Extracellular Calmodulin-Induced Stomatal Closure Is Mediated by Heterotrimeric G Protein and H_2O_2[1]

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Extracellular calmodulin (ExtCaM) exerts multiple functions in animals and plants, but the mode of ExtCaM action is not well understood. In this paper, we provide evidence that ExtCaM stimulates a cascade of intracellular signaling events to regulate stomatal movement. Analysis of the changes of cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) and H_2O_2 in *Vicia faba* guard cells combined with epidermal strip bioassay suggests that ExtCaM induces an increase in both H_2O_2 levels and $[Ca^{2+}]_{cyt}$, leading to a reduction in stomatal aperture. Pharmacological studies implicate heterotrimeric G protein in transmitting the ExtCaM signal, acting upstream of $[Ca^{2+}]_{cyt}$ elevation, and generating H_2O_2 in guard cell responses. To further test the role of heterotrimeric G protein in ExtCaM signaling in stomatal closure, we checked guard cell responses in the Arabidopsis (*Arabidopsis thaliana*) Gα-subunit-null *gpa1* mutants and cGα overexpression lines. We found that *gpa1* mutants were insensitive to ExtCaM stimulation of stomatal closure, whereas cGα overexpression enhanced the guard cell response to ExtCaM. Furthermore, *gpa1* mutants are impaired in ExtCaM induction of H_2O_2 generation in guard cells. Taken together, our results strongly suggest that ExtCaM activates an intracellular signaling pathway involving activation of a heterotrimeric G protein, H_2O_2 generation, and changes in $[Ca^{2+}]_{cyt}$ in the regulation of stomatal movements.

Changes in cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) have been observed during the signal transduction in response to abiotic and biotic stresses (Sanders et al., 2002). In guard cells, $[Ca^{2+}]_{cyt}$ elevations have been shown to be early events in the signaling cascade that results in abscisic acid (ABA)-induced stomatal closure in a number of plant species (McAinsh et al., 1995; Grabov and Blatt, 1998). Accumulating evidence indicates that many stimuli enhance $[Ca^{2+}]_{cyt}$ increase in guard cells (Rudd and Franklin-Tong, 2001); however, the upstream components of calcium signaling are not well understood.

Heterotrimeric G proteins composed of α-, β-, and γ-subunits are a key intracellular signaling molecule in eukaryotic cells. The activation by G-protein-coupled receptor results in conformation change of the Gα protein due to GTP binding and the separation of Gα from the G$\beta\gamma$ dimer. GTP hydrolysis by GTPase activity of Gα results in the reassociation of Gα with G$\beta\gamma$ (Jones and Assmann, 2004). In plants, G protein has been found to be involved in ion-channel regulation (Aharon et al., 1998; Wang et al., 2001), control of seed germination (Ullah et al., 2002), pollen tube elongation (Ma et al., 1999), and responses to ABA (Wang et al., 2001). Genome sequencing revealed the existence of only one prototypical Gα (*GPA1*) in Arabidopsis (*Arabidopsis thaliana*; Ma et al., 1990). It was reported that Gα-subunit-null mutants, *gpa1-1* and *gpa1-2*, were insensitive to ABA inhibition of whole-cell inward K^+ currents and pH-independent ABA-activation of anion channels (Wang et al., 2001), suggesting Gα is a key component in ABA signaling. It is unknown whether Gα-subunit participates in calcium signaling in ABA regulation of guard cell responses.

Recently, reactive oxygen species (ROS) has been shown to be an important second messenger in signaling to developmental processes, such as polar growth of Fucus rhizoid cells (Coelho et al., 2002) and cell elongation in root growth (Demidchik et al., 2003), responses to environmental stresses (Baxter-Burrell et al., 2002), and guard cell movement (Pei et al., 2000). Evidence indicates that homeostasis of ROS depends on the activity of several enzymes involved in ROS generation as well as the activity of ROS scavenging enzymes (for review, see Mittler, 2002). Recently, guard cell-specific NADPH oxidases AtrbohD (Arabidopsis respiratory burst oxidase homologs D) and AtrbohF have been identified, and the double mutants of *atrbohD/F* are impaired in ABA-induced ROS generation, $[Ca^{2+}]_{cyt}$ increases, and stomatal closing (Kwak et al., 2003), suggesting that AtrbohD and AtrbohF NADPH oxidases and ROS play an important

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role in ABA signal transduction in guard cells. The evidence that Ca^{2+}-sensing receptor (CAS) in Arabidopsis plasma membrane, which mediates extracellular Ca^{2+} induced cytosolic Ca^{2+} increase in guard cells (Han et al., 2003) suggests that CAS may regulate $[Ca^{2+}]_{cyt}$ status through functioning together with the regulation of Ca^{2+} influx and release from intracellular Ca^{2+} stores, Ca^{2+}-ATPase, and Ca^{2+}/H^+ antiporter. However, it is unclear whether ROS also acts as a mediator in extracellular Ca^{2+} receptor mediated stomatal movement.

Calmodulin (CaM), a ubiquitous and abundant intracellular Ca^{2+} receptor, exists in all eukaryotic cells (for review, see Vetter and Leclerc, 2003). Recently, it has been found that CaM exists extracellularly to exert many functions in both animals and plants. In animals, extracellular CaM (ExtCaM) is present in body fluids, saliva, urine, and milk (Houston et al., 1997), stimulating the proliferation of cultured hepatocytes, melanoma cells, and fibroblasts (Goberdhan et al., 1993). In addition, ExtCaM inhibits tumor necrosis factor-α release and augments neutrophil elastase release, preventing further cytotoxicity (Houston et al., 1997). In plants, extracellular peptides, such as systemin, CLAVATA3, and ENOD40, may act as intercellular signals, regulating some important processes, e.g. wound defense reaction, maintenance of shoot apical meristem, and nodule formation (Pearce et al., 1993; Yang et al., 1993; van de Sande et al., 1996; Trotochaud et al., 2000). ExtCaM has been found in many plant species. For example, ExtCaM was detected in the medium of suspension-cultured cells from *Angelica dahurica*, carrot, and tobacco (Sun et al., 1994, 1995). The existence of ExtCaM in plants suggests that it may have important functions. Indeed, ExtCaM stimulates proliferation of suspension-cultured cells of *A. dahurica*, *Fenistum typhoides*, and *Sataria italica* by enhancing cell wall regeneration and protoplast division (Sun et al., 1994) and accelerates pollen germination and tube growth (Ma and Sun, 1997; Ma et al., 1999; Shang et al., 2001). Pharmacological studies have implicated several signaling molecules, including heterotrimeric G protein (Ma et al., 1999), phosphoinositide, and cytosolic Ca^{2+} (Shang et al., 2001) in the signal transduction pathway of ExtCaM-enhanced pollen germination.

Stomatal movements regulate the loss of water to the atmosphere and the entry of CO_2 into the plants for photosynthetic carbon fixation. Many factors, such as ABA, CO_2, light/darkness, and temperature, are known to modulate stomatal movements (Schroeder et al., 2001). The involvement of intracellular CaM in stomatal movements has also been studied (Cottele et al., 1996). However, the effects of ExtCaM on stomatal movements are not well addressed. In our previous report, we demonstrated that ExtCaM existed in the walls of guard cells and that its exogenous application promoted stomatal closure (Chen et al., 2003). In this report, we investigate the intracellular signaling mechanism by which ExtCaM mediates stomatal movement using combined pharmacological, physiological, and genetic approaches. We have provided convincing evidence that ExtCaM triggers a cascade of intercellular signaling events involving heterotrimeric G protein, H_2O_2, and Ca^{2+}in the regulation of stomatal closure.

RESULTS AND DISCUSSION

ExtCaM Induces $[Ca^{2+}]_{cyt}$ Increase in Guard Cells

Since $[Ca^{2+}]_{cyt}$ levels and oscillation have been shown to be a key mediator of guard cell movement (Allen et al., 1999, 2001), we were interested in whether ExtCaM has an effect on the dynamic changes of $[Ca^{2+}]_{cyt}$ in guard cells. *Vicia faba* guard cells have been a favorite model for the study of guard cell movement (Assmann, 1993). In this study, we applied 10^{-8} M CaM to induce stomata closure (Chen et al., 2003) and used confocal laser scanning microscopy (CLSM) to visualize the fluorescence of fluo-3, which was loaded into guard cells. Among 27 *V. faba* guard cells, 63% of the cells showed the typical $[Ca^{2+}]_{cyt}$ changes responsive to ExtCaM (Fig. 1A). ExtCaM-induced dramatic elevation in $[Ca^{2+}]_{cyt}$ was found after 280 s incubation of CaM (Fig. 1D), while the control treatment (10^{-8} M bovine serum albumin) did not cause obvious fluorescent changes in guard cells (data not shown).

Multiple factors such as ABA, CO_2, light/darkness, and temperature regulate stomatal movements and cause guard cell $[Ca^{2+}]_{cyt}$ changes; for example, ABA induces increase in $[Ca^{2+}]_{cyt}$ and subsequently stomatal closure (Grabov and Blatt, 1998; Hamilton et al., 2000). A plasma membrane-localized extracellular CAS has been shown to regulate guard cell $[Ca^{2+}]_{cyt}$ (Han et al., 2003). Our results demonstrate that an apoplast-localized protein, ExtCaM, can regulate $[Ca^{2+}]_{cyt}$ elevation in guard cells. This finding is quite exciting because it supports the hypothesis that guard cells may sense extracellular Ca^{2+} and regulate intracellular Ca^{2+} levels via Ca^{2+}-CaM complex. A very important and interesting future question is whether Ca^{2+}-CaM interacts with or acts independent of CAS to regulate $[Ca^{2+}]_{cyt}$ during guard cell movement. Furthermore, considering that the ExtCaM induction of $[Ca^{2+}]_{cyt}$ elevation is similar to that of ABA, it might be possible that a new regulatory factor naturally existing in guard cell walls regulates stomatal movements together with ABA. These findings not only extend the functions of ExtCaM, but also provide clues to understanding the regulatory mechanisms for stomatal movements.

Heterotrimeric G Protein Might Be Involved in ExtCaM Promotion of Stomatal Closure

Having observed ExtCaM induction of $[Ca^{2+}]_{cyt}$ elevation and stomatal closure, we next sought to investigate whether other important signaling components might transduce this signal input. Heterotrimeric

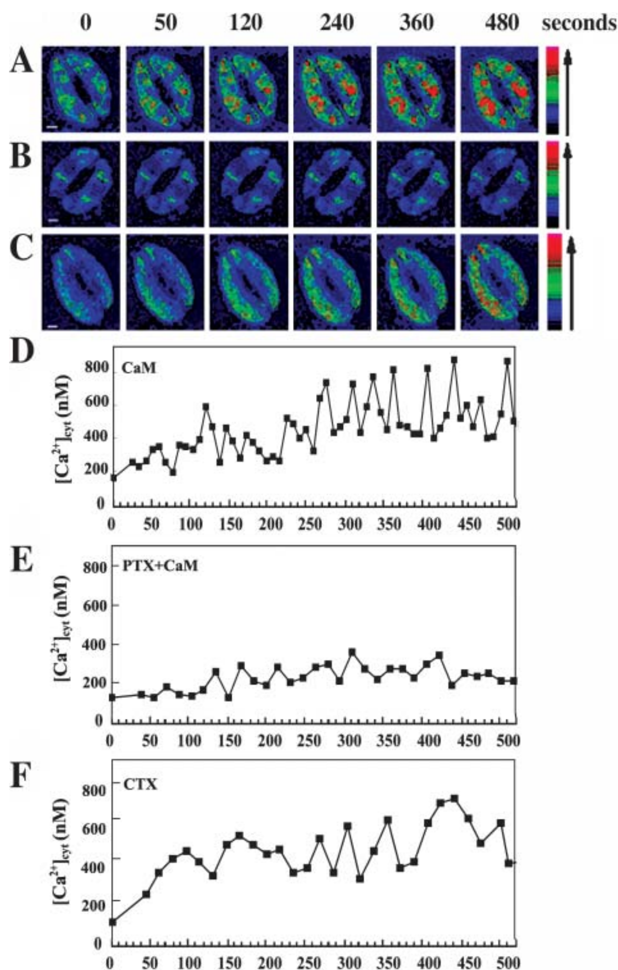

Figure 1. Application of ExtCaM causes the changes of $[Ca^{2+}]_{cyt}$ through the function of G protein in *V. faba* guard cells. A to C, Fluorescent changes in guard cells preloaded with 10 μM fluo-3 AM and (D–F) quantitative curve of $[Ca^{2+}]_{cyt}$ responsive to the induction of 10^{-8} M CaM (A and D), pretreatment of 400 ng/mL PTX plus CaM (B and E), and 400 ng/mL CTX (C and F), respectively. $[Ca^{2+}]_{cyt}$ was quantified based the relative fluorescent intensity referred to the standard serial calcium concentrations as described in "Material and Methods." All the start times in the following figures of this paper reorganize the time point of chemicals/protein treatment as zero time. Bar = 10 μm.

G proteins have been shown to regulate $[Ca^{2+}]_{cyt}$ by modulating Ca^{2+} channels in the plasma membrane of animal cells. It was reported that heterotrimeric G protein mediated ExtCaM stimulation of pollen germination (Ma et al., 1999). In guard cells, G protein activators such as cholera toxin (CTX; van Corven et al., 1993) and GTPγS inhibited the influx of K^+, and the effect of GTPγS is prevented by buffering cytosolic Ca^{2+} to a low level, suggesting that activated G proteins may inhibit inward K^+ channels via elevation of $[Ca^{2+}]_{cyt}$ (Fairley-Grenot and Assmann, 1991; Wu and Assmann, 1994). Using genetic approaches, Gα has been shown to mediate ABA signaling in regulating inward K^+ channels and slow anion channels and stomatal movements in Arabidopsis (Wang et al., 2001; Coursol et al., 2003). Thus, we speculated that heterotrimeric G proteins may also mediate ExtCaM signaling in guard cells. In this study, we used pertussis toxin (PTX), an inhibitor of heterotrimeric G protein α-subunit (Kuryshev et al., 1993), and CTX, an activator of heterotrimeric G protein α-subunit (van Corven et al., 1993) to assess whether heterotrimeric G proteins act as a mediator in ExtCaM promotion of $[Ca^{2+}]_{cyt}$ elevation. As shown in Figure 2, CaM promotion of *V. faba* stomatal closure was greatly impaired when leaf epidermal strips were pretreated with PTX. In parallel with this effect, when *V. faba* guard cells were pretreated with PTX, 21 guard cells (n = 30) failed to trigger increase of $[Ca^{2+}]_{cyt}$ responsive to ExtCaM (Fig. 1, B and E). Meanwhile, CTX, an activator of heterotrimeric G protein, induced both stomatal closure (Fig. 2) and $[Ca^{2+}]_{cyt}$ increase in 16 of 22 guard cells during 480-s CTX treatment (Fig. 1, C and F). The effect of CTX on stomatal apertures and elevation in $[Ca^{2+}]_{cyt}$ resembled that of ExtCaM, further supporting the hypothesis that ExtCaM acts through $[Ca^{2+}]_{cyt}$ to regulate stomatal movement. Taken together, these results indicate that heterotrimeric G protein may mediate ExtCaM induction of *V. faba* stomatal closure by tuning $[Ca^{2+}]_{cyt}$ in guard cells.

To confirm these pharmacological data, we further used Arabidopsis mutants *gpa1-1* and *gpa1-2* harboring the recessive T-DNA knockout alleles of *AtGPA1*, the only one prototypical Gα gene in Arabidopsis genome (Ullah et al., 2001), and Arabidopsis transgenic lines overexpressing cGα (*AtGPA1* with a point mutation of Glu-222 to Leu, which locks Gα in the active state once activated; Okamoto et al., 2001), and wild-type Arabidopsis ecotype Wassilewskija (Ws). In

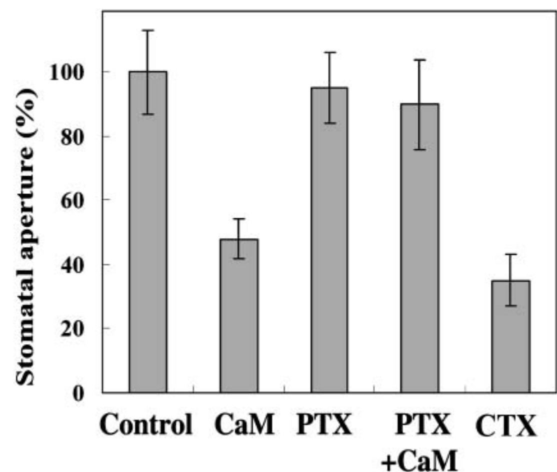

Figure 2. Effect of PTX or CTX on *V. faba* stomatal closure. Control, Open stomata were kept in MES buffer but minus CaM for 2 h, then the stomatal aperture was treated as 100%; CaM, open stomata were treated with 10^{-8} M CaM solution for 2 h; PTX, open stomata were treated with 400 ng/mL PTX solution for 2 h; PTX + CaM, open stomata were pretreated with 400 ng/mL PTX for 30 min, washed with MES buffer, then kept in CaM solution for 2 h; CTX, open stomata were treated with 400 ng/mL CTX for 2 h.

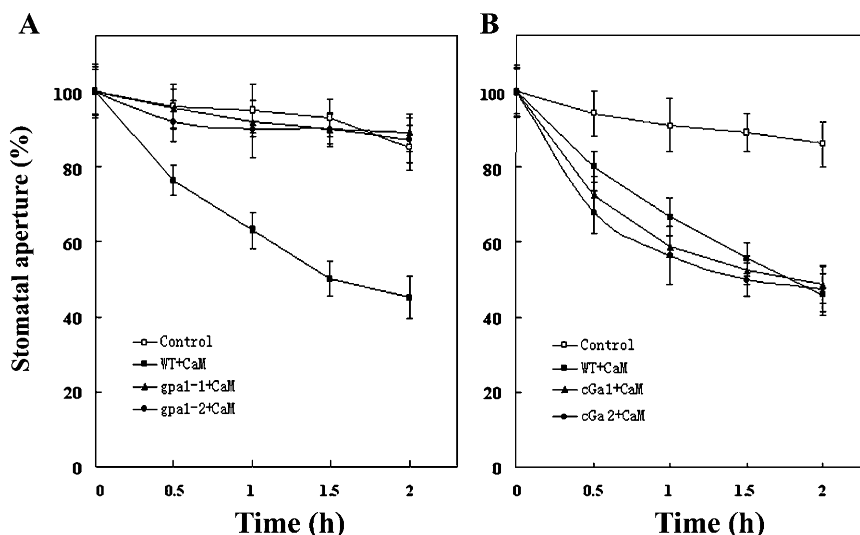

Figure 3. Application of ExtCaM causes stomatal closure through the function of G protein in Arabidopsis guard cells. A, Mutants *gpa1-1 and gpa1-2* impaired stomatal closure induced by 10^{-8} M CaM, but not in wild type. Control, Epidermal strips of Ws plants were kept in MES buffer; WT (wild type) + CaM, epidermal strips of Ws plants were treated with 10^{-8} M CaM solution; *gpa1-1* + CaM, epidermal strips of *gpa1-1* plants were treated with CaM solution; *gpa1-2* + CaM, epidermal strips of *gpa1-2* plants were kept in 10^{-8} M CaM solution. B, cGα constitutively overexpressing heterotrimeric G protein α-subunit *AtGPA1* stimulated stomatal closure induced by 10^{-8} M CaM. Control, Epidermal strips of Ws plants were kept in MES buffer; WT + CaM, epidermal strips of Ws plants were treated with 10^{-8} M CaM solution; cGα1 + CaM, epidermal strips of cGα1 plants were treated with 10^{-8} M CaM solution; cGα2 + CaM, epidermal strips of cGα2 plants were kept in 10^{-8} M CaM solution. Each assay was repeated three times. The data were presented as mean ± SE (n = 150).

Arabidopsis wild-type leaf epidermal strips, ExtCaM induced stomatal closure as in *V. faba* (Fig. 3A). ExtCaM induction of stomatal closure was completely impaired in the mutants of *gpa1-1* and *gpa1-2* (Fig. 3A), as the mutant stomata in the presence of ExtCaM behaved exactly like wild-type control in the absence of ExtCaM. In contrast, cGα overexpressing lines showed faster stomatal closure induced by ExtCaM than they did in the wild type, although the final stomatal aperture of the cGα lines was not significantly different from that in the wild type (Fig. 3B). In the meantime, we also checked the effects of PTX and CTX on guard cell responses in *gpa1* mutants. Consistent with the above results, our data showed that *gpa1* mutants were insensitive to these drugs (data not shown). Therefore our results provide the genetic evidence that Gα is involved in the regulation of ExtCaM action in animals and plants. Together with the pharmacological experiment described above, these results indicate that heterotrimeric G protein acts as a positive regulator of guard cell responses to ExtCaM.

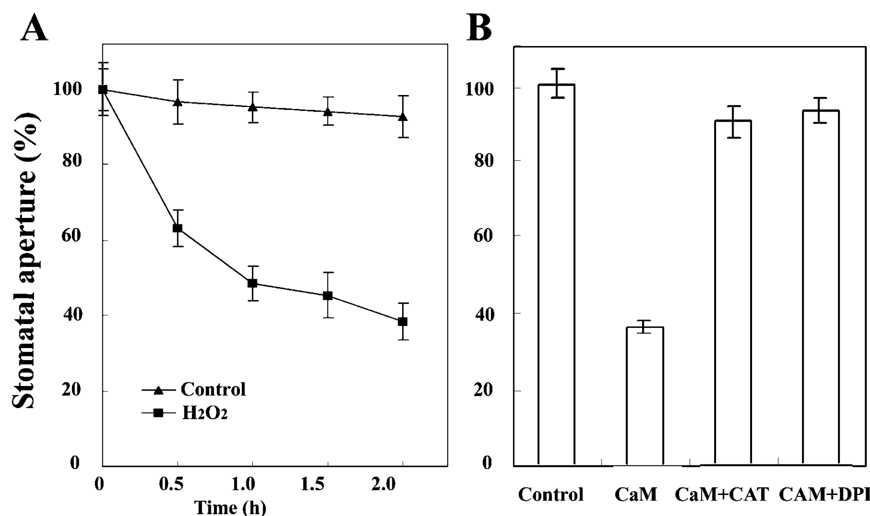

Figure 4. H_2O_2 mediates ExtCaM-induced *V. faba* stomatal closure. A, Effect of 5×10^{-5} M H_2O_2 in MES buffer on stomatal closure within 2 h. Control indicates no addition of H_2O_2 except MES buffer. B, Effects of DPI or CAT on stomatal closure-induced by CaM for 2 h. Control, Open stomata were kept in MES buffer under light for 2 h, then the stomatal aperture was treated as 100%; CaM, open stomata were treated with 10^{-8} M CaM solution; CaM + CAT, open stomata were treated with 100 units/mL CAT plus 10^{-8} M CaM; CaM + DPI, open stomata were kept in 10 μM DPI plus 10^{-8} M CaM. Each assay was repeated three times. The data were presented as mean ± SE (n = 150).

Based on the above results we propose that ExtCaM, perhaps acting as extracellular Ca^{2+} sensor and activating the receptor of CaM, activates G protein α-subunit, leading to stomatal closure. ExtCaM activation of heterotrimeric G proteins seems to be a common mechanism for the action of ExtCaM, as a similar mechanism was reported for ExtCaM promotion of pollen tube elongation (Ma et al., 1999). In *gpa1-1* and *gpa1-2* mutants, because of the T-DNA insertion in the predicted seventh intron (*gpa 1-1*) and in the eighth exon (*gpa1-2*), four of its five polypeptide loops required for GTP binding, GTPase, and the effector loop have been eliminated (Ullah et al., 2001). The transduction pathway of ExtCaM to stomatal closure has been interrupted in G protein, as the result, stomata failed to close in response to ExtCaM. Thus, Gα is required for ExtCaM-mediated stomatal closure. In *AtGPA1* cGα overexpression lines, ExtCaM induction of stomatal closure was accelerated but not constitutive, suggesting that Gα activation is not sufficient for ExtCaM induction of stomatal closure. An interesting question to be addressed in the future is whether there is a functional link between ExtCaM, G protein, and ABA, which is also known to regulate G protein in the regulation of stomatal movement (Wang et al., 2001).

Involvement of H_2O_2 in ExtCaM-Induced Stomatal Closure

It has been reported that ROS is a key regulator of stomatal movements (Purohit et al., 1994). For instance, H_2O_2 caused an increase in guard cell $[Ca^{2+}]_{cyt}$, which was abolished in the presence of EGTA (McAinsh et al., 1996). H_2O_2 has been shown to be a signal molecule in ABA induction of stomatal closure. In this process, ABA induces H_2O_2 production in guard cells by activating NADPH oxidases, and then H_2O_2 causes a $[Ca^{2+}]_{cyt}$ increase by activating Ca^{2+} channels in the plasma membrane (Pei et al., 2000; Murata et al., 2001; Kwak et al., 2003). In this study, we observed that H_2O_2 promoted stomatal closure in *V. faba* (Fig. 4A), which is consistent with the previous reports in Arabidopsis (Pei et al., 2000). To investigate whether H_2O_2 is involved in ExtCaM-induced stomatal closure, *V. faba* epidermal strips were incubated in ExtCaM solution containing diphenylene iodonium (DPI) or catalase (CAT), which was either an inhibitor of NADPH oxidases, the key enzyme in the production of H_2O_2, or the scavenger of H_2O_2 (Zhang et al., 2001; Qin et al., 2004). Under these conditions, both DPI and CAT abolished the stimulation of stomatal closure by ExtCaM (Fig. 4B), suggesting that stomatal closure induced by ExtCaM requires the production of H_2O_2, and NADPH oxidases might be involved in the generation of H_2O_2.

It has been previously evidenced that H_2DCF-DA-based assays are suitable for measurement of H_2O_2 production in guard cells (Zhang et al., 2001). Using this method we showed that ExtCaM-induced H_2O_2 production in *V. faba* guard cells. Among the 25 guard cells, 64% of the cells showed the typical H_2O_2 response curve to 10^{-8} M CaM and CTX but not 10^{-8} M bovine serum albumin (data not shown). The generation rate of H_2O_2 was rapid during the first 5 min of ExtCaM treatment (Fig. 5, A and C), further suggesting that H_2O_2 might be a signal molecule involved in the signal transduction pathway of ExtCaM-induced stomatal closure.

Given that CaM-induced H_2O_2 production might be a crucial element in the signal transduction pathway of ExtCaM in guard cells, we next assessed H_2O_2-induced changes in $[Ca^{2+}]_{cyt}$ in *V. faba* guard cells. Our results showed that 65% guard cells (n = 26) had dramatic elevations of $[Ca^{2+}]_{cyt}$ triggered by 50 μM H_2O_2 during a 500-s treatment of H_2O_2 (Fig. 5, B and D).

Heterotrimeric G Protein Mediates ExtCaM-Induced H_2O_2 Increase in Guard Cells

To investigate the relationship among heterotrimeric G protein, H_2O_2, and Ca^{2+}, we performed the follow-

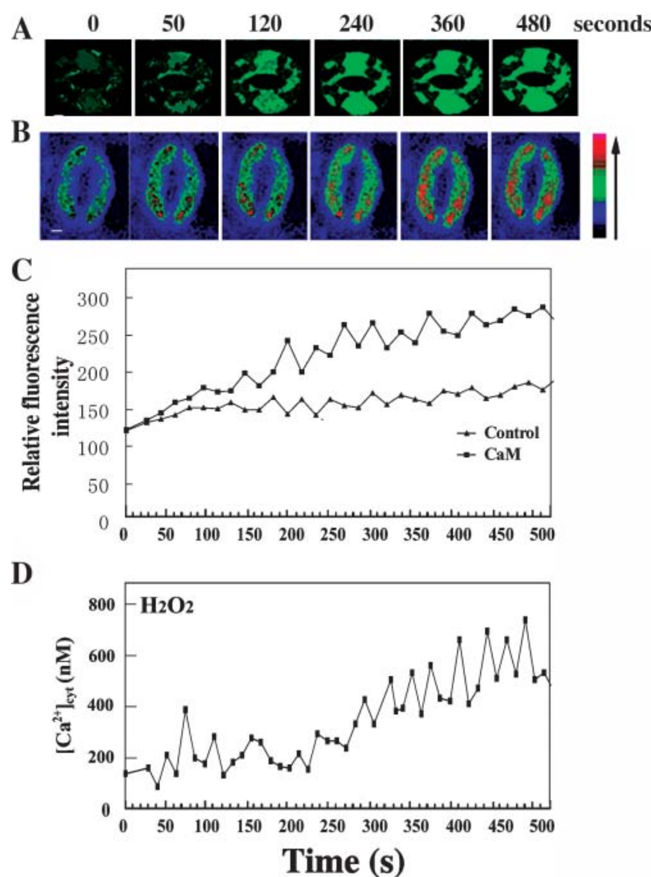

Figure 5. ExtCaM promotes production of H_2O_2 that further induces changes of $[Ca^{2+}]_{cyt}$ in *V. faba* guard cells. A, Fluorescence and (C) quantitative curve of H_2O_2 changes reflected with 50 μM H_2DCF-DA after addition of 10^{-8} M CaM. B, Fluorescence and (D) quantitative curve of $[Ca^{2+}]_{cyt}$ in *V. faba* guard cells preloaded with 10 μM fluo-3 AM responsive to the induction of 5×10^{-5} M H_2O_2. Control indicates no chemical addition in the buffer solution. Bar = 10 μm.

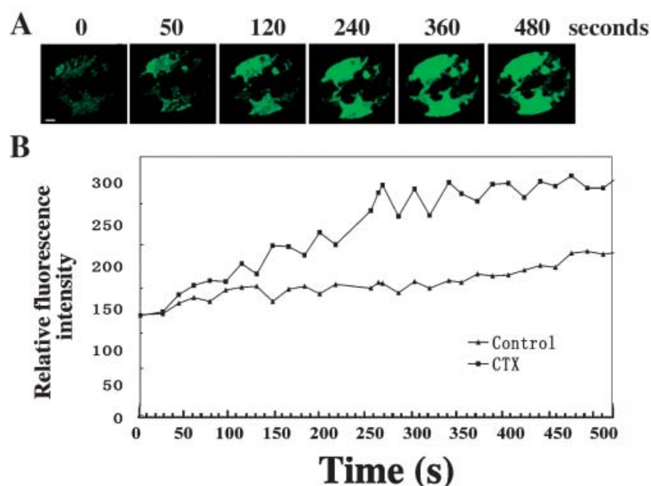

Figure 6. Heterotrimeric G protein activator CTX enhanced H_2O_2 production in *V. faba* guard cells. A, Fluorescent changes and (B) quantitative curve of H_2O_2 reflected with 50 μM H_2DCF-DA after addition of 400 ng/mL CTX. Control indicates no chemical addition in the buffer solution. Bar = 10 μm.

ing experiments. First we tested this pathway in *V. faba* epidermal strips. As shown in Figure 6, A and B, 17 guard cells (n = 25) displayed an increase of H_2O_2 production induced by CTX. Similarly, CTX induction of stomatal closure was also blocked by either DPI or CAT (Fig. 7A), suggesting that heterotrimeric G protein may act upstream of H_2O_2 production of the regulation of stomatal closure. Once extracellular Ca^{2+} was chelated by EGTA, CTX failed to induce H_2O_2 increase in guard cells (data not shown), suggesting a likely requirement for Ca^{2+} in the H_2O_2 production induced by G protein.

To confirm the above results, we next used two Gα-subunit-null lines, *gpa1-1* and *gpa1-2*, to assess the role of G protein in the regulation of H_2O_2 levels in response to ExtCaM. Under the same conditions, *gpa1-1* and *gpa1-2* showed lower levels of H_2O_2 than wild-type plants did. Furthermore, *gpa1-1* and *gpa1-2* showed almost no increase in H_2O_2 levels when treated with CaM, whereas the wild type had significantly increased in fluorescent intensity within 5 min in the presence of 10^{-8} CaM (Fig. 7, B and C), indicating that G protein is required for the generation of H_2O_2.

CONCLUSION

In this study, we have provided strong evidence that ExtCaM stimulates stomatal closure through the activation of heterotrimeric G protein and subsequent promotion of H_2O_2 production and $[Ca^{2+}]_{cyt}$ elevation. Both genetic and pharmacological studies consistently support the hypothesis that ExtCaM mediating G protein activates the production of H_2O_2. Pharmacological data also support G protein regulation of ExtCaM-dependent $[Ca^{2+}]_{cyt}$ elevation; this remains to be confirmed by genetic studies. In ABA promotion of stomatal closure, it has been shown that ABA activates the production of H_2O_2, which in turn activates plasma membrane-localized calcium channels, leading to $[Ca^{2+}]_{cyt}$ elevation (Pei et al., 2000). Given that ExtCaM and G protein activate both H_2O_2 production and $[Ca^{2+}]_{cyt}$ elevation, it is tempting to propose that in ExtCaM mediated stomatal closure,

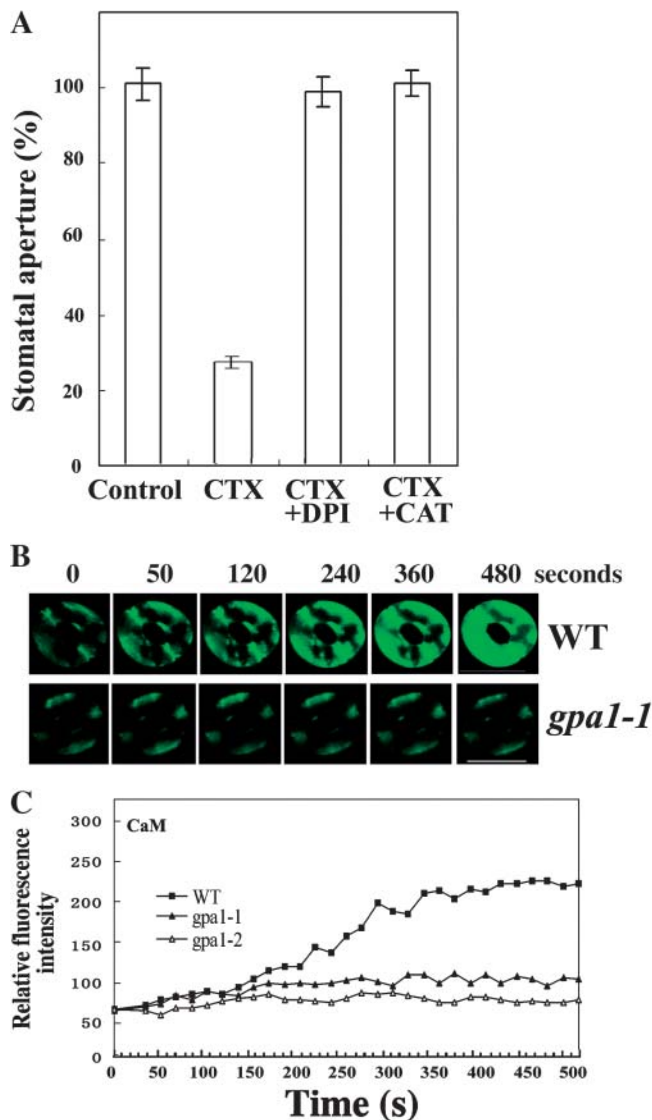

Figure 7. Analysis of functional relationship among heterotrimeric G protein, H_2O_2, and ExtCaM in guard cells. A, Effects of activator of G protein (400 ng/mL CTX) and inhibitor of NADPH oxidase (10 μM DPI) or scavenger of H_2O_2 (100 units/mL CAT) on *V. faba* stomatal closure. Control, Open stomata were kept in MES buffer for 2 h under light, then the stomatal aperture was treated as 100%; CTX, open stomata were treated with 10^{-8} M CTX solution; CTX + DPI, open stomata were treated with 400 ng/mL CTX plus 10 μM DPI; CTX + CAT, open stomata were pretreated with 400 ng/mL CTX plus 100 units/mL CAT. Each assay was repeated three times. The data were presented as mean ± SE (n = 150). B, Fluorescent changes and (C) quantitative curve of H_2O_2 reflected with 50 μM H_2DCF-DA after addition CaM in WT and *gap1*. Bar = 40 μm.

H_2O_2 too acts as a second messenger to activate plasma membrane-localized calcium channels and $[Ca^{2+}]_{cyt}$ elevation. However, it is also possible that Ca^{2+} might also regulate H_2O_2 production. It has been reported that there are Ca^{2+} binding sites in NADPH oxidases and that this enzyme may be regulated by heterotrimeric G protein (Keller et al., 1998). The induction of ROS generated by oligogalacturonic acid involves a series of processes including receptor binding (Horn et al., 1989), activation of a heterotrimeric G protein (Legendre et al., 1992), influx of Ca^{2+} (Chandra et al., 1997), stimulation of phospholipase C (Legendre et al., 1993), and induction of a number of kinases (Chandra and Low, 1995). Nonetheless, future studies should be directed at understanding the functional relationship among G protein, H_2O_2, and calcium in ExtCaM-mediated stomatal movement.

MATERIALS AND METHODS

Plant Materials

Vicia faba plants were grown in potting mix in a growth chamber under a 12-h-light and 12-h-dark cycle, with a photon flux density of 0.30 mmol m^{-2} s^{-1}, and day/night temperature cycle of 25°C ± 2°C and 20°C ± 2°C, respectively. Lower epidermis of fully expanded leaves from 3- to 4-week-old *V. faba* seedlings was used for bioassay and the measurements of cytosolic calcium and ROS. Arabidopsis (*Arabidopsis thaliana*) plants of cGα overexpressing constitutively active form mutant of heterotrimeric G protein α-subunit *AtGPA1*, which were obtained from Dr. L.G. Ma (Yale University), were grown in the presence of 70 nM dexamethasone (DEX; Sigma, St. Louis) according to the methods described by Okamoto et al. (2001). T-DNA insertion mutants *gpa1-1* and *gpa1-2* (from Nottingham Arabidopsis Stock Center) that lack function of G-protein α-subunit (Ullah et al., 2001), and wild-type Ws were cultured as described by Wang et al. (2001). Fully expanded leaves of 4- to 6-week-old Arabidopsis plants were used for epidermal strip bioassay and ROS measurement.

Ca^{2+}-Sensitive Fluorescent Dye Loading

The abaxial epidermal strips from *V. faba* were peeled gently and incubated in 10 μM 1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxo-9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-*N*,*N*,*N′*,*N′*-tetraacetic acid, pentaacetoxymethyl ester (fluo-3 AM) loading buffer (10 mM MES-Tris, pH 6.1) at 4°C for 2 h in darkness. Because the activities of esterases at 4°C were low, fluo-3 AM permeated through the membranes without being hydrolyzed by esterases in cell walls. After washed three times with MES buffer, strips were kept at room temperature for 1 h. During this time, fluo-3 AM inside the cell was hydrolyzed by intracellular esterases and the hydrolyzed form of fluo-3 AM bound to free Ca^{2+} to indicate dynamic Ca^{2+} changes in guard cells (Shang et al., 2001).

H_2O_2-Sensitive Fluorescent Dye Loading

The abaxial epidermal strips from *V. faba* or Arabidopsis were peeled gently and incubated in 50 μM H_2DCF-DA buffer (10 mM MES-Tris, pH 6.1) for 15 min at room temperature and then washed three times before measurement.

CLSM Microscopy

The fluorescence in guard cells was measured using CLSM (Bio-Rad CLSM 1024, Hercules, CA) with the following settings: excitation = 488 nm, emission = 535 nm, frame 512 × 512. Images were recorded every 10 s. When the fluorescence stabilized around 100 s after scanning, the reagents were added directly to the buffer in which the strips were placed, and we treated this agent addition point as zero time in all assays, and fluorescence changes were recorded and the calcium or ROS relative fluorescence intensity was figured by subtracting the basal signal at different time points indicated in figure legends. Using pixel intensity standard curves created by calcium calibration kit (Molecular Probes, Eugene, OR), the calcium concentrations in cells was quantified (Shang et al., 2001). The experiments were repeated at least three times with 7 to 10 cells each time, and one time data were presented to illustrate the changes of fluorescence intensity.

Epidermal Strip Bioassay

After incubated in MES buffer (10 mM MES-Tris, pH 6.1, containing 30 mM KCl and 0.1 mM $CaCl_2$) for 90 min under light to open the stomata, the strips from *V. faba* or Arabidopsis were treated with the following procedures. For studying the effects of ExtCaM, CTX, or H_2O_2 on stomatal closure, the strips with open stomata were transferred to the above buffer containing 10^{-8} M CaM, 400 ng/mL CTX, or 5×10^{-5} M H_2O_2 solution, separately. For studying the effects of PTX, DPI, or CAT on CaM induction of stomatal closure, the strips with open stomata were either pretreated with 400 ng/mL PTX solution for 30 min and the strips transferred to and incubated in 10^{-8} M CaM solution for 2 h, or the strips were transferred to and incubated in 10^{-8} M CaM solution plus10 μM DPI or plus 100 units/mL CAT for 2 h, respectively. For investigating the effect of G protein on the stimulation of H_2O_2 production by ExtCaM, the strips with open stomata were transferred to and incubated in 400 ng/mL CTX solution plus 10 μM DPI or plus 100 units/mL CAT, respectively. Stomatal apertures were measured under microscope at indicated times with 50 randomly selected stomata. Each assay was repeated three times. The data were presented as mean ± SE ($n = 150$).

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