**CO₂ signaling in guard cells: Calcium sensitivity response modulation, a Ca²⁺-independent phase, and CO₂ insensitivity of the gca2 mutant**

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Leaf stomata close in response to high carbon dioxide levels and open at low CO₂. CO₂ concentrations in leaves are altered by daily dark/light cycles, as well as the continuing rise in atmospheric CO₂. Relative to abscisic acid and blue light signaling, little is known about the molecular, cellular, and genetic mechanisms of CO₂ signaling in guard cells. Interestingly, we report that repetitive Ca²⁺ transients were observed during the stomatal opening stimulus, low [CO₂]. Furthermore, low/high [CO₂] transitions modulated the cytosolic Ca²⁺ transient pattern in *Arabidopsis* guard cells (Landsberg erecta). Inhibition of cytosolic Ca²⁺ transients, achieved by loading guard cells with the chemical chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid and not adding external Ca²⁺, attenuated both high CO₂-induced stomatal closing and low CO₂-induced stomatal opening, and also revealed a Ca²⁺-independent phase of the CO₂ response. Furthermore, the mutant, *growth controlled by abscisic acid* (gca2) shows impairment in [CO₂] modulation of the cytosolic Ca²⁺ transient rate and strong impairment in high CO₂-induced stomatal closing. Our findings provide insights into guard cell CO₂ signaling mechanisms, reveal Ca²⁺-independent events, and demonstrate that calcium elevations can participate in opposed signaling events during stomatal opening and closing. A model is proposed in which CO₂ concentrations prime Ca²⁺ sensors, which could mediate specificity in Ca²⁺ signaling.

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tomatopores in aerial parts of plants close in response to high carbon dioxide concentrations and open at low [CO₂]. These changes in leaf CO₂ concentrations occur as a result of photosynthesis and respiration. Furthermore, atmospheric CO₂ is predicted to double in the present century (1). Many studies have been carried out to determine the effects of atmospheric CO₂ increases on plant gas exchange, carbon fixation, and growth and the resulting impact this will have on natural and agricultural ecosystems (2–6). One of the mechanisms by which increased atmospheric CO₂ affects plants is CO₂ regulation of stomatal apertures. Reports show that a doubling of atmospheric [CO₂] causes significant stomatal closure by 20–40% in diverse plant species (7–9).

Stomata experience diurnal changes in [CO₂] inside the leaf during light/dark transitions. In the dark, CO₂ is produced in leaves by cellular respiration. The [CO₂] shifts caused by illumination changes are rapid and large, with [CO₂] in the stomatal cavity ranging from 200 to 650 ppm (10).

Stomatal aperture is controlled by the turgor pressure in the guard cells surrounding the stomatal pore. Guard cell turgor pressure is mediated by the ion and organic solute concentration in guard cells. Elevated CO₂ has been shown to enhance potassium efflux channel and S type anion channel activities that mediate extrusion of ions during stomatal closure (11, 12). In correlation with these findings, chloride release from guard cells is triggered by CO₂ elevation (13), and high CO₂ causes depolarization of guard cells (14, 15). Furthermore, CO₂ activation of R type anion channel currents was found in a subset of *Vicia faba* guard cells (12).

Little is known about the CO₂ signal transduction mechanisms that function upstream of ion channels in guard cells (16–19). CO₂-induced stomatal movements were previously found to be absent in two mutants that affect the stomatal closure signaling network for the drought stress hormone abscisic acid (ABA): *abi1-1* and *abi2-1* (20). Conditional CO₂ responsiveness was reported in these mutants by using different experimental conditions (21, 22). Another ABA-insensitive mutant, *ost1*, shows a wild-type response to [CO₂] (23). Although the *abi1-1* and *abi2-1* mutants can show a conditional effect, strong CO₂-insensitive stomatal movement response mutants have not been reported until recently.

Studies have suggested a role for Ca²⁺ in mediating CO₂-induced stomatal closing (24, 25). High [CO₂] induces cytosolic Ca²⁺ elevation in Commelina communis guard cells (24). Many studies have established that cytosolic calcium functions in mechanisms that mediate stomatal closing (16–18, 24, 26–32). Furthermore, some studies have also indicated a role for calcium increases in the opposing response of stomatal opening (33–35). It is unclear how these opposite responses could be directed via elevations in the same second messenger, Ca²⁺.

CO₂ is a particularly interesting stomatal stimulus with respect to cytosolic Ca²⁺ responses, as demonstrated in the present study. CO₂ can induce stomatal opening or closing depending on its concentration (36). Studies in animal and plant cells have shown that different patterns of repetitive calcium transients modulate responses (29, 37–39). Although previous studies have shown a role for calcium in CO₂ signaling in guard cells (24, 25), changes in repetitive calcium transient patterns have not yet been studied in guard cells in response to CO₂. The present study demonstrates CO₂ modulation of repetitive calcium transient patterns in *Arabidopsis* guard cells and characterizes roles of Ca²⁺ in CO₂-regulated stomatal opening and stomatal closing, revealing both calcium-independent and calcium-dependent components. In addition, we show that the ABA-insensitive mutant, *growth controlled by abscisic acid* (gca2), exhibits a strong CO₂ insensitivity in cytosolic Ca²⁺ pattern regulation, in stomatal aperture regulation, and in CO₂ regulation of stomatal conductance changes in leaves. Furthermore, findings including repetitive cytosolic Ca²⁺ elevations in response to low CO₂ lead us to propose a new model in CO₂ and stomatal signaling, in which signal transduction occurs via stimulus-dependent priming of guard cell Ca²⁺ sensors.

**Results**

**Cytosolic Calcium Responses to Changes in [CO₂].** Calcium imaging was performed on *Arabidopsis* (cv. Landsberg erecta) guard cells

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Abbreviations: ABA, abscisic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; BAPTA-AM, BAPTA-acetoxymethyl ester.

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expressing the GFP-based calcium indicator yellow cameleon 2.1 (40), which allows long-term (>90-min) ratiometric measurements of changes in free $[\text{Ca}^{2+}]_{\text{cyt}}$ in guard cells (41, 42). Leaf epidermides were preexposed to low CO$_2$ buffer (115 ppm), and guard cells were analyzed in low CO$_2$ buffer and then exposed to elevated CO$_2$ buffer (508 ppm). Unexpectedly, more $[\text{Ca}^{2+}]_{\text{cyt}}$ transients were produced in the low CO$_2$ (stomatal opening) buffer than in the elevated CO$_2$ (stomatal closing) buffer (Fig. 1 A and D and Fig. 4, which is published as supporting information on the PNAS web site). This CO$_2$-induced shift in the $[\text{Ca}^{2+}]_{\text{cyt}}$ transient rate was observed in the majority of guard cells (36 of 41; 88%). Leaf epidermides were subsequently returned to low CO$_2$-free buffer (115 ppm) again, and the transient rate increased (Fig. 1 A and D). This reversibility in the $[\text{Ca}^{2+}]_{\text{cyt}}$ transient rate was observed in 78% of cells (32 of 41). Thus in guard cells, the rate of $[\text{Ca}^{2+}]_{\text{cyt}}$ transient production decreased after the switch from low CO$_2$ buffer to high CO$_2$ and increased after the switch back to low CO$_2$ buffer (Fig. 1 A), showing that the $[\text{Ca}^{2+}]_{\text{cyt}}$ transient rate is modulated by CO$_2$ concentration changes. Furthermore, repetitive $[\text{Ca}^{2+}]_{\text{cyt}}$ transients occur at low CO$_2$, a condition that mediates stomatal opening. Gas exchange experiments in which a similar series of $[\text{CO}_2]$ changes were imposed showed a reduction in stomatal conductance in response to high CO$_2$ and increased after the switch back to low CO$_2$ buffer (508 ppm) CO$_2$ buffer. Where two CO$_2$ concentrations are listed, the first value represents the $[\text{CO}_2]$ measured in the buffer after perfusion, whereas the second value, listed in parentheses, represents the $[\text{CO}_2]$ of the air source bubbled into the buffer prior to perfusion (see Materials and Methods).

Additional approaches were pursued to further analyze CO$_2$ modulation of $[\text{Ca}^{2+}]_{\text{cyt}}$ transient patterns. Wavelet transformation allows data analyses at a range of widths of wavelet functions (43). This ensures that the results found with the transient detection software do not depend on the specific function width chosen. Independent wavelet analyses also showed clear reversible changes in the $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation pattern in response to CO$_2$ concentration changes as shown in Fig. 1 D. $[\text{Ca}^{2+}]_{\text{cyt}}$ imaging traces were transformed with a Morlet wavelet (44). The goodness of fits of the waveform to the imaging trace is depicted by the color scale. The weaker colors in high CO$_2$ indicate there are fewer transients in high CO$_2$ across all function widths (scales) shown. Thus transitions from low [CO$_2$] to high [CO$_2$] cause a significant change in the $[\text{Ca}^{2+}]_{\text{cyt}}$ transient pattern in response to CO$_2$ concentration changes as shown in Fig. 1 D. Amplitudes of $[\text{Ca}^{2+}]_{\text{cyt}}$ transients were compared at low CO$_2$ and high CO$_2$ by analyzing ratio changes during the initial two CO$_2$ exposures, before substantial bleaching of the yellow fluorescent protein acceptor occurred (40, 41). $[\text{Ca}^{2+}]_{\text{cyt}}$ transient ratios were 0.26 ± 0.02 ratio units at low CO$_2$ and 0.21 ± 0.02 ratio units at high CO$_2$ ($P > 0.1$, $n = 11$ guard cells), suggesting no dramatic effects of CO$_2$ on $[\text{Ca}^{2+}]_{\text{cyt}}$ transient amplitudes under the imposed conditions. The average width of calcium transients was also calculated. Average transient half-amplitude widths were 61 ± 3 seconds at low CO$_2$ and 69 ± 4 seconds at high CO$_2$ ($P = 0.06$, $n = 41$ guard cells). Average transient width measured at the base of transients was 129 ± 6 seconds at low CO$_2$ and 150 ± 8 seconds at high CO$_2$ ($P = 0.04$, $n = 41$ guard cells). These analyses suggest that the calcium transients in high CO$_2$ have a slightly longer duration than those in low CO$_2$, but this difference was not as dramatic as the difference in $[\text{Ca}^{2+}]_{\text{cyt}}$ transient rates in high and low CO$_2$ (Fig. 1 C).

To further investigate CO$_2$ modulation of the $[\text{Ca}^{2+}]_{\text{cyt}}$ transient rate, calcium imaging was also performed on cells exposed to the
opposite series of [CO₂] regimes. Cells were first exposed to elevated [CO₂], switched to low CO₂, and finally switched back to elevated [CO₂]. In these experiments, 69% (18 of 26) showed an increase in the transient rate upon switching from elevated [CO₂] to low CO₂, and the majority of those cells (15 of 18, 88% of 26 guard cells) showed a decrease in the transient rate when they were returned to elevated CO₂. Automated analyses show a statistically significant difference in the [Ca^{2+}]_cyt transient rates produced in high and low CO₂ using this protocol (high CO₂, 0.95 ± 0.13; low CO₂, 1.55 ± 0.16 transients per 10 min; n = 26 guard cells; P = 0.005). Note that, for a given cell, the relative change in [Ca^{2+}]_cyt transient rate in response to a physiological stimulus may be more relevant than the absolute observed [Ca^{2+}]_cyt transient rate during stimulation, which can depend on experimental conditions (45–47). Experiments were also performed by using ambient CO₂. At ambient CO₂, spontaneous Ca^{2+} transients were observed, as described (41, 47). Transient rates in ambient CO₂ determined by using automated analysis, including traces without transients, were compared to transient rates in low and high CO₂, and a significant difference was found in both cases (Fig. 1E, P < 0.001 for both ambient vs. low CO₂ and ambient vs. high CO₂).

Analysis of CO₂ Concentrations in Calcium Imaging Experiments. During calcium imaging, epidermes were placed in a well and perfused via a peristaltic pump and Teflon tubing with buffers equilibrated by bubbling with air containing either 0 ppm CO₂ or high CO₂ (740 or 800 ppm). During travel of the buffer through the Teflon tubing and residence of the buffer in the well, a significant degree of equilibration of the [CO₂] with the ambient air should occur (48). The CO₂ content of the buffers bathing the epidermes during calcium imaging was determined (see Materials and Methods). [CO₂] of buffer equilibrated with 0 ppm CO₂ was 115 ± 11 ppm, [CO₂] of buffer equilibrated with 740 ppm CO₂ was interpolated to 508 ppm, and [CO₂] of buffer equilibrated with 800 ppm CO₂ was 535 ± 23 ppm (±SEM, n = 3).

Stomatal Responses to [CO₂] Shifts in the Absence of [Ca^{2+}]_cyt Transients. Interestingly, commencement of significant stomatal aperture responses was measured within 10 min during low to high CO₂ shifts (P ≤ 0.05; Fig. 1B; Fig. S, which is published as supporting information on the PNAS web site), whereas calcium transients were produced, on average, once every 10 min in high CO₂ (Fig. 1A, D, and E). Thus it appears that calcium transient pattern changes would occur too slowly to directly function in early guard cell CO₂ responses (see Discussion). Therefore, to test whether [Ca^{2+}]_cyt transients function in CO₂ regulation of stomatal movements, we sought a condition under which these transients are abolished.

Guard cells were loaded with the calcium chelator 1,2-bis(2-amino-5-phenoxo)ethane-N,N,N',N'-tetraacetic acid (BAPTA), by using an esterified form, BAPTA–acetoxymethyl ester (BAPTA-AM). Incubation of leaf epidermes with a buffer containing 25 μM BAPTA-AM and no added extracellular calcium caused inhibition of [Ca^{2+}]_cyt transients in 12 of 12 cells in 115 ppm CO₂ buffer (Fig. 2, additional traces in Fig. 6, which is published as supporting information on the PNAS web site). After [Ca^{2+}]_cyt inhibition, baseline ratios changed by only −0.015 ± 0.01 (n = 12) on average, and in 5 of 12 cells, no baseline reduction was observed (Fig. 6), indicating no dramatic changes in baseline [Ca^{2+}]_cyt, which contrasts with dramatic reduction in baseline [Ca^{2+}]_cyt induced by nicotinamide (47). Note that esterified BAPTA-AM does not chelate Ca^{2+} (see Materials and Methods). Thus the combination of BAPTA loading into cells without addition of Ca^{2+} to the external buffer inhibited repetitive [Ca^{2+}]_cyt transients in guard cells (Fig. 2A).

We then analyzed whether CO₂-induced stomatal movements are affected when repetitive cytosolic Ca^{2+} transients are inhibited. We determined that measurement of robust CO₂-induced stomatal movement responses in leaf epidermes requires intact epidermal pavement cells surrounding the analyzed stomata. Thus we added a viability stain (fluorescein diacetate) at the end of incubations immediately before aperture measurements to allow collection of stomatal aperture data exclusively from guard cells surrounded by live pavement cells. In control experiments with 50 μM Ca^{2+} added to the bath solution, shifts from ambient [CO₂] to low [CO₂] caused stomatal opening and shifts from ambient [CO₂] to high [CO₂] caused stomatal closing (Fig. 2B; P = 0.01 for 0 ppm CO₂ vs. ambient CO₂, P < 0.001 for 800 ppm CO₂ vs. ambient CO₂). CO₂-induced stomatal movements from ambient [CO₂] did not occur when cells were treated under conditions in which calcium transients were abolished (Fig. 2C; P > 0.42 for 0 ppm CO₂ vs. ambient CO₂, P > 0.28 for 800 ppm CO₂ vs. ambient CO₂).
Identification and Characterization of a CO₂-Insensitive Mutant. Strong CO₂-insensitive mutants in stomatal movement responses have not been identified until recently (see Introduction). To further analyze the significance of CO₂-regulated [Ca²⁺]cyt transient responses to stomatal movements, we analyzed CO₂ signal transduction in stomatal response mutants. Guard cells from the dominant ABA-insensitive protein phosphatase 2C mutant abil-1 showed a reduction in the [Ca²⁺]cyt transient rate upon elevation of CO₂ in 19 of 23 cells and a significantly different average number of transients produced in low and elevated CO₂, showing a CO₂ response (Fig. 3A, P = 0.001). Guard cells of the recessive ABA-insensitive gca2 mutant have been previously documented to show an aberrant pattern of [Ca²⁺]cyt transient production compared to wild type in ABA signal transduction (29). Interestingly, when guard cells of the gca2 mutant were exposed to low CO₂ buffer, high CO₂ buffer, and then low CO₂ buffer again, no marked change in the average [Ca²⁺]cyt transient rate was observed, whereas parallel wild-type controls (Ler) showed typical changes in the transient rate (Fig. 3A and B; additional traces are in Fig. 8, which is published as supporting information on the PNAS web site). Upon the shift from low to high CO₂, the [Ca²⁺]cyt transient rate decreased in only 31% of gca2 guard cells (9 of 29). Only 24% of gca2 guard cells (7 of 29) reversibly produced more transients in the low CO₂ buffer than in the high CO₂ buffer. Furthermore, the average rates of [Ca²⁺]cyt transients in gca2 guard cells exposed to low and high CO₂ were similar (Fig. 3A, P = 0.88).

The physiological relevance of the impairment of high CO₂ modulation of the [Ca²⁺]cyt transient rate in gca2 was investigated by analyzing CO₂-induced stomatal closure responses in leaf epidermal strips of the gca2 mutant and wild-type (Ler) controls. In contrast to wild type, significant high CO₂-induced stomatal closure was not observed in gca2 (Fig. 3C, P = 0.001 for wild type, P = 0.20 for gca2). Next, gas exchange analyses were pursued to determine whether the gca2 mutant impairs CO₂ responses in intact leaves of growing plants. A small CO₂-induced stomatal closure response was resolved in gca2 in response to CO₂ elevation (Fig. 3D). However, CO₂-induced reduction in stomatal conductance was dramatically weaker in gca2 than in wild-type leaves (Fig. 3D, P < 0.001). In contrast, gca2 had a more robust stomatal opening response to low CO₂ (Fig. 3E). The stomatal density in leaves of gca2 plants did not differ from wild-type controls (wild type, 90 ± 19 stomata mm⁻²; gca2, 89 ± 2 stomata mm⁻², SEM, n = 3, P = 0.9). Thus guard cells of the gca2 mutant did not show statistically significant shifts in the [Ca²⁺]cyt transient rate in response to [CO₂] shifts (Fig. 3A and B) and showed strongly attenuated stomatal closure in response to [CO₂] elevation in leaf epidermes and in intact leaves of plants (Fig. 3C and D). These data show that gca2 is a strong high CO₂-insensitive mutant.

Discussion

Understanding the molecular mechanisms by which CO₂ modulates stomatal apertures is fundamental to understanding the regulation of gas exchange between plants and the atmosphere and is important for elucidating effects of the combined diurnal changes in leaf [CO₂] (10) and the continuing atmospheric CO₂ elevation on stomatal movements (3-5, 7, 8, 18). Our findings build upon an earlier study showing that [Ca²⁺]cyt elevations function in CO₂-induced stomatal movements (24) and in addition unexpectedly demonstrate the presence of repetitive [Ca²⁺]cyt transients at low CO₂, which opens stomatal pores (Figs. 1 A and D, 2A, and 3B). Furthermore, guard cell [Ca²⁺]cyt imaging experiments for >90 min
show that CO₂ concentration changes modulate the [Ca²⁺]_{cyt} transient pattern in *Arabidopsis* (Ler) guard cells, as determined by automatic transient detection and wavelet analyses (Fig. 1 C, D, and E). Moreover both CO₂-induced stomatal closing and CO₂-induced stomatal opening are attenuated when [Ca²⁺]_{cyt} transients are experimentally repressed (Fig. 2). In addition, the gca2 mutant shows insensitivity to elevated CO₂ in modulation of the [Ca²⁺]_{cyt} transient rate, stomatal closing, and stomatal conductance responses in intact leaves showing that gca2 is a strong high-CO₂ insensitive mutant (Fig. 3).

**Ca²⁺ Sensor Priming Model.** Our data show that elevated CO₂ causes slow repetitive [Ca²⁺]_{cyt} transients in guard cells, similar to those that function in ABA signaling (29–31, 42), whereas low CO₂ causes rapid [Ca²⁺]_{cyt} transients (Figs. 1 and 2A). It has been shown that experimentally imposed cytosolic calcium transients in guard cells will trigger immediate (Ca²⁺ reactive) stomatal closure, regardless of the Ca²⁺ pattern (29, 49, 50). Such a stomatal closing response would seem unlikely when the calcium transients are produced in response to a stomatal opening stimulus such as low CO₂. Indeed, the rapid [Ca²⁺]_{cyt} transients induced by low CO₂ in guard cells (Figs. 1, 2A, and 3B) did not cause measurable short-term Ca²⁺ reactive stomatal closure responses based on time-resolved stomatal movement imaging analyses (J.J.Y., unpublished results). These findings indicate that the stomatal opening signal, low CO₂, produces a signal transduction state or “physiological address” (51) in guard cells that does not permit strong Ca²⁺ reactive stomatal closing events to proceed. These results lead us to propose a new model in which, rather than solely causing different patterns of [Ca²⁺]_{cyt} increases, physiological stimuli may modulate the appropriate calcium sensors (Ca²⁺ sensor priming), affecting when they can respond to [Ca²⁺]_{cyt} transients.

Previous studies showing spontaneous [Ca²⁺]_{cyt} elevations in guard cells and ABA-induced dampering of these [Ca²⁺]_{cyt} transients (41, 47) indicate that ABA-induced stomatal closing may also make use of Ca²⁺ sensor modulation (priming) during stomatal closing. Consistent with this model, a previous study demonstrated that [Ca²⁺]_{cyt} activation of guard cell anion channels can be turned off or “deprimed” by prior incubation conditions of guard cells (see figure 3 in ref. 52). Future studies of Ca²⁺ sensor functions and modifications in guard cells could provide a novel approach and valuable insights into the mechanisms by which opposing cellular responses can be mediated by Ca²⁺ in the same cell type.

**CO₂ [Ca²⁺]_{cyt} Modulation and Membrane Polarization.** Interestingly, the present study provides evidence that [CO₂] modulation of the cytosolic Ca²⁺ transient rate itself is not involved in the earliest stomatal movements in response to [CO₂] shifts (Figs. 2E and 7). Consistent with these findings, measurable stomatal conductance and aperture changes appear to occur more rapidly than the establishment of a new calcium transient pattern (Figs. 1 A, B, and D; 2E; 3D; 4; 5; and 7). This suggests the existence of events downstream of initial CO₂ responses that trigger modulation of the calcium spike frequency. One such event could be a change in the membrane potential. Reports have shown that decreases in [CO₂] cause hyperpolarization, whereas increases in [CO₂] cause depolarization of the guard cell plasma membrane (11, 14, 15, 53). Low CO₂-induced hyperpolarization would activate voltage-gated K⁺ influx channels (54) and provide the driving force for K⁺ uptake, contributing to stomatal opening (55). Hyperpolarization would also lead to an increase in calcium transient frequency (31, 47). Conversely, high CO₂-induced depolarization via CO₂ activation of anion channels (11, 12) would cause a decrease in calcium transient frequency (31, 47).

This suppression of calcium transients by depolarization likely contributes to the absence of repetitive calcium transients in response to CO₂ changes in the report of Webb *et al.* (24), in which depolarizing (50 mM extracellular KCl) conditions were used, allowing identification of high CO₂ induction of a [Ca²⁺]_{cyt} increase.

The slowing in the [Ca²⁺]_{cyt} pattern upon depolarization would be predicted to occur after an initial high CO₂-induced stomatal closing response, as was found in the present study (Figs. 1A, B, and D; 2E; 3D and E; 4; 5; and 7). Priming of Ca²⁺ sensors by stomatal closing signals, as proposed above, would cause Ca²⁺-induced stomatal closure. The subsequent slowing of the [Ca²⁺]_{cyt} transient pattern in response to elevated CO₂ (Figs. 1A, C, and D; 3A; 4; and 7) could contribute to maintaining long-term “Ca²⁺ programmed” stomatal closure (Fig. 2E) (29, 49, 50). Note that, as highlighted by Figs. 2D and 7, Ca²⁺-independent mechanisms are also likely to be important for CO₂ regulation of stomatal opening and stomatal closing.

**High CO₂ Insensitivity of gca2 Mutant.** *abi1-1* and *abi2-1* have been shown to exhibit a degree of CO₂ insensitivity (20), with the extent of CO₂ sensitivity proposed to depend on plasma membrane polarization (21, 22). However, apart from the conditional *abi1-1* and *abi2-1* mutants, no strong CO₂-insensitive mutant had been described that affects CO₂ regulation of stomatal movements in plants until recently. The gca2 mutant does not exhibit significant changes in the [Ca²⁺]_{cyt} transient rate in response to CO₂ changes and has a strongly attenuated stomatal movement response to elevated CO₂ in leaf epidermis and in intact leaves (Fig. 3).

Another ABA-insensitive mutant *ost1*, which encodes a protein kinase that is activated during early ABA signal transduction, did not affect stomatal CO₂ responses (23), further suggesting that initial ABA and CO₂ signaling networks differ but converge at the level of gca2 and downstream ion channel regulation (11, 12).

*gca2* mutation impairs reactive oxygen species activation of Ca²⁺-permeable channels (IC₅a) in guard cells (56), gca2 also exhibits an aberrant ABA-induced [Ca²⁺]_{cyt} pattern in guard cells (29), which may be due to a hyperpolarized state based on the model derived here. Thus CO₂-insensitive mutants or plants with a weaker CO₂ response (discussed in ref. 12) would be predicted to show no or less robust [CO₂] modulation of the [Ca²⁺]_{cyt} pattern (Fig. 3). Previous findings indicate gca2 regulation of the [Ca²⁺]_{cyt} elevation pattern via Ca²⁺ feedback regulation of IC₅a Ca²⁺ channels in ABA signaling (29, 56), and this may be a point of cross talk with CO₂ signal transduction. The present and previous findings and the Ca²⁺ sensor priming model proposed here suggest that the gca2 mutant may affect mechanisms that function close to the priming of Ca²⁺ sensors during stomatal movements. Isolation of the gca2 mutant gene and further molecular characterization of GCA2 functions may thus shed light into the model proposed here that stomatal movement stimuli prime and deprime Ca²⁺ sensors.

In summary, we have found that repetitive [Ca²⁺]_{cyt} transients are observed even during low CO₂-induced stomatal opening. CO₂ concentration changes modulate the [Ca²⁺]_{cyt} transient pattern in guard cells, and these occur after initial Ca²⁺-independent stomatal movements. Furthermore, experimental repression of CO₂-regulated [Ca²⁺]_{cyt} transients impairs CO₂-induced stomatal opening and closing. The gca2 mutant causes a strong high CO₂ insensitivity. The present findings lead us to propose a new model of cellular calcium signaling specificity in eukaryotes in which priming and depriming of calcium sensors by physiological stimuli may provide a basis for mediating distinct responses to cytosolic calcium elevations in the same cell type.

**Materials and Methods**

**Stomatal Aperture Measurements.** Stomatal movements were analyzed as described in Supporting Text, which is published as supporting information on the PNAS web site. Assays were performed as double-blind experiments, in which both the plant genotype and the CO₂ concentration were unknown or both the ±BAPTA-AM treatment and the [CO₂] were unknown to the experimenter. Time course assays performed in Fig. 2E were single-blind, in which the
±BAPTA-AM treatment was unknown to the experimenter (time course assays presented in Figs. 5 and 7 were not blind). Just prior (~2 min) to stomatal aperture measurements, fluorescent diacetate (dissolved in acetone) was added at 0.0007% (wt/vol) to visualize live cells. Only live stomatal guard cells that were completely surrounded by live pavement cells were chosen for measurements.

**Determination of [CO₂] in Perfused Buffers.** Buffers equilibrated by bubbling with CO₂-free or high CO₂ air were perfused via Teflon tubing into the well used for calcium imaging (see Supporting Text), then collected 3–5 seconds later in an airtight glass syringe (Li-Cor, Lincoln, NE). The CO₂ content of the buffer was calculated by performing calibration measurements and a mass balance as described (57). The [CO₂] content of buffer equilibrated with 740 ppm [CO₂] was estimated by interpolation from 800 and 0 ppm CO₂ concentrations in buffers. M.I. is a Formas postdoctoral fellow. This work was supported by National Science Foundation Grant MCB 0417118, National Institutes of Health Grant R01GM060396, and in part by Department of Energy Grant DE-FG02-03ER15449 (to J.I.S.).

**Note Added in Proof.** In addition to the positive transducers of CO₂ signaling described here, a recent paper reported genetic isolation of a negative regulator of CO₂ signaling encoded by the HT1 protein kinase (59).

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