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Inhibition of inward K^+ channels and stomatal response by abscisic acid: An intracellular locus of phytohormone action

(*Commelina communis* L./guard cell/patch clamp/*Vicia faba* L.)

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ABSTRACT **Abscisic acid (ABA), a plant hormone whose production is stimulated by water stress, reduces the apertures of stomatal pores in the leaf surface, thereby lessening transpirational water loss. It has been thought that inhibition of stomatal opening and promotion of stomatal closure by ABA are initiated by the binding of extracellular ABA to a receptor located in the guard-cell plasma membrane. However, in the present research, we employ three distinct experimental approaches to demonstrate that ABA can act from within guard cells to regulate stomatal apertures. (*i*) The extent to which ABA inhibits stomatal opening and promotes stomatal closure in *Commelina communis* L. is proportional to the extent of ABA uptake, as assayed with [^{3}H]ABA. (*ii*) Direct microinjection of ABA into the cytoplasm of *Commelina* guard cells precipitates stomatal closure. (*iii*) Application of ABA to the cytosol of *Vicia faba* L. guard-cell protoplasts via patch-clamp techniques inhibits inward K^+ currents, an effect sufficient to inhibit stomatal opening. These results demonstrate an intracellular locus of phytohormone action and imply that the search for hormone receptor proteins should be extended to include intracellular compartments.**

Plant hormones play crucial roles in the regulation of plant growth and development and in plant responses to changing environmental conditions (1). The phytohormone abscisic acid (ABA) is synthesized and redistributed within plants in response to stress, particularly water stress (2, 3). ABA reduces stomatal apertures, and thus lessens transpirational water loss (4, 5). Stomatal opening normally occurs when an increase in guard-cell osmotica, caused in large part by uptake of K^+ through inward rectifying K^+ channels in the plasma membrane, drives water influx, cell swelling, and a bowing out of the guard-cell pair. K^+ uptake is promoted by a negative or hyperpolarized membrane potential. ABA depolarizes the membrane potential, thus favoring K^+ efflux and opposing osmotic build-up. ABA-stimulated membrane depolarization (6) may be caused by Ca^{2+} influx (7), anion efflux (8, 9), and/or inhibition of a H^+-extruding ATPase (10). ABA additionally inhibits stomatal opening by inhibiting the inward K^+ channels that mediate K^+ uptake (11) and may additionally promote stomatal closure by increasing the availability or activity of the outward K^+ channels that mediate K^+ efflux (11).

In general, hormones either bind to plasma membrane receptors and initiate a second messenger cascade or cross the cell membrane and bind to internal targets (12). For the past decade, it has been accepted (for review, see, e.g., refs. 12 and 13) that extracellular ABA interacts with a plasma membrane receptor in guard cells (14, 15). This conclusion has been based on two observations. (*i*) In epidermal peels of *Valerianella locusta*, stomatal closure was observed to occur similarly in medium of pH 8 and pH 5, despite the fact that at pH 8 zero ABA uptake was recorded (14). (*ii*) Binding of ABA by intact guard-cell protoplasts of *Vicia faba* was reported to be eliminated by addition of trypsin (15). SDS/PAGE analysis of whole protoplast proteins revealed three proteins that were photoaffinity-labeled with *cis*-(+)-[^{3}H]ABA but their cellular localization was not determined. In fact, progress in the identification of ABA receptors in any plant material has been slow (16, 17). In the present study, we provide evidence that ABA localized within guard cells regulates stomatal apertures. These results suggest that attention to intracellular compartments may be fruitful in the search for ABA receptor proteins.

MATERIALS AND METHODS

Plant Material. Plants of *Commelina communis* L. and *Vicia faba* L. were grown as described (18, 19). Young expanded leaves from plants 3–4 weeks old were used in all experiments.

Epidermal Peel Experiments. The protonated form of ABA (ABAH) readily permeates the lipid bilayer of the cell membrane, and ABA(H) partitions passively across that membrane according to the pH gradient (20, 21). Therefore, various pH values were used to control the extent of ABA uptake by guard cells in epidermal peels. For *Commelina communis*, solutions were prepared with pH values ranging from 5.0 to 8.0, by using 10 mM Mes buffered with KOH or Bistris propane (BTP) (pH 5 and 6) or 10 mM Hepes buffered with KOH or BTP (pH 7 and 8). KCl was added as necessary to achieve 100 mM K^+. For *Vicia faba*, incubation solutions were designed to match bath solutions used in patch-clamp experiments and contained 100 mM KCl, 5 mM Mes, 5 mM Hepes, 1 mM $MgCl_2$, and 1 mM $CaCl_2$, titrated with Tris to the desired pH. ABA [(±)-*cis*/*trans*, 99%$^+$ purity; Sigma A-1012] was added to final concentrations as reported in *Results*.

Experiments to assay stomatal apertures were performed as described (18, 22, 23). Apertures were measured after incubation in white light at 0.120 mmol·m^{-2}·s^{-1} for 3 h (opening experiments) or 1 h (closing experiments).

[^{3}H]ABA Uptake Experiments. Epidermes of *Commelina communis* were peeled and placed in a pH 3.9 medium (10 mM KCl/10 mM Mes·HCl, pH 3.9) for 60 min to kill epidermal cells while preserving viable guard cells (24, 25). Peels were then transferred to uptake medium, consisting of 10 μM unlabeled ABA and 100 mM KCl, with the solution pH adjusted as described for epidermal peel experiments. [^{3}H]ABA [Amersham; 600 nCi (9.0 pmol; 1 Ci = 37 GBq)] was added to each treatment mixture.

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Abbreviations: ABA, abscisic acid; CF, carboxyfluorescein.

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Peels were incubated for 60 min at 20–22°C with gentle shaking. Peels were then quickly (<5 min) washed in four changes of ice-cold 50 mM KCl (pH 8.0) and transferred to scintillation vials containing 4.5 ml of Quicksafe (Zimsser Analytic, Maidenhead, U.K.). Vials were agitated for 2 h before ^3H was measured. Apoplastic ABA was determined by measuring radioactivity in peels in which all cells had been killed by twice freezing and thawing after the wash step. Apoplastic ABA was subtracted from all values presented, which express ABA uptake on an epidermal area basis. Typical apoplastic levels of ABA were ≤0.18 pmol of ABA per cm^2.

Electrophysiology. Guard-cell protoplasts were isolated as described (19), except that sorbitol instead of mannitol was used as an osmoticum, because of reports that some commercial sources of mannitol are contaminated with ABA (26). Until use, protoplasts were maintained in the dark at 0–2°C in a solution containing 5 mM Mes (KOH, pH 5.5), 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.45 M sorbitol.

For patch-clamp analysis, the external (bath) solution contained 100 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM Mes, and 5 mM Hepes. The solution was titrated with Tris to the desired pH and adjusted to 460 mmol/kg with sorbitol. Glass microelectrodes (Kimax 51) were filled with a solution containing 10 mM Hepes (KOH, pH 7.2), 100 mM potassium glutamate, 2 mM $MgCl_2$, 2 mM EGTA, and 2 mM MgATP, adjusted to 500 mmol/kg with sorbitol. The final K^+ concentration for this solution was 107 mM. ABA was added to the bath or pipette solution as indicated; this addition did not alter the solution pH.

Whole-cell patch-clamp analysis was performed at 20 ± 2°C under green light (no. 874 filter; Roscolene, Woburn, MA). Seal resistances were >2 GΩ in all experiments. Cell capacitance was between 4.8 and 8.0 pF and was measured for each cell using a compensation device on the patch-clamp amplifier (Axopatch 1-D; Axon Instruments, Foster City, CA). Whole-cell currents were acquired to disk (486/33c PC; Gateway 2000, North Sioux City, SD) at 2 ms per sample via a TL-1 DMA interface (Axon Instruments). PCLAMP software (version 5.5.1, Axon Instruments) was used for data acquisition and analysis. The voltage-pulse protocol is shown in Fig. 4. During the 6 s between adjacent voltage pulses, membrane potential was held at −52 mV. Under our conditions, whole-cell currents from control cells remained stable for 1–2 h.

To derive the magnitude of time-activated current for each cell, instantaneous current at 2 ms into the voltage pulse was subtracted from steady-state current, defined as the average current value from 3.1 to 3.3 s into the voltage pulse. Time-activated current was divided by cell capacitance to normalize for variations in cell surface area (Fig. 3). Omitting this normalization has no significant effect on the results (Fig. 3 *Insets*).

Microinjection Experiments. Guard cells were microinjected as described (27). Leaves of *Commelina communis* were submerged, abaxial-side up, in distilled water and illuminated with white light at 0.150 $mmol \cdot m^{-2} \cdot s^{-1}$ for 2 h to open stomata to 8–15 μm. Peels were attached cuticle-side down to a microscope slide with double-sided sticky tape and submerged in 100 mM KCl/1 μM ABA, pH 8.0, throughout the experiment. Stomata with apertures of 10–12 μm (Fig. 5) were chosen for microinjection. Microinjection was into the pore side of a single guard cell of the stomatal pair. Injection pipettes contained 100 mM KCl, 500 μM carboxyfluorescein (CF), and 10 mM Mes·KOH (pH 7.0) with or without 100 μM ABA. Fluorescence intensity was used to estimate the cytosolic concentration of CF and thus of ABA (27). Intracellular CF concentrations varied from 12.7 to 73.3 μM, giving cytosolic ABA concentrations of 2.5–14.7 μM, which are physiological (28, 29).

The intracellular distribution and diffusion rate of CF indicated whether the injection had been into the cytosol, producing a rapid fluorescence in nucleus and chloroplasts, or into the vacuole, resulting in a weak diffuse fluorescence. Cells showing the latter pattern usually deflated within about 10 s as did cells that exhibited extrusion of cytoplasm from the injection location. Such cells were discarded, as were cells with any visible morphological changes. To impose the most stringent criteria for cell viability, cells that looked morphologically normal but failed to recover cytoplasmic streaming were also excluded. These are the same criteria that have been used for other microinjection studies on guard cells (30). After injection, the epidermal peel was maintained under white light at 0.060 $mmol \cdot m^{-2} \cdot s^{-1}$ for up to 30 min, and stomatal apertures were recorded continuously using a video camera and video recorder. Stomatal apertures were subsequently measured from these video images.

RESULTS

ABA Effectiveness Correlates with the Extent of ABA Uptake. Reduced opening by control stomata of *Vicia faba* (Fig. 1*B*) compared to control stomata of *Commelina communis* (Fig. 1*A*) can presumably be attributed (31) to the presence of 1 mM $CaCl_2$ in the *Vicia* incubation solution, provided to keep the *Vicia* solution consistent with the bath solution used in subsequent patch-clamp experiments. Under the incubation conditions used, little effect of external pH on stomatal opening could be observed in *Commelina*, and as was noted (32), no significant effects could be detected in *Vicia* (Fig. 1). However, the ability of ABA to inhibit stomatal opening was clearly greater at low pH in both *Commelina* and *Vicia* (Fig.

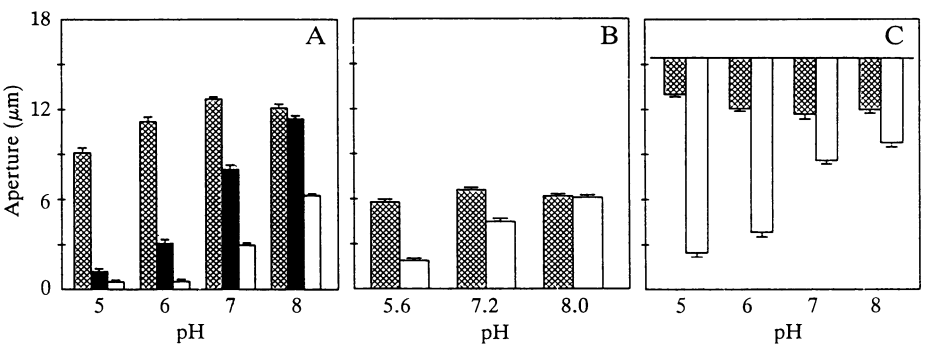

FIG. 1. pH dependency of ABA inhibition of stomatal opening in *Commelina communis* (*A*) and *Vicia faba* (*B*) and of ABA promotion of stomatal closure in *Commelina* (*C*). Bars: cross-hatched, control; solid, 1 μM ABA; open, 10 μM ABA. Each data point represents the mean ± 1 SEM of at least 60 apertures, obtained in at least three experiments.

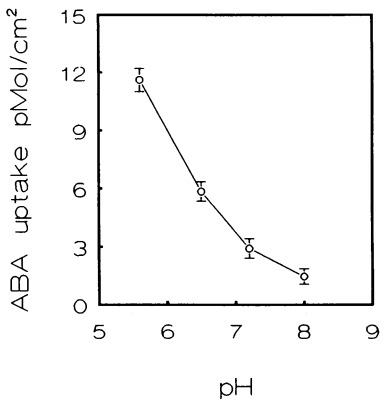

FIG. 2. pH dependency of ABA uptake by isolated guard cells in epidermal peels of *Commelina communis*. ABA uptake in a 1-h period is plotted as a function of extracellular pH. ($\bar{x} \pm 1$ SEM; n = 5 experiments).

1 *A* and *B*). A similar pH dependency was observed for closing responses of *Commelina* to 10 μM ABA (Fig. 1*C*).

The significantly greater effectiveness of ABA at low pH in both opening and closing experiments correlated with significantly greater ABA uptake at low pH than at high pH (Fig. 2). Since low pH promotes passive uptake of ABA(H), this pH effect was expected and is consistent with previous reports in guard cells and other tissues (14, 32–35). Unlike in *Valerianella* (14), a small amount of ABA uptake at pH 8.0, possibly carrier-mediated (refs. 21, 33, and 34; A.S., unpublished data), was exhibited by *Commelina* guard cells. This uptake may contribute to the partial effectiveness of 10 μM ABA at pH 8.0 in this species (Fig. 1).

Internal ABA Mediates Inward K^+ Current Inhibition. Alteration of external pH in the range of pH 5.6–pH 8.0 had no effect on the magnitude of inward K^+ currents (Fig. 3 *A* and *C*). In some experiments (Fig. 3*A*), acidic external pH somewhat reduced the magnitude of outward K^+ currents (36). When ABA was applied to the external solution, its effect was strongly pH-dependent. External ABA had no effect at pH 8.0 (compare current magnitude at pH 8.0 ± ABA) and became increasingly effective as pH was lowered (Fig. 3*B*), analogous to the ABA effects on stomatal aperture (Fig. 1). Since low pH promotes ABA uptake (Fig. 2), these data are consistent with an internal locus of ABA action on inward K^+ currents. In isolation, however, these results could not preclude an alternate explanation, for example, pH dependence of ABA binding to an externally facing plasma membrane receptor. It was therefore crucial to apply ABA directly to the cytosol via the patch pipette solution. As shown in Fig. 3*D*, this internal ABA was effective, independent of external pH. At all pH values tested, internal ABA strongly reduced the magnitude of inward K^+ current. Results shown in Fig. 3*D* are from 1 μM internal ABA application; the magnitude of current reduction in response to 10 μM internal ABA was similar (data not shown). These data confirm that ABA acts from the cytosolic side to regulate inward K^+ channels; if ABA was acting from the external side, it should have been ineffective at pH 8.0 (compare Fig. 3 *B* and *D*). ABA alters the magnitude and not the overall shape of the K^+ current vs. time kinetics (Fig. 4).

Internal ABA Causes Stomatal Closure. Microinjection was used to address the locus of ABA action in intact guard cells. With 1 μM ABA (pH 8.0) in the external medium, uninjected stomata of *Commelina communis* remained open (Figs. 5 and 6), consistent with Fig. 1. Stomata where one of the guard cells was sham-injected with CF-containing buffer showed slight closure, but apertures of buffer-injected and uninjected cells did not differ significantly (Fig. 6). Since CF has the same pKa as ABA and a similar molecular weight, the absence of a significant effect of CF microinjection indicates that the effects of intracellular ABA application in the microinjection and patch-clamp experiments cannot be attributed to a nonspecific weak-acid effect.

In contrast to the control injections, microinjection of ABA into the guard-cell cytosol stimulated marked stomatal clo-

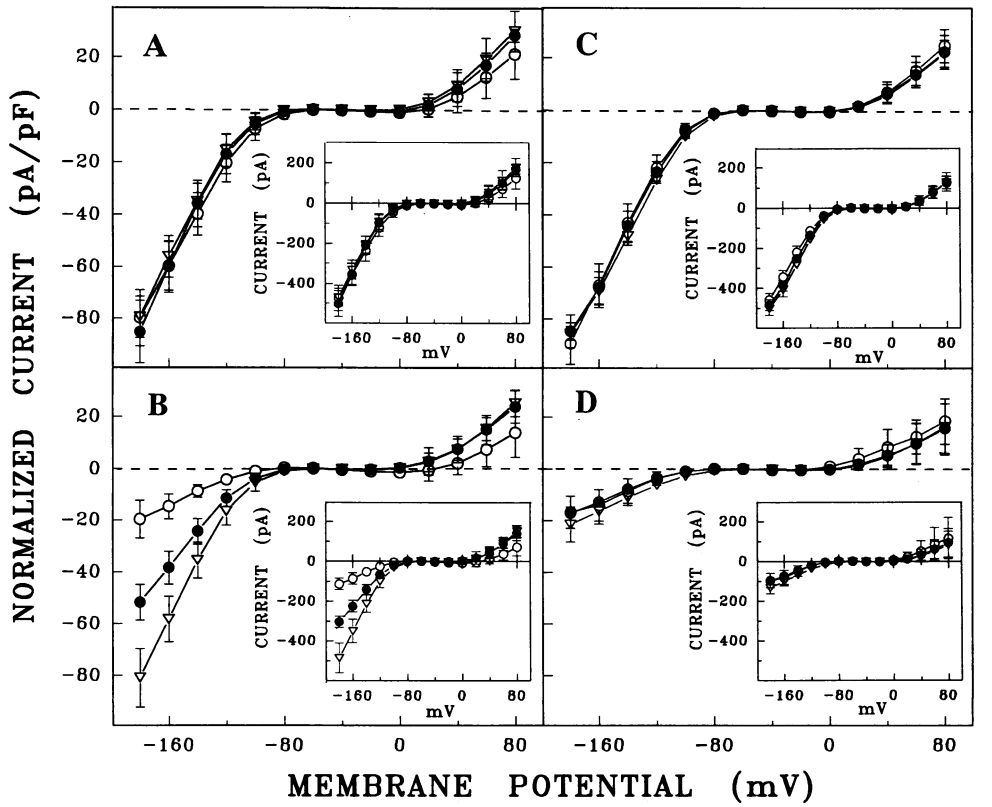

FIG. 3. Effects on whole-cell K^+ currents of *Vicia* guard-cell protoplasts of extracellular (*B*) or intracellular (*D*) ABA application at external pH values of pH 5.6 (○), pH 7.2 (●), and pH 8.0 (▽). (*A* and *B*) Current–voltage relationship of time-activated current in the absence (*A*) and presence (*B*) of 10 μM ABA in the bath solution. Data were acquired 20 min after achieving the whole-cell configuration. Onset of ABA inhibition was gradual, and maximal effects were observed by 20 min. (*C* and *D*) Current–voltage relationship in the absence (*C*) or presence (*D*) of 1 μM ABA in the patch pipette solution. Data were acquired 10 min after achieving the whole-cell configuration. Inhibition was evident within 2 min and was maximal by 10 min. Each data point in *A–D* is the mean ± 1 SEM (n = 8 for each treatment). (*Insets*) Same data as in the main figure but without normalization by capacitance.

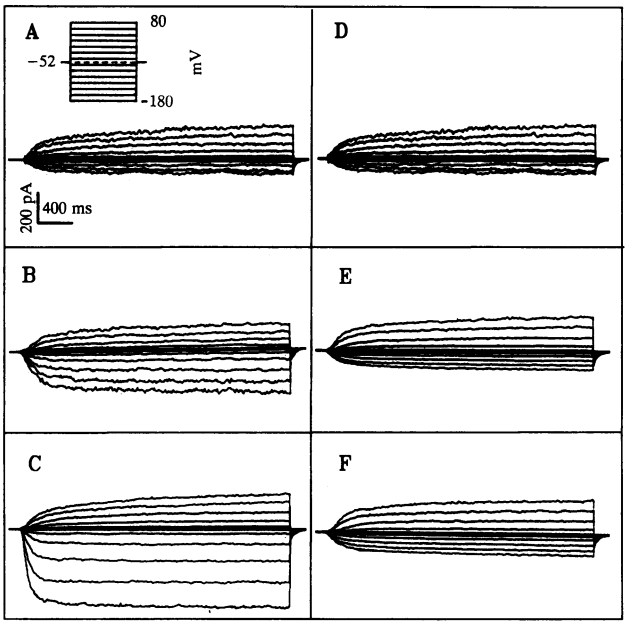

FIG. 4. Whole-cell K^+ currents from guard-cell protoplasts of *Vicia faba*. External pH was 5.6 (*A* and *D*), 7.2 (*B* and *E*), or 8.0 (*C* and *F*). ABA (10 μM) was added in the bath solution (*A–C*) or 1 μM ABA was added in the pipette solution (*D–F*). Recordings were taken 20 min after the addition of ABA (*A–C*) or 10 min after formation of the whole-cell configuration (*D–F*). Whole-cell capacitance for these six protoplasts was similar (5.5 ± 0.45 pF; $\bar{x}$ ± 1 SEM). Voltage protocol and scale bars, shown in *A*, are the same for *B–F*.

sure (Figs. 5 and 6). Whereas external application of 10 μM ABA at pH 8.0 (Fig. 1*C*) caused stomatal closure of only 2.3 μm (i.e., 1.15 μm for "half-apertures"), stomata microinjected with 2.5–14.7 μM ABA showed treatment-specific average closure of 5 μm for half-apertures. Therefore, leakage of microinjected ABA to the apoplast cannot be invoked as an explanation for the extent of closure observed.

DISCUSSION

Our data demonstrate that ABA acts from within the guard cell to regulate stomatal apertures. As also reported by others (32, 37, 38), the effectiveness of ABA in inhibiting stomatal opening and promoting stomatal closure is enhanced at acidic external pH values (Fig. 1) that promote ABA(H) uptake

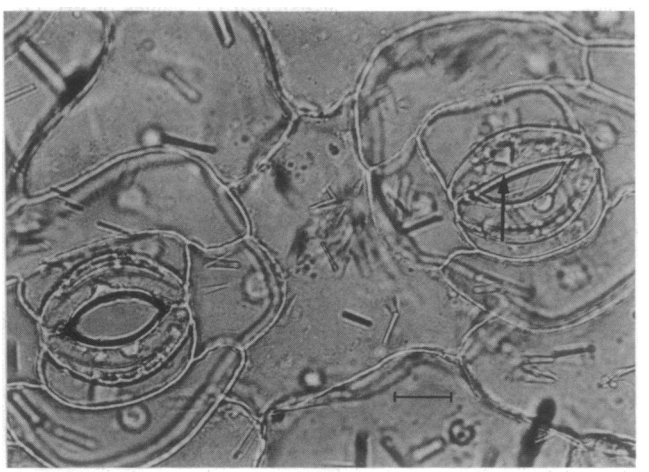

FIG. 5. Photomicrograph of *Commelina communis* guard cells 30 min after the indicated (arrow) guard cell was microinjected with ABA. Other cells visible were not injected. (Bar = 20 μm.)

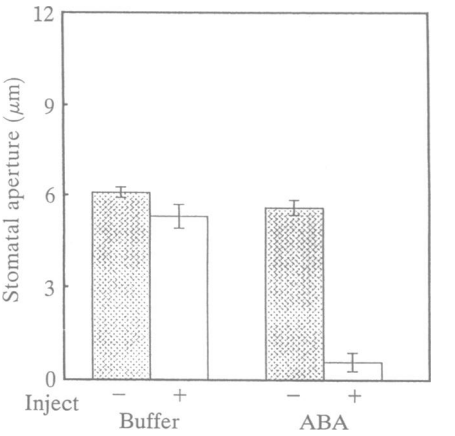

FIG. 6. Effect of ABA or buffer microinjection on stomatal aperture in *Commelina communis*. One guard cell in a pair was injected. In each case, cross-hatched bars represent the half-apertures of the uninjected guard cells in the pairs and open bars represent the half-apertures of the injected guard cells in the pairs. Values shown are the mean ± 1 SEM (n = 8 for each treatment).

(Fig. 2), consistent with a mechanism involving an internal target for ABA. The extent of ABA loss from preloaded *Commelina* guard cells also correlates well with the extent of subsequent stomatal opening (29), consistent with an internal locus of ABA action. While these correlative studies fall short of proving causation, observation of stomatal closure or K^+ channel inhibition after direct application of ABA to the guard-cell cytosol by microinjection or patch-clamp techniques (Figs. 3–6) clearly demonstrates a role of internal ABA in regulating stomatal apertures. Effective internal ABA concentrations in *Commelina* and *Vicia* were in the micromolar range, consistent with measured ABA levels in guard cells of these species (28, 29). Significantly higher ABA levels have been reported in *Valerianella* (39), and possible reasons for these differences have been discussed (40).

In agreement with our microinjection data, stomatal closure occurs after photolytic release of caged ABA injected into guard cells; nonphotolyzable caged ABA is ineffective (A. Trewavas, personal communication). Although further research will be required to determine which of the several ion transport processes involved in stomatal closure is altered by internal ABA, insight into the mechanism by which internal ABA inhibits stomatal opening is provided by patch-clamp measurements. Application of ABA to the cytosol via the patch pipette inhibits inward K^+ currents, an effect sufficient to prevent the K^+ uptake required for stomatal opening. Other researchers have also reported ABA inhibition of inward K^+ currents (6, 11, 48). In their experiments, ABA was applied extracellularly at external pH values of 7.4 (11), 6.1 (6), and 5.5–6.1 (48), which allow some inhibition of inward K^+ current (cf. Figs. 3 and 4). Contrary to other reports (11, 48), we did not observe enhancement of outward K^+ currents by either internally applied or externally applied ABA. Differences in plant growth conditions may explain the differences between our results and those of others. For example, in cotton, water stress, nitrogen deficiency, and leaf aging all increase stomatal closure in response to exogenous ABA (41). In contrast, we utilized the youngest mature leaves from plants that were well-watered with nutrient solution and raised under growth chamber conditions.

Our results imply that internal receptors exist for ABA in guard cells. Given these data, it will be of interest to determine the subcellular locations of previously reported ABA binding proteins (15). Our data also have implications for the effects of ABA on gene expression (42, 43), including the possibility that ABA directly regulates transcription factors.

However, while our data do demonstrate an internal locus of ABA action in regulating both stomatal opening and stomatal closure, these data do not preclude the existence of additional external loci of ABA action, in guard cells or other cell types. For example, under the high external Cl^- concentrations of our experiments, if ABA acted from the outside to activate anion channels, this would not result in Cl^- efflux and thus would not affect stomatal apertures.

Our data provide physiological relevance to the observation that water stress stimulates an increase in guard-cell ABA levels (44) with a time course of minutes (28). This accumulation results from ABA synthesis by the guard cells (45), release of ABA by other leaf cells (4, 41, 46), and increased ABA delivery in some species (4, 5). According to the theory that ABA acts only from outside the guard cell, this stress-induced ABA accumulation within the guard cell is paradoxical. It has been theorized that guard cells first accumulate ABA, then release the hormone to the medium so that it can access the ABA receptor (39), although in at least one study stomatal closure preceded the increase in external ABA (47). Our results do not preclude such a repartitioning mechanism but do offer a simpler scheme in which internal ABA directly influences stomatal apertures.

Other major phytohormones, auxins and gibberellins, are also weak acids and partition across cell membranes (21). Further experimentation will be required to ascertain whether an intracellular locus of hormone action, as reported here for ABA, is a typical pathway for the transduction of plant hormone signals.

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