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 calcium chelator 1,2-bis(2-amino-phenoxy)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.

calcium signaling | cytoplasmic calcium | calcium specificity | stomata | *Arabidopsis thaliana*

Stomatal pores 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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Fig. 1. The rate of cytosolic calcium transient production in guard cells is modulated by the CO₂ concentration. (A) Cytosolic calcium changes in a guard cell preexposed to low CO₂, switched to high CO₂, then returned to low CO₂, as indicated. (B) Stomatal conductance changes in response to similar [CO₂] changes as in A ($n = 4$ leaves $\pm$ SEM). (C) Automated [Ca²⁺]_{cyt} transient detection program analysis of [Ca²⁺]_{cyt} transient rates in guard cells first exposed to low CO₂ buffer, then to high CO₂, and subsequently again to low CO₂ ($n = 41$ cells, values are means $\pm$ SEM). A. *thaliana* plants (Landsberg *erecta* ecotype) were grown in Conviron growth chambers with a 16-h light/8-h dark cycle at 75 μ E m⁻²s⁻¹ and 20°C. (D) Wavelet analysis shows CO₂-induced alteration of cytosolic Ca²⁺ elevation pattern in guard cells. (Upper) Raw data trace; y axis depicts the cameleon ratio as in A. (Lower) Corresponding wavelet transformation of the data trace above shows change in Morlet wavelet parameters at high CO₂. Colors reflect the strength of the frequency component at a given coordinate. (E) Automated [Ca²⁺]_{cyt} transient detection program analysis of [Ca²⁺]_{cyt} transient rates in guard cells recorded in low (115 ppm) CO₂ buffer, ambient (365 ppm) CO₂ buffer ($n = 24$ cells), or high (508 ppm) CO₂ buffer. Where two CO₂ concentrations are listed, the first value represents the [CO₂] measured in the buffer after perfusion, whereas the second value, listed in parentheses, represents the [CO₂] of the air source bubbled into the buffer prior to perfusion (see Materials and Methods).

expressing the GFP-based calcium indicator yellow cameleon 2.1 (40), which allows long-term (>90-min) ratiometric measurements of changes in free [Ca²⁺]_{cyt} in guard cells (41, 42). Leaf epidermes were preexposed to low CO₂ buffer (115 ppm), and guard cells were analyzed in low CO₂ buffer and then exposed to elevated CO₂ buffer (508 ppm). Unexpectedly, more [Ca²⁺]_{cyt} transients were produced in the low CO₂ (stomatal opening) buffer than in the elevated CO₂ (stomatal closing) buffer (Fig. 1A and D and Fig. 4, which is published as supporting information on the PNAS web site). This CO₂-induced shift in the [Ca²⁺]_{cyt} transient rate was observed in the majority of guard cells (36 of 41; 88%). Leaf epidermes were subsequently returned to low CO₂-free buffer (115 ppm) again, and the transient rate increased (Fig. 1A and D). This reversibility in the [Ca²⁺]_{cyt} transient rate was observed in 78% of cells (32 of 41). Thus in guard cells, the rate of [Ca²⁺]_{cyt} transient production decreased after the switch from low CO₂ buffer to high CO₂ and increased after the switch back to low CO₂ buffer (Fig. 1A), showing that the [Ca²⁺]_{cyt} transient rate is modulated by CO₂ concentration changes. Furthermore, repetitive [Ca²⁺]_{cyt} transients occur at low CO₂, a condition that mediates stomatal opening. Gas exchange experiments in which a similar series of [CO₂] changes were imposed showed a reduction in stomatal conductance in response to high CO₂ and a subsequent reopening of stomata upon the return to CO₂-free air, showing robust CO₂ responses in *Arabidopsis* (cv. Landsberg *erecta*) leaves (Fig. 1B).

To pursue standardized analyses of [Ca²⁺]_{cyt} data, software was developed to analyze [Ca²⁺]_{cyt} transient rates. [Ca²⁺]_{cyt} transients were automatically detected based on predefined search parameters. This approach allows an automated consistent basis for [Ca²⁺]_{cyt} transient analysis throughout experiments. The [Ca²⁺]_{cyt} transient rate was calculated by determining the number of [Ca²⁺]_{cyt} transients detected within the time windows analyzed. The average [Ca²⁺]_{cyt} transient rate under low and elevated CO₂

concentrations was determined for all cells and found to be significantly different (Fig. 1C; $P < 0.001$, $n = 41$).

Additional approaches were pursued to further analyze CO₂ modulation of [Ca²⁺]_{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 Ca²⁺ elevation pattern in response to CO₂ concentration changes as shown in Fig. 1D. Ca²⁺ 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 CO₂ indicate there are fewer transients in high CO₂ across all function widths (scales) shown. Thus transitions from low [CO₂] to high [CO₂] cause a significant change in the [Ca²⁺]_{cyt} transient pattern in guard cells (Fig. 1C and D). Amplitudes of Ca²⁺ transients were compared at low CO₂ and high CO₂ by analyzing ratio changes during the initial two CO₂ exposures, before substantial bleaching of the yellow fluorescent protein acceptor occurred (40, 41). Ca²⁺ transient ratios were 0.26 ± 0.02 ratio units at low CO₂ and 0.21 ± 0.02 ratio units at high CO₂ ($P > 0.1$, $n = 11$ guard cells), suggesting no dramatic effects of CO₂ on [Ca²⁺]_{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 CO₂ and 69 ± 4 seconds at high CO₂ ($P = 0.06$, $n = 41$ guard cells). Average transient width measured at the base of transients was 129 ± 6 seconds at low CO₂ and 150 ± 8 seconds at high CO₂ ($P = 0.04$, $n = 41$ guard cells). These analyses suggest that the calcium transients in high CO₂ have a slightly longer duration than those in low CO₂, but this difference was not as dramatic as the difference in [Ca²⁺]_{cyt} transient rates in high and low CO₂ (Fig. 1C).

To further investigate [CO₂] modulation of the [Ca²⁺]_{cyt} transient rate, calcium imaging was also performed on cells exposed to the

opposite series of $[\text{CO}_2]$ regimes. Cells were first exposed to elevated $[\text{CO}_2]$, switched to low CO_2 , and finally switched back to elevated $[\text{CO}_2]$. In these experiments, 69% (18 of 26) showed an increase in the transient rate upon switching from elevated $[\text{CO}_2]$ to low CO_2 , and the majority of those cells (15 of 18, 58% of 26 guard cells) showed a decrease in the transient rate when they were returned to elevated CO_2 . Automated analyses show a statistically significant difference in the $[\text{Ca}^{2+}]_{\text{cyt}}$ transient rates produced in high and low CO_2 using this protocol (high CO_2 , 0.95 ± 0.13 ; low CO_2 , 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 $[\text{Ca}^{2+}]_{\text{cyt}}$ transient rate in response to a physiological stimulus may be more relevant than the absolute observed $[\text{Ca}^{2+}]_{\text{cyt}}$ transient rate during stimulation, which can depend on experimental conditions (45–47). Experiments were also performed by using ambient CO_2 . At ambient CO_2 , spontaneous Ca^{2+} transients were observed, as described (41, 47). Transient rates in ambient CO_2 determined by using automated analysis, including traces without transients, were compared to transient rates in low and high CO_2 , and a significant difference was found in both cases (Fig. 1E, $P < 0.001$ for both ambient vs. low CO_2 and ambient vs. high CO_2).

Analysis of CO_2 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 CO_2 or high CO_2 (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 $[\text{CO}_2]$ with the ambient air should occur (48). The CO_2 content of the buffers bathing the epidermes during calcium imaging was determined (see *Materials and Methods*). $[\text{CO}_2]$ of buffer equilibrated with 0 ppm CO_2 was 115 ± 11 ppm, $[\text{CO}_2]$ of buffer equilibrated with 740 ppm CO_2 was interpolated to 508 ppm, and $[\text{CO}_2]$ of buffer equilibrated with 800 ppm was 535 ± 23 ppm ($\pm \text{SEM}$, $n = 3$).

Stomatal Responses to $[\text{CO}_2]$ Shifts in the Absence of $[\text{Ca}^{2+}]_{\text{cyt}}$ Transients. Interestingly, commencement of significant stomatal aperture responses was measured within 10 min during low to high CO_2 shifts ($P \leq 0.05$; Fig. 1B; Fig. 5, which is published as supporting information on the PNAS web site), whereas calcium transients were produced, on average, once every 10 min in high CO_2 (Fig. 1A, D, and E). Thus it appears that calcium transient pattern changes would occur too slowly to directly function in early guard cell CO_2 responses (see *Discussion*). Therefore, to test whether $[\text{Ca}^{2+}]_{\text{cyt}}$ transients function in CO_2 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-aminophenoxy)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 $[\text{Ca}^{2+}]_{\text{cyt}}$ transients in 12 of 12 cells in 115 ppm CO_2 buffer (Fig. 2A, additional traces in Fig. 6, which is published as supporting information on the PNAS web site). After $[\text{Ca}^{2+}]_{\text{cyt}}$ 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 $[\text{Ca}^{2+}]_{\text{cyt}}$, which contrasts with dramatic reduction in baseline $[\text{Ca}^{2+}]_{\text{cyt}}$ 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 $[\text{Ca}^{2+}]_{\text{cyt}}$ transients in guard cells (Fig. 2A).

We then analyzed whether CO_2 -induced stomatal movements are affected when repetitive cytosolic Ca^{2+} transients are inhibited. We determined that measurement of robust CO_2 -induced stomatal movement responses in leaf epidermes requires intact epidermal

Fig. 2. Loading guard cells with the calcium chelator BAPTA and removing external calcium inhibits both repetitive $[\text{Ca}^{2+}]_{\text{cyt}}$ transients in guard cells and CO_2 regulation of stomatal movements. (A) Repetitive $[\text{Ca}^{2+}]_{\text{cyt}}$ transients recorded in guard cells exposed first to buffer with 50 μM added Ca^{2+} and low CO_2 , then switched to buffer with 25 μM BAPTA-AM without added calcium at ambient CO_2 (striped bar), and finally switched to buffer with 25 μM BAPTA-AM without added calcium at low CO_2 . (B and C) Stomatal apertures after preincubation at ambient CO_2 and incubation in buffers containing 0 ppm CO_2 , ambient ≈ 365 ppm CO_2 , or elevated 800 ppm CO_2 with 50 μM external calcium (B) or no added calcium and 25 μM BAPTA-AM (C) (five experiments, $n = 200$ stomata per condition in B; three experiments, $n = 120$ stomata per condition in C). (D) Stomatal apertures after preincubation at 800 ppm CO_2 followed by incubation in buffers containing 0 or 800 ppm CO_2 with 50 μM external calcium (on the left) or with no added calcium and 25 μM BAPTA-AM (on the right) (eight experiments, $n = 320$ stomata per condition). (E) Time course of stomatal aperture response in intact epidermal layers to a shift from ambient CO_2 (365 ppm) to high CO_2 (800 ppm) with 50 μM external calcium (gray circles) or with no added calcium and BAPTA-AM (black squares) [$n =$ four experiments, >109 total stomata per time point per condition (B–E were blind assays)]. Values in B–E are means $\pm$ SEM.

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 $[\text{CO}_2]$ to low $[\text{CO}_2]$ caused stomatal opening and shifts from ambient $[\text{CO}_2]$ to high $[\text{CO}_2]$ caused stomatal closing (Fig. 2B; $P = 0.01$ for 0 ppm CO_2 vs. ambient CO_2 , $P < 0.001$ for 800 ppm CO_2 vs. ambient CO_2). CO_2 -induced stomatal responses to shifts from ambient $[\text{CO}_2]$ did not occur when cells were treated under conditions in which calcium transients were abolished (Fig. 2C; $P > 0.42$ for 0 ppm CO_2 vs. ambient CO_2 , $P > 0.28$ for 800 ppm CO_2 vs. ambient CO_2).

To increase the dynamic range of the low CO_2 -induced stomatal responses, shifts from 800 to 0 ppm CO_2 were analyzed (Fig. 2D). Interestingly, the low CO_2 -induced stomatal opening response was smaller in samples in which $[\text{Ca}^{2+}]_{\text{cyt}}$ transients were inhibited than in control stomata, suggesting the contribution of both calcium-dependent and -independent pathways for low CO_2 -induced stomatal opening (Fig. 2D). We further tested whether Ca^{2+} transient inhibition would prevent early CO_2 -regulated stomatal movements. Rapid stomatal closure induced by shifting stomata from ambient CO_2 to high CO_2 was still robust in intact epidermal layers treated with BAPTA-AM, but interestingly, stomata subsequently reopened (Fig. 2E and Fig. 7, which is published as supporting information on the PNAS web site). These findings suggest that $[\text{Ca}^{2+}]_{\text{cyt}}$ elevations are not required for early stomatal CO_2 -induced responses but are important for maintaining an adjusted aperture after a $[\text{CO}_2]$ change. Thus, chelation of internal calcium coupled with an absence of added external calcium silenced $[\text{Ca}^{2+}]_{\text{cyt}}$ transients (Fig. 2A), prevented long-term CO_2 -regulated stomatal closing, and reduced low CO_2 -regulated stomatal opening (Fig. 2C and D), suggesting that $[\text{Ca}^{2+}]_{\text{cyt}}$ functions in the opposing CO_2 -regulated guard cell stomatal closing and opening responses. In addition, the data provide evidence that Ca^{2+} -independent mechanisms also function in the CO_2 responses.

Identification and Characterization of a CO_2 -Insensitive Mutant.

Strong CO_2 -insensitive mutants in stomatal movement responses have not been identified until recently (see Introduction). To further analyze the significance of CO_2 -regulated $[\text{Ca}^{2+}]_{\text{cyt}}$ transient rate changes for stomatal movements, we analyzed CO_2 signal transduction in stomatal response mutants. Guard cells from the dominant ABA-insensitive protein phosphatase 2C mutant *abi1-1* showed a reduction in the $[\text{Ca}^{2+}]_{\text{cyt}}$ transient rate upon elevation of CO_2 in 19 of 23 cells and a significantly different average number of transients produced in low and elevated CO_2 , showing a CO_2 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 $[\text{Ca}^{2+}]_{\text{cyt}}$ transient production compared to wild type in ABA signal transduction (29). Interestingly, when guard cells of the *gca2* mutant were exposed to low CO_2 buffer, high CO_2 buffer, and then low CO_2 buffer again, no marked change in the average $[\text{Ca}^{2+}]_{\text{cyt}}$ 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 CO_2 , the $[\text{Ca}^{2+}]_{\text{cyt}}$ 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 CO_2 buffer than in the high CO_2 buffer. Furthermore, the average rates of $[\text{Ca}^{2+}]_{\text{cyt}}$ transients in *gca2* guard cells exposed to low and high CO_2 were similar (Fig. 3A, $n = 29$, $P = 0.88$).

The physiological relevance of the impairment of high CO_2 modulation of the $[\text{Ca}^{2+}]_{\text{cyt}}$ transient rate in *gca2* was investigated by analyzing CO_2 -induced stomatal closure responses in leaf epidermes of the *gca2* mutant and wild-type (*Ler*) controls. In contrast to wild type, significant high CO_2 -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 CO_2 responses in intact leaves of growing plants. A small CO_2 -induced stomatal closure response was resolved in *gca2* in response to $[\text{CO}_2]$ elevation (Fig. 3D). However, CO_2 -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 CO_2 (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} , $\pm \text{SEM}$, $n = 3$, $P = 0.9$). Thus guard cells of the *gca2* mutant did not show statistically significant shifts in the $[\text{Ca}^{2+}]_{\text{cyt}}$ transient rate in response to $[\text{CO}_2]$ shifts (Fig. 3A

Fig. 3. CO_2 regulation of $[\text{Ca}^{2+}]_{\text{cyt}}$ transients in guard cells and CO_2 -induced stomatal closure are impaired in the *gca2* mutant. (A) Automated analysis of $[\text{Ca}^{2+}]_{\text{cyt}}$ transient rates in low CO_2 buffer (white bars) and high CO_2 (black bars) in *gca2* guard cells ($n = 29$), wild-type guard cells ($n = 11$) recorded in parallel, and *abi1-1* guard cells ($n = 23$) exposed to low CO_2 , then high CO_2 , then low CO_2 again. (B) Repetitive $[\text{Ca}^{2+}]_{\text{cyt}}$ transients in a *gca2* guard cell using the protocol described in A. (C) Stomatal apertures of wild-type and *gca2* after 2-h incubations in buffers containing ambient (≈ 365 ppm) CO_2 (white bars) or elevated (800 ppm) CO_2 (black bars; three experiments, $n = 120$ total stomata per condition). (D) Stomatal conductance changes in intact leaves of whole plants from wild-type (open circles) and *gca2* (closed squares) in response to an increase in $[\text{CO}_2]$. Conductance was normalized to the average conductance at the last 365 ppm data point, averages were $0.0975 \pm 0.004 \text{ mol m}^{-2} \text{ s}^{-1}$ in wild-type and $0.126 \pm 0.009 \text{ mol m}^{-2} \text{ s}^{-1}$ in *gca2* ($n = 4$ leaves per genotype). (E) Stomatal conductance changes in intact leaves of whole plants from wild-type (open circles) and *gca2* (closed squares) in response to a decrease in $[\text{CO}_2]$. Conductance was normalized to the average conductance at the last 365 ppm data point, averages were $0.136 \pm 0.02 \text{ mol m}^{-2} \text{ s}^{-1}$ in wild type, and $0.104 \pm 0.02 \text{ mol m}^{-2} \text{ s}^{-1}$ in *gca2* ($n = 3$ leaves per genotype). A and C were blind assays. Values in A, C, D, and E are means $\pm \text{SEM}$.

and B) and showed strongly attenuated stomatal closure in response to $[\text{CO}_2]$ elevation in leaf epidermes and in intact leaves of plants (Fig. 3C and D). These data show that *gca2* is a strong high CO_2 -insensitive mutant.

Discussion

Understanding the molecular mechanisms by which CO_2 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 $[\text{CO}_2]$ (10) and the continuing atmospheric CO_2 elevation on stomatal movements (3–5, 7, 8, 18). Our findings build upon an earlier study showing that $[\text{Ca}^{2+}]_{\text{cyt}}$ elevations function in CO_2 -induced stomatal movements (24) and in addition unexpectedly demonstrate the presence of repetitive $[\text{Ca}^{2+}]_{\text{cyt}}$ transients at low CO_2 , which opens stomatal pores (Figs. 1A and D, 2A, and 3B). Furthermore, guard cell $[\text{Ca}^{2+}]_{\text{cyt}}$ 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 2*A*). 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, 2*A*, and 3*B*) 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 dampening 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. 2*E* 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*; 2*E*; 3*D*; 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. 1 *A, B*, and *D*; 2*E*; 3 *D* 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. 1 *A, C*, and *D*; 3*A*; and 4) could contribute to maintaining long-term “Ca²⁺ programmed” stomatal closure (Fig. 2*E*) (29, 49, 50). Note that, as highlighted by Figs. 2*D* and *E* 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 epidermes 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 (I_{Ca}) 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 I_{Ca} 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 presented in Fig. 2*E* were single-blind, in which the

$\pm$ 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, fluorescein 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 $[CO_2]$ in Perfused Buffers. Buffers equilibrated by bubbling with CO_2 -free or high CO_2 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, to estimate the final CO_2 concentration to which stomata were exposed (48, 57). CO_2 -free air (6–9 ml) was introduced into the syringe, and the $[CO_2]$ in the buffer and air was equilibrated by shaking for 5 min. The $[CO_2]$ of the equilibrated air was then measured by using a Li-6400 gas exchange analyzer (Li-Cor, Lincoln, NE). The CO_2 content of the buffer was calculated by performing calibration measurements and a mass balance as described (57). The $[CO_2]$ content of buffer equilibrated with 740 ppm $[CO_2]$ was estimated by interpolation from 800 and 0 ppm CO_2 measurements.

Plant Growth and Calcium Imaging. *Arabidopsis* plants (*Landsberg erecta*) were grown and Ca^{2+} imaging performed by using standard methods (40, 41) as described in *Supporting Text* (see Fig. 9, which is published as supporting information on the PNAS web site).

Automated $[Ca^{2+}]$ Transient Analysis. $[Ca^{2+}]_{\text{cyt}}$ transients were automatically detected by using a modified version of a MATLAB

(MathWorks, Natick, MA) program (58). The first derivative of 535/480-nm cameleon ratio data were convolved with a Haar function with a width of 186 seconds. The transformed data traces produced from the convolution were then analyzed for areas with high correlation; i.e., areas that matched the shape of the function. A detection threshold was set for data traces, thus times at which the transformed data exceeded the detection threshold were counted as $[Ca^{2+}]_{\text{cyt}}$ transients. A reset threshold of -0.1 was also used for all data traces, requiring that the transients returned to low values before another transient could be counted; this prevented high-frequency noise near the detection threshold from being registered as $[Ca^{2+}]_{\text{cyt}}$ transients. Programs are available upon request. For wavelet analysis, raw data traces were transformed with the Morlet wavelet by using the MATLAB WAVELET TOOLBOX (44). A larger-scale value in wavelet analyses corresponds to both wider spikes and longer periodicities. The maximum scale illustrated (Fig. 1D) corresponds to a waveform spanning ≈ 10 min.

Note Added in Proof. In addition to the positive transducers of CO_2 signaling described here, a recent paper reported genetic isolation of a negative regulator of CO_2 signaling encoded by the HT1 protein kinase (59).

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