub>i</sub>.

Fura-2-loaded guard cells of stomata open to 6 to 10 μm were perfused with either 0.1 (hatched bar) or 1.0 mM (open bar) [Ca<sup>2+</sup>]<sub>e</sub>. After the characteristic oscillations in [Ca<sup>2+</sup>]<sub>i</sub> were established, guard cells were perfused with the same concentration of [Ca<sup>2+</sup>]<sub>e</sub> containing 0.01 mM verapamil (dotted bars). Resting [Ca<sup>2+</sup>]<sub>i</sub> was determined at the start of each experiment during perfusion with 0.01 mM [Ca<sup>2+</sup>]<sub>e</sub> (solid bars). Representative responses are shown in (A) and (B).

(A) The effect of 0.01 mM verapamil on oscillations in [Ca<sup>2+</sup>]<sub>i</sub> induced by 0.1 mM [Ca<sup>2+</sup>]<sub>e</sub> (*n* = 10 cells).

(B) The effect of 0.01 mM verapamil on oscillations in [Ca<sup>2+</sup>]<sub>i</sub> induced by 1.0 mM [Ca<sup>2+</sup>]<sub>e</sub> (*n* = 5 cells).

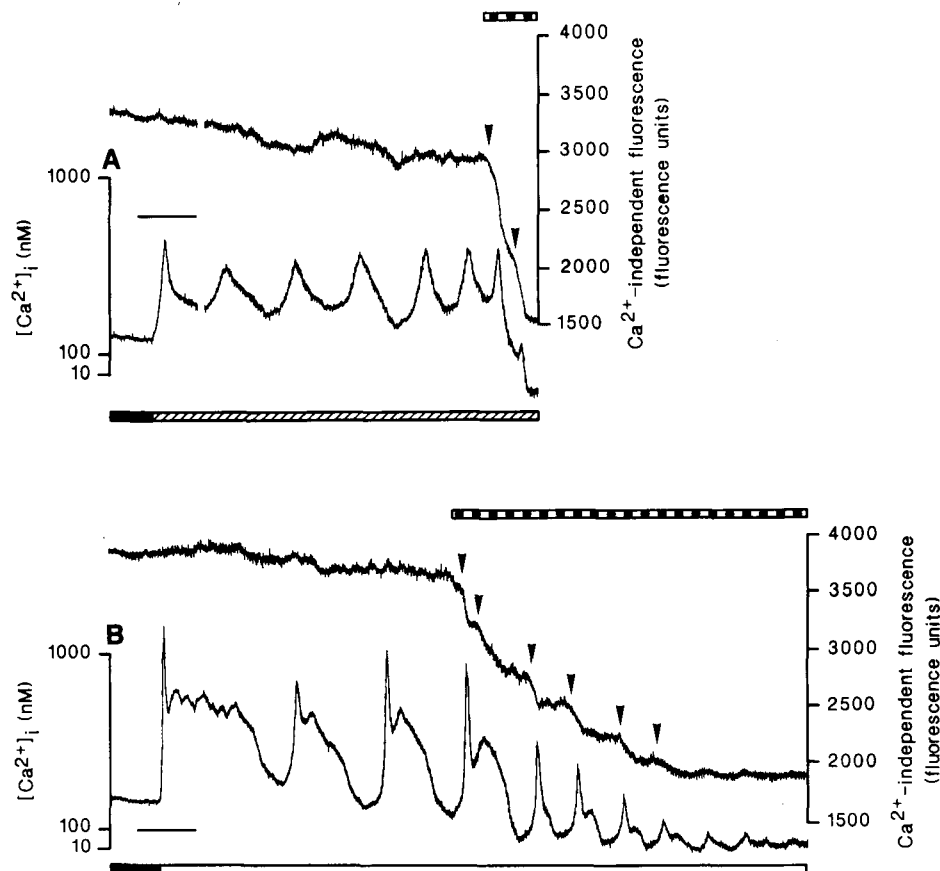
Bars = 10 min.

technique does not exclude the possibility of  $Mn^{2+}$  entry through nonselective channels or  $Ca^{2+}$  influx via an  $Mn^{2+}$ -impermeable route.

Figure 6 shows that  $Mn^{2+}$  had a marked effect on both the  $Ca^{2+}$ -independent fluorescence and the  $Ca^{2+}$ -dependent fluorescence ratio. When guard cells exhibiting characteristic oscillations in response to 0.1 mM ( $n = 7$  cells; Figure 6A) or 1.0 mM ( $n = 6$  cells; Figure 6B)  $[Ca^{2+}]_e$  were perfused with the same concentration of  $[Ca^{2+}]_e$  containing 1.0 mM  $MnCl_2$ , there was an immediate decrease in the  $Ca^{2+}$ -independent

fura-2 fluorescence. Decreases occurred in a series of rapid drops followed by periods in which the fluorescence remained relatively constant. The rapid drops in fluorescence corresponded exactly with the rising phase of oscillations. These data indicate that  $Mn^{2+}$ , and by inference  $Ca^{2+}$ , enters guard cells during the rising phase of oscillations in  $[Ca^{2+}]_i$ .

There was also damping of the oscillatory behavior following the addition of 1.0 mM  $MnCl_2$ . Although less striking, this effect is similar to the effect of  $Ca^{2+}$  channel blockers on  $[Ca^{2+}]_e$ -induced oscillations in  $[Ca^{2+}]_i$  (Figure 5) and may



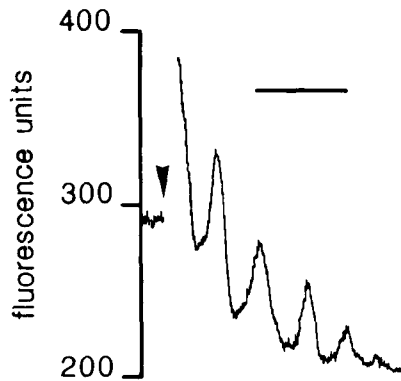
**Figure 6.** The Use of  $Mn^{2+}$  as a Tracer of  $Ca^{2+}$  Influx during Oscillations in  $[Ca^{2+}]_i$ .

Guard cells of stomata open to between 6 and 10  $\mu m$  and loaded with fura-2 in the cytosol were perfused with either 0.1 (hatched bar) or 1.0 mM (open bar)  $[Ca^{2+}]_e$ . After the characteristic oscillations in  $[Ca^{2+}]_i$  were established, guard cells were perfused with the same concentration of  $[Ca^{2+}]_e$  containing 1.0 mM  $MnCl_2$  (dotted bars).  $Mn^{2+}$ , which can be used as a tracer for  $Ca^{2+}$  channel-mediated  $Ca^{2+}$  influx, quenches the fluorescence emissions of fura-2 at all excitation wavelengths. Changes in both the  $Ca^{2+}$ -dependent fura-2 fluorescence (used to calculate  $[Ca^{2+}]_i$ ) and the  $Ca^{2+}$ -independent fluorescence (used to follow  $Ca^{2+}$ -independent changes in the fura-2 fluorescence due to quenching that indicated the entry of  $Mn^{2+}$  into cells) were monitored photometrically.  $Mn^{2+}$  quenching of the  $Ca^{2+}$ -independent fura-2 fluorescence occurred in a series of steps (arrowheads marking rapid drops in fluorescence). Resting  $[Ca^{2+}]_i$  was determined at the start of each experiment during perfusion with 0.01 mM  $[Ca^{2+}]_e$  (solid bars). Representative responses are shown in (A) and (B).

(A) Changes in the  $Ca^{2+}$ -dependent fluorescence ratio and  $Ca^{2+}$ -independent fura-2 fluorescence in response to 1.0 mM external  $Mn^{2+}$  during oscillations in  $[Ca^{2+}]_i$  induced by 0.1 mM  $[Ca^{2+}]_e$  ( $n = 7$  cells).

(B) Changes in the  $Ca^{2+}$ -dependent fluorescence ratio and  $Ca^{2+}$ -independent fura-2 fluorescence in response to 1.0 mM external  $Mn^{2+}$  during oscillations in  $[Ca^{2+}]_i$  induced by 1.0 mM  $[Ca^{2+}]_e$  ( $n = 6$  cells).

Bars = 10 min.



**Figure 7.** Oscillations in  $[\text{Ca}^{2+}]_i$  Induced by the Intracellular Release of  $\text{Ca}^{2+}$  from Caged  $\text{Ca}^{2+}$ .

Guard cells of closed stomata perfused with 0.01 mM  $[\text{Ca}^{2+}]_e$  were microinjected with 0.1 mM Calcium Green-1 (a nonratiometric  $\text{Ca}^{2+}$ -sensitive dye) and 10 mM Nitr-5 (caged  $\text{Ca}^{2+}$ ) into the cytosol, and the stoma subsequently opened to 6 to 10  $\mu\text{m}$ . Photoactivation of the Nitr-5 to release  $\text{Ca}^{2+}$  was achieved by exposure to a 10- to 20-sec pulse of 380-nm light (arrowhead). Changes in  $[\text{Ca}^{2+}]_i$  were monitored photometrically and expressed in fluorescence units (high values represent high  $[\text{Ca}^{2+}]_i$ ). The response shown is representative of measurements made with three cells. Bar = 10 min.

reflect the inhibition of  $\text{Ca}^{2+}$  transport across the plasma membrane by  $\text{Mn}^{2+}$  in a manner analogous to that reported in membrane vesicles of maize (Marshall et al., 1994).

#### Increases in $[\text{Ca}^{2+}]_i$ Trigger Oscillatory Behavior

The photolytic release of  $\text{Ca}^{2+}$  from caged  $\text{Ca}^{2+}$  in guard cells perfused with 0.01 mM  $[\text{Ca}^{2+}]_e$ , a concentration that has no effect on  $[\text{Ca}^{2+}]_i$  (Figure 2A), induced oscillatory behavior. Figure 7 shows that oscillations in  $[\text{Ca}^{2+}]_i$  were symmetrical with a mean period of  $4.5 \pm 0.3$  min ( $n = 3$  cells). The period of the oscillations in a given cell remained constant. However, oscillations became rapidly damped and were abolished within 30 min following photoactivation of caged  $\text{Ca}^{2+}$ . This damped oscillatory behavior may represent a  $\text{Ca}^{2+}$  homeostatic response in which  $[\text{Ca}^{2+}]_i$  "hunts" before a new steady resting level of  $[\text{Ca}^{2+}]_i$  is established. The falling phase of the oscillations may result from  $\text{Ca}^{2+}$  being pumped out of the cell or sequestered into intracellular stores, whereas the rising phase may reflect either  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release from intracellular  $\text{Ca}^{2+}$  stores (Berridge et al., 1988; Berridge, 1990, 1993; Eichwald and Kaiser, 1993; Fewtrell, 1993) or  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  influx across the plasma membrane.

#### Spatial Distributions of $[\text{Ca}^{2+}]_i$ during Oscillations in Response to $[\text{Ca}^{2+}]_e$ Are Uneven

Spatial heterogeneities in increases in  $[\text{Ca}^{2+}]_i$  may play an important role during  $\text{Ca}^{2+}$ -mediated signal transduction (Tsien

and Tsien, 1990). Therefore, simultaneous imaging and photometric studies were conducted on the same guard cell to investigate the spatial and temporal dynamics of the oscillatory changes in  $[\text{Ca}^{2+}]_i$ . Ratio images obtained using this technique show the spatial distribution of  $[\text{Ca}^{2+}]_i$   $\sim 2.5$  sec after the last photometric determination of  $[\text{Ca}^{2+}]_i$  made on-line before the images were acquired. Figure 8 shows that increases in  $[\text{Ca}^{2+}]_i$  were unevenly distributed throughout cells during the oscillatory behavior characteristic of both 0.1 mM ( $n = 6$  cells; Figures 8A and 8B) and 1.0 mM ( $n = 5$  cells; Figures 8C and 8D)  $[\text{Ca}^{2+}]_e$ . In addition, during the transitory rise and minor oscillations that precede the characteristic oscillations induced by 1.0 mM  $[\text{Ca}^{2+}]_e$ , increases in  $[\text{Ca}^{2+}]_i$  appeared to occur in two distinct stages (Figures 8C and 8D). Initially, increases were observed in the cytoplasmic regions around the periphery of cells (Figure 8C, image 2), followed by an increase in the cytoplasm surrounding the vacuolar region (Figure 8C, image 3). However, to date, we have been unable to demonstrate a similar pattern of spatial-temporal localization in any of the characteristic oscillations in response to 0.1 or 1.0 mM  $[\text{Ca}^{2+}]_e$ . This may reflect the discrete intervals at which images are acquired. Consequently, increased temporal resolution may be required before it is possible to assess the significance of these observations.

## DISCUSSION

### The Response of Guard Cells to $[\text{Ca}^{2+}]_e$ Provides a Model for the Investigation of $\text{Ca}^{2+}$ -Based Signal Transduction

We used stomatal responses to  $[\text{Ca}^{2+}]_e$  as a model to address one of the outstanding questions in the area of  $\text{Ca}^{2+}$ -based cell signaling, namely, the mechanism by which stimuli of different strengths are transduced into a graded physiological response through increases in  $[\text{Ca}^{2+}]_i$ . Figure 1 demonstrates clearly the ability of stomatal guard cells to discriminate between different strengths of an external signal when formulating their final response, that is, a reduction of guard cell turgor leading to stomatal closure. In addition, these data show that differences in signal strength were not simply translated into differences in the rate of stomatal closure (Figure 1B). In light of these observations, we investigated the potential for the  $\text{Ca}^{2+}$  signal to contain the stimulus-specific information required to produce a specific stimulus-dependent response.

### The Strength of the External Stimulus Determines the Nature of the $\text{Ca}^{2+}$ Signal

The results described in this study show conclusively that the nature of the  $\text{Ca}^{2+}$  signal is absolutely dependent on the strength of the external stimulus (Figure 2). These data also demonstrate that it is possible to switch between different

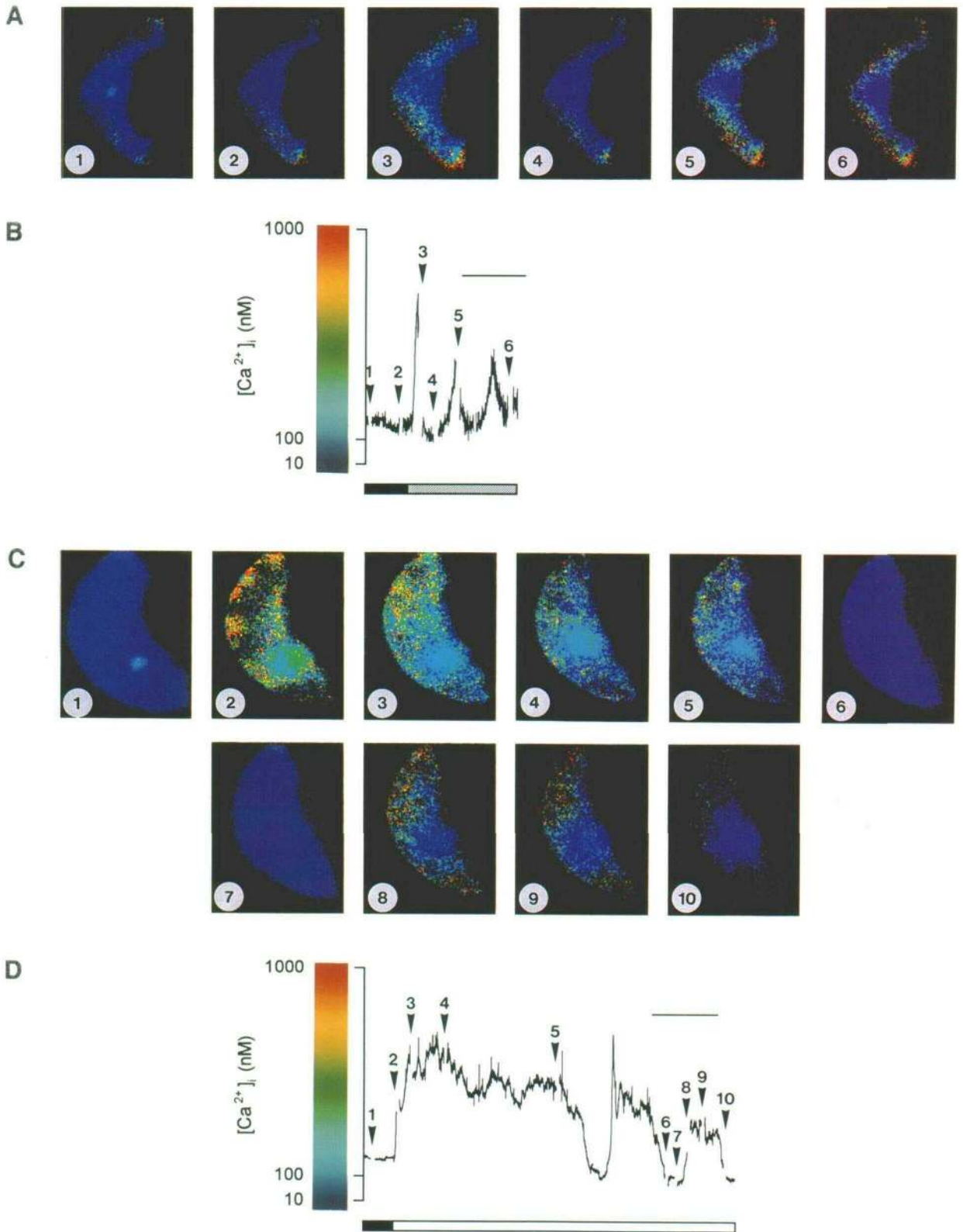


Figure 8. Simultaneous Ratio Imaging and Ratio Photometry of Oscillations in  $[Ca^{2+}]$ .



Ca<sup>2+</sup> signals by changing the strength of the external stimulus and that this switch is reversible (Figures 3 and 4). The generation of stimulus-specific patterns of oscillations in [Ca<sup>2+</sup>]<sub>i</sub> may provide a mechanism for coupling signals of different strengths to their characteristic responses. Previously, changes in [Ca<sup>2+</sup>]<sub>i</sub> akin to oscillations have been reported in a minority of guard cells (McAinsh et al., 1990; Gilroy et al., 1991) and in oat protoplast (Volotovski et al., 1993) and maize epidermal cells (Felle, 1988). However, in many studies, it has been concluded that these represent artifacts of signal detection (for review, see Read et al., 1993). Our data illustrate the direct relationship between the strength of the external stimulus (that is, the concentration of [Ca<sup>2+</sup>]<sub>e</sub>), the pattern of the oscillations, and the magnitude of the physiological response (that is, the degree of stomatal closure).

In animals, oscillations in [Ca<sup>2+</sup>]<sub>i</sub> occur in a wide range of cell types in response to many different agonists (for reviews, see Berridge et al., 1988; Berridge, 1990; Fewtrell, 1993). For a given cell type, the pattern of agonist-induced oscillations in [Ca<sup>2+</sup>]<sub>i</sub> is a direct function of both the agonist applied and its concentration (Berridge et al., 1988; Meyer and Stryer, 1991; Fewtrell, 1993). Typically, oscillations with periods varying from <1 sec in cardiac muscle up to 30 min in mouse oocytes have been reported (Berridge et al., 1988). Therefore, the mean period of oscillations in [Ca<sup>2+</sup>]<sub>i</sub> observed in guard cells (0.1 mM [Ca<sup>2+</sup>]<sub>e</sub>, 8.3 ± 0.8 min; 1.0 mM [Ca<sup>2+</sup>]<sub>e</sub>, 13.6 ± 0.6 min) falls within the range observed in animals. On the basis of the data presented in this article, the generation of stimulus-specific oscillations in [Ca<sup>2+</sup>]<sub>i</sub> that are modulated in a manner dependent on the strength of the stimulus, creating a stimulus-specific "Ca<sup>2+</sup> fingerprint," appears to be an important feature of Ca<sup>2+</sup>-based cell signaling, which is conserved between plants and animals.

### The Generation of Oscillations in [Ca<sup>2+</sup>]<sub>i</sub>

Several models have been proposed for the generation and maintenance of oscillations in [Ca<sup>2+</sup>]<sub>i</sub> in animal cells, and these have been reviewed extensively (Berridge and Galione, 1988; Berridge et al., 1988; Berridge, 1990, 1993; Eichwald and Kaiser, 1993; Fewtrell, 1993). We have used two approaches

to examine the origin of the Ca<sup>2+</sup> required for the generation of oscillations in [Ca<sup>2+</sup>]<sub>i</sub>: (1) Ca<sup>2+</sup> channel blockers that are known to inhibit Ca<sup>2+</sup> entry into plant cells (Hetherington et al., 1992) and that have been shown previously to have activity in stomatal guard cells (MacRobbie, 1989; McAinsh et al., 1991), and (2) the Mn<sup>2+</sup>-quenching technique (Sage et al., 1989; Kass et al., 1990; Fasolato et al., 1993). The Ca<sup>2+</sup> channel blocker verapamil caused a reduction in the amplitude of the oscillations in [Ca<sup>2+</sup>]<sub>i</sub> induced by 0.1 mM [Ca<sup>2+</sup>]<sub>e</sub>, leading to abolition of the oscillatory behavior (Figure 5A). In addition, Figure 6A shows clearly that the rapid entry of Mn<sup>2+</sup>, and by inference Ca<sup>2+</sup> influx, coincided with the rising phase of oscillations. These data may suggest that Ca<sup>2+</sup> influx is responsible for the increases in [Ca<sup>2+</sup>]<sub>i</sub> during 0.1 mM [Ca<sup>2+</sup>]<sub>e</sub>-induced oscillations, implying that an oscillator, located in the plasma membrane, is responsible for regulating Ca<sup>2+</sup> influx. A plasma membrane Ca<sup>2+</sup> channel, which is regulated either directly by [Ca<sup>2+</sup>]<sub>e</sub> or indirectly by another factor such as membrane potential, could constitute such an oscillator. Patch clamp data have revealed several different classes of plasma membrane channels that can allow the entry of Ca<sup>2+</sup> into stomatal guard cells: stretch-activated Ca<sup>2+</sup>-selective channels; the inwardly rectifying K<sup>+</sup> channel, which exhibits a limited permeability to Ca<sup>2+</sup>; and nonselective Ca<sup>2+</sup>-permeable channels (for a review, see Schroeder, 1992). However, until recently there has been little electrophysiological evidence for a specific voltage-dependent Ca<sup>2+</sup> channel in the plasma membrane of plants. Membrane depolarization is an early event in the signal transduction cascade triggered by many physiological stimuli in plants (for review, see Tester, 1990). Recently, voltage-dependent Ca<sup>2+</sup>-permeable channels have been reported in the plasma membrane of carrot cells (Thuleau et al., 1994a, 1994b) and in plasma membrane fractions from wheat (Piñeros and Tester, 1995) and rye (White, 1994) roots. If these Ca<sup>2+</sup>-permeable channels exist in stomatal guard cells, they could constitute a regulated pathway for the entry of Ca<sup>2+</sup> into the cytosol during oscillations in [Ca<sup>2+</sup>]<sub>i</sub>.

The falling phase of oscillations in response to 0.1 mM [Ca<sup>2+</sup>]<sub>e</sub> can be explained most simply through the action of Ca<sup>2+</sup>-sensitive Ca<sup>2+</sup> efflux mechanisms (for reviews, see Evans et al., 1991; Hetherington et al., 1992; Johannes et al., 1992; Maathuis and Sanders, 1992; Evans, 1994; Webb et al.,

**Figure 8.** (continued).

Guard cells of closed stomata were microinjected with fura-2 into the cytosol, and the stoma opened to 6 to 10 μm. Resting [Ca<sup>2+</sup>]<sub>i</sub> was determined during perfusion with 0.01 mM [Ca<sup>2+</sup>]<sub>e</sub> (solid bars), after which guard cells were perfused with either 0.1 (hatched bar) or 1.0 mM (open bar) [Ca<sup>2+</sup>]<sub>e</sub>, stimulating characteristic oscillatory behavior. Oscillations were continuously monitored by ratio photometry (**B**) and (**D**). Ratio images were obtained at discrete intervals (**A**) and (**C**), depending on the changes in [Ca<sup>2+</sup>]<sub>i</sub> reported by the ratio calculated on-line using photometry. The numbered images shown in (**A**) and (**C**) indicate the spatial distribution of [Ca<sup>2+</sup>]<sub>i</sub> ~2.5 sec after the numbered points shown in (**B**) and (**D**) indicated by arrowheads.

**(A)** Ratio images of oscillations in [Ca<sup>2+</sup>]<sub>i</sub> induced by 0.1 mM [Ca<sup>2+</sup>]<sub>e</sub> (*n* = 6 cells).

**(B)** Oscillations in [Ca<sup>2+</sup>]<sub>i</sub> induced by 0.1 mM [Ca<sup>2+</sup>]<sub>e</sub> monitored by ratio photometry (*n* = the same 6 cells as in **(A)**).

**(C)** Ratio images of oscillations in [Ca<sup>2+</sup>]<sub>i</sub> induced by 1.0 mM [Ca<sup>2+</sup>]<sub>e</sub> (*n* = 5 cells).

**(D)** Oscillations in [Ca<sup>2+</sup>]<sub>i</sub> induced by 1.0 mM [Ca<sup>2+</sup>]<sub>e</sub> monitored by ratio photometry (*n* = the same 5 cells as in **(C)**).

Bars = 10 min.

1995).  $\text{Ca}^{2+}$ -ATPases, identified in the plasma membrane, and the tonoplast and endoplasmic reticulum of plant cells (Evans et al., 1991; Evans, 1994), together with  $\text{H}^+/\text{Ca}^{2+}$  antiporters (Johannes et al., 1992) and outward-rectifying  $\text{Ca}^{2+}$  channels reported in the vacuolar membrane (Pantoja et al., 1992), may provide such a mechanism, allowing  $\text{Ca}^{2+}$  to be expelled from the cytosol into the apoplast or to be sequestered into intracellular stores such as the vacuole.

In contrast, oscillations in response to 1.0 mM  $[\text{Ca}^{2+}]_e$  are more complex than those elicited by 0.1 mM  $[\text{Ca}^{2+}]_e$ ; consequently their generation requires additional explanation. Figure 5B shows that verapamil converts the oscillatory behavior from an asymmetrical pattern to a symmetrical one, whereas Figure 6B shows that  $\text{Mn}^{2+}$ , and by inference  $\text{Ca}^{2+}$ , enters cells during the rising phase of oscillations in  $[\text{Ca}^{2+}]_i$  in response to 1.0 mM  $[\text{Ca}^{2+}]_e$ . Therefore, our data suggest that  $\text{Ca}^{2+}$  influx, regulated by an oscillator(s) located in the plasma membrane, contributes to the generation of 1.0 mM  $[\text{Ca}^{2+}]_e$ -induced oscillations in  $[\text{Ca}^{2+}]_i$ . In addition, the inability of verapamil to abolish totally the oscillatory behavior and the apparent absence of  $\text{Mn}^{2+}$  influx during the second half of the asymmetrical oscillatory pattern raise the possibility that a second source of  $\text{Ca}^{2+}$  may contribute to the generation of this oscillatory behavior. This source might be  $\text{Ca}^{2+}$  released from intracellular stores through the action of an intracellular oscillator. However, our data do not exclude the involvement of  $\text{Ca}^{2+}$  influx through either verapamil-insensitive and/or  $\text{Mn}^{2+}$ -impermeable  $\text{Ca}^{2+}$  channels in the plasma membrane. A vacuolar membrane  $\text{Ca}^{2+}$  channel would constitute a prime candidate for an intracellular oscillator. Three categories of  $\text{Ca}^{2+}$ -selective channels have been characterized in the vacuolar membrane of plants: inward-rectifying voltage-gated channels, inositol 1,4,5-trisphosphate-gated  $\text{Ca}^{2+}$  release channels, and outward-rectifying  $\text{Ca}^{2+}$  channels (for a review, see Allen and Sanders, 1994). Recently, the voltage-dependent slow vacuolar cation channel, which has been characterized previously as a nonselective  $\text{Ca}^{2+}$ -activated voltage-dependent channel (for a review, see Ward and Schroeder, 1994), has also been implicated in the release of  $\text{Ca}^{2+}$  from the vacuole in guard cells (Ward and Schroeder, 1994).

### The Significance of Oscillations in $[\text{Ca}^{2+}]_i$

Our data demonstrate clearly that the pattern of oscillations in guard cell  $[\text{Ca}^{2+}]_i$  is directly related to the strength of the external stimulus, both of which correlate with the magnitude of the physiological response. This suggests strongly that in plants, as in animals, oscillations in  $[\text{Ca}^{2+}]_i$  provide the potential for encoding stimulus-specific information in the  $\text{Ca}^{2+}$  signal (Berridge and Galione, 1988; Berridge et al., 1988; Meyer and Stryer, 1991; Fewtrell, 1993). Whether this represents a universal mechanism employed by all plant cells for a range of stimuli requires further investigation. In addition, we can only speculate about the signaling machinery downstream of  $\text{Ca}^{2+}$  that is responsible for decoding this information. It has

been proposed that in animals, protein phosphorylation may provide a mechanism by which this decoding can occur via the coupled action of a phosphatase and a kinase (Goldbeter et al., 1990). For example, oscillatory changes in  $[\text{Ca}^{2+}]_i$  would modify the activity of a  $\text{Ca}^{2+}$ -activated protein phosphatase while having no effect on that of a  $\text{Ca}^{2+}$ -independent protein kinase, causing the level of the dephosphorylated form of a target protein to go up and down. The presence of phosphatases and kinases, which exhibit different  $\text{Ca}^{2+}$  activation kinetics, could allow differential decoding of stimulus-specific patterns of oscillations in  $[\text{Ca}^{2+}]_i$  into a range of physiological responses that can occur downstream of phosphorylation events.

### Conclusions

Our studies indicate a role for stimulus-induced oscillations in  $[\text{Ca}^{2+}]_i$  during  $\text{Ca}^{2+}$ -based signal transduction. The pattern of stimulus-induced oscillations is directly related to the strength of the external stimulus, both of which correlate with the degree of stomatal closure. This suggests that oscillations are a feature of  $\text{Ca}^{2+}$ -based signaling that is conserved between plants and animals and that they provide a possible mechanism for encoding the information required to distinguish between a number of different  $\text{Ca}^{2+}$ -mobilizing stimuli. However, it is also possible to imagine a more prosaic but equally important explanation to account for their existence. Oscillations may be a feature of the machinery responsible for the maintenance of cellular  $\text{Ca}^{2+}$  homeostasis, representing cycles of  $\text{Ca}^{2+}$  efflux and release in response to " $\text{Ca}^{2+}$  overload" as the cell establishes a new resting level of  $[\text{Ca}^{2+}]_i$ . Nevertheless, the results presented in this study highlight the importance of establishing the concentration of apoplastic free  $\text{Ca}^{2+}$  that is perceived by plant cells, because, on the basis of these data, the concentration of  $[\text{Ca}^{2+}]_e$  selected for experimental manipulations would be expected to influence  $[\text{Ca}^{2+}]_i$  in a profound manner.

### METHODS

#### Plant Material

Plants (*Commelina communis*) were grown from seed (McAinsh et al., 1991). Immediately prior to each experiment, the epidermis was peeled carefully from the abaxial surface of the youngest fully expanded leaf of 4-week-old plants and floated on  $\text{CO}_2$ -free 10 mM KCl, 10 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes)-KOH, pH 6.15, at 25°C (McAinsh et al., 1992).

#### Epidermal Strip Bioassay

Freshly prepared epidermis was cut into 5-mm lengths and incubated under conditions promoting stomatal opening for 3 hr (McAinsh et al.,

1991) and then transferred to CO<sub>2</sub>-free 50 mM KCl, 10 mM Mes-KOH, pH 6.15 (KCl-Mes), containing either 2 mM EGTA or 0.01, 0.1, or 1.0 mM CaCl<sub>2</sub>. The strips were incubated for an additional 1 hr, after which they were examined under the microscope to determine the aperture of the stomatal pores.

### Video Microscopy

Epidermal strips (>2 cm in length) were incubated under conditions promoting stomatal opening (McAinsh et al., 1991) and then mounted, cuticle downward, in a perfusion chamber (McAinsh et al., 1990, 1992). Strips were illuminated (photon flux density of 100 μmol m<sup>-2</sup> sec<sup>-1</sup>) from the transmitted light source (12 V tungsten lamp) of a Nikon (Sussex, UK) Diaphot inverted microscope and perfused with CO<sub>2</sub>-free KCl-Mes containing 0.01, 0.1, or 1.0 mM CaCl<sub>2</sub>. Stomatal movements were monitored continuously using a JVC (Sussex, UK) color CCD camera and recorded using a SVHS video recorder (JVC). Apertures were subsequently measured from the video monitor calibrated using a Graticule (Dorset, UK) slide.

### Microinjection of Fluorescent Dyes and Caged Ca<sup>2+</sup>

Freshly prepared epidermis (>2 cm in length) in which stomata were open to <1 μm were mounted cuticle side downward in a perfusion chamber (McAinsh et al., 1990, 1992). Guard cells were impaled with filamented glass microelectrodes (<0.25 μm tip diameter) containing 10 mM fura-2 pentapotassium salt or 0.1 mM Calcium Green-1 and 10 mM Nitr-5 (caged Ca<sup>2+</sup>; Calbiochem-Novabiochem, Nottingham, UK) in their tips. Dyes and caged probes were microinjected into the cytosol iontophoretically (McAinsh et al., 1990, 1992). Injected cells were maintained under conditions promoting stomatal opening for 45 min (McAinsh et al., 1992). Fluorescence measurements were only made on stomata that opened to the same aperture as those on the rest of the epidermal strip (6 to 10 μm) and in which both the injected and noninjected cells of a single stoma exhibited the same increase in turgor. Epidermal strips in which injected guard cells met all the criteria for estimating viability (Gilroy et al., 1991; McAinsh et al., 1992) were perfused with CO<sub>2</sub>-free KCl-Mes, containing 0.01, 0.1, or 1.0 mM CaCl<sub>2</sub> at 25°C in the presence or absence of 1 mM MnCl<sub>2</sub> or 0.01 mM verapamil (Calbiochem-Novabiochem), and the fluorescence was monitored.

### Fluorescence Microscopy

The perfusion chamber was mounted on the stage of a Nikon Diaphot inverted epifluorescence microscope (McAinsh et al., 1992). Excitation light was provided by a 100 W Nikon xenon light source. Excitation wavelengths were specified using 10-nm bandwidth interference filters (fura-2, 340 and 380 nm, 400-nm dichroic mirror; Calcium Green-1, 495 nm, 515-nm dichroic mirror [Cairn Research Ltd., Kent, UK]). These were selected using a spinning filter changer in combination with three metal gauze neutral density filters (reducing the excitation intensity to 3%) and transmitted to the microscope via a liquid light guide (Cairn Research Ltd.). The field of excitation was limited to the area of a single stoma using the excitation diaphragm of the microscope. A Nikon CF Fluor DL 40× oil immersion lens (1.30 numerical aperture) and nonfluorescent immersion oil (Fluka, Dorset, UK) were used for all measurements. Fluorescent light was passed to the side camera port of

the microscope and focused with a 1× television relay lens (Nikon). A Nikon PFX shutter assembly and rectangular emission aperture were used to limit the area of the specimen studied to one stoma. Fluorescence emissions (fura-2, 510 nm with a 20-nm bandwidth interference filter [Nikon]; Calcium Green-1, 530 nm with a 10-nm bandwidth interference filter [Cairn Research Ltd.]) were quantified using the appropriate filter and detector combination.

### Photometry

Fluorescence measurements were made using a Cairn spectrophotometer system. The autofluorescence of each guard cell was determined at each excitation wavelength prior to microinjection (McAinsh et al., 1990, 1992). Autofluorescence subtraction was calculated on-line. The spinning filter changer was run at 64 revolutions per second in spin mode, allowing fluorescence measurements to be obtained at a rate of 64 readings per second. Values were calculated as the mean of 64 individual readings to increase the signal-to-noise ratio. A data point was given each second. For fura-2, the ratio (340/380 nm) was calculated each second on-line. Ratios were converted into measurements of whole-cell cytosolic free Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) using a predetermined calibration curve (see later section).

### Ratio Imaging

Ratio imaging and ratio photometric studies were conducted simultaneously on the same cell. Excitation light was provided by a Cairn spinning filter changer operating in a step mode, which enabled the excitation wavelengths to be changed manually between 340 and 380 nm, for imaging and in a spin mode for photometric measurements. The emission wavelength (fura-2, 510 nm with a 20 nm bandwidth) was specified using a Nikon interference filter. A mechanical detector changer (built by Peter Williams, Lancaster University, Lancaster, UK) was used to switch rapidly between detectors. A switch from photometry to imaging took less than a second (with the return to photometry taking up to 15 sec, depending on the final speed of the spinning filter changer, during which time no photometric measurements were obtained). Fluorescence images were recorded using a cooled extended ISIS-M intensified CCD camera (Photonic Science, Sussex, UK). Images were acquired using an ARGUS-50 image analysis system (Hamamatsu Photonics Ltd., Middlesex, UK). Typically, images were captured at a resolution of 512 × 512 pixels and digitized to 256 gray levels. Each image recorded was the mean of eight individual frames. The time taken to acquire a pair of 340- and 380-nm images, including the step between filter positions, was ~1.5 sec. Image pairs were obtained at discrete intervals, depending on the changes in [Ca<sup>2+</sup>]<sub>i</sub> reported by the ratio calculated on-line using photometry. The camera dark signal was recorded prior to each experiment and subtracted on-line. The average autofluorescence value, calculated from the cytoplasmic region of an unloaded guard cell at both excitation wavelengths, was subtracted from images at the end of each experiment (Gilroy et al., 1991). After autofluorescence subtraction, the 340-nm image was divided by the 380-nm image on a pixel-by-pixel basis to produce a ratio image. Ratio images obtained using this technique show the spatial distribution of [Ca<sup>2+</sup>]<sub>i</sub> ~2.5 sec after the last photometric determination of [Ca<sup>2+</sup>]<sub>i</sub> made on-line before the pair of 340- and 380-nm images were acquired. The [Ca<sup>2+</sup>]<sub>i</sub> was coded as different pseudocolors using a predetermined calibration curve (see later section).

### Calibration

Similar results were obtained for both in vitro and in vivo calibration of the  $\text{Ca}^{2+}$ -dependent ratio in guard cells (Gilroy et al., 1991; McAinsh et al., 1992). Consequently, an in vitro calibration is routinely used. Calibration of the  $\text{Ca}^{2+}$ -dependent fluorescence of Calcium Green-1 is complex and only yields approximate values. Therefore, measurements of  $[\text{Ca}^{2+}]_i$  using Calcium Green-1 were expressed in relative fluorescence units (Allan et al., 1994).

### $\text{Mn}^{2+}$ -Quenching Experiments

The influx of  $\text{Ca}^{2+}$  into fura-2-loaded guard cells was assessed through the quenching of the  $\text{Ca}^{2+}$ -independent fura-2 fluorescence signal (excitation isosbestic, 360 nm with a 10-nm bandwidth interference filter [Cairn Research Ltd.]; emission, 510 nm with a 20-nm bandwidth interference filter [Nikon]) by  $\text{Mn}^{2+}$  (1.0 mM  $\text{MnCl}_2$  in  $\text{CO}_2$ -free KCl-Mes containing either 0.1 mM or 1.0 mM  $\text{CaCl}_2$ ) (Sage et al., 1989; Kass et al., 1990; Fasolato et al., 1993).

### Photoactivation of Caged $\text{Ca}^{2+}$

Photoactivation of Nitr-5 (caged  $\text{Ca}^{2+}$ ) was achieved by exposure to a 10- to 20-sec pulse of 380-nm light (10-nm bandwidth interference filter; no neutral density filters; 400-nm dichroic mirror [Cairn Research Ltd.]) from the 100 W xenon light source of a Nikon Diaphot epifluorescence microscope (Gilroy et al., 1990) via a liquid light guide (Cairn Research Ltd.) and a Nikon CF Fluor DL 40x oil immersion lens (1.30 numerical aperture) using nonfluorescent immersion oil.

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