Cytoskeletal inhibitors suppress the stomatal opening of *Vicia faba* L. induced by fusicoccin and IAA

Rong Feng Huang a,c, Xue Chen Wang b,c,*, Cheng Hou Lou b,c

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**Abstract**

Stomatal movement is governed by osmotic potential, in which K⁺ concentration plays the dominant role. Our previous work has shown that both microtubules and microfilaments are involved in regulating stomatal movement. In the present investigation the relationships between cytoskeletal components and K⁺ fluxes in stomatal opening were addressed by using fusicoccin (FC), indoleacetic acid (IAA), and cytoskeletal inhibitors to treat both epidermal strips and protoplasts of guard cells. The results revealed that FC and IAA induced stomatal opening with or without KCl in the dark. Also FC or IAA induced guard cell protoplast swelling in the dark even without added KCl. However, the induction was partially suppressed when strips and protoplasts were pretreated with cytochalasin B (CB), an inhibitor of F-actin polymerization, or oryzalin, an inhibitor of plant microtubule polymerization. Thus our preliminary results indicate for the first time that microtubules and microfilaments can affect stomatal opening independently of K⁺ fluxes. © 2000 Published by Elsevier Science Ireland Ltd. All rights reserved.

**Keywords**: Microtubules; Microfilaments; Fusicoccin (FC); Indoleacetic acid (IAA); Stomatal opening

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**1. Introduction**

Various environmental and internal signals, working through different signal transduction pathways, control the process of stomatal movement. Changes of turgor pressure, caused by the osmotic potential of guard cells, trigger stomatal movement, and a central role of K⁺ in stomatal movements is undisputed in the current literature [1]. Stomatal aperture depends on the turgor differences between guard cells and the surrounding subsidiary or epidermal cells, as well as on the mechanical properties of the cell walls of guard cells. However, there is recent evidence indicating that microfilaments [2,3] and microtubules [4,5] are involved in both stomatal opening and closing. For example, the effects of actin antagonists on stomatal opening parallel their effects on hyperpolarization-dependent inward K⁺ current, suggesting that activities of inward K⁺ rectified channels in guard cells are regulated by structural changes in actin filament [6].

Though K⁺ has a central role in stomatal opening, other factors must also be important. For instance, when there is no K⁺ in the incubation medium the stomatal aperture can increase in response to blue light, without any K⁺ accumulation or starch loss [7]. And for this reason there has been discussion of the role of the dynamics of cortical microtubules and microfilaments in stomatal movement [2,8,9]. Thus, to understand stomatal movement it is very important to investigate the relationships between cytoskeletal components and K⁺ fluxes in stomatal opening. In the present study this point was addressed by using fusicoccin (FC) and indoleacetic acid (IAA) separately in order to induce stomatal movement [10–14] and protoplast swelling in combinations with cy-
toskeletal inhibitors to treat both epidermal strips and guard cell protoplasts.

2. Materials and methods

2.1. Plant materials

Plants of *Vicia faba* L. were grown in a greenhouse with 13–16 h of natural light per day at temperatures of 20–25°C. Plants were kept free from water stress at all stages of development. The two youngest fully expanded leaves were used from plants 3–4-weeks-old. Epidermal peels were in MAC buffer containing 10 mM 2-[N-morpholino] ethanesulfonic acid (Mes)-tris-(hydroxymethyl) aminomethane (Tris), pH 6.1, 1 mM AlCl₃ and 0.1 mM CaCl₂. And all experiments were conducted in the dark at a temperature of 25 ± 2°C.

2.2. Isolation of guard cell protoplasts

Eight to ten leaflets were put in cold distilled water. The midveins and veins were removed using a razor blade, and the leaves were cut into small pieces. Then the leaf pieces were placed in 100 ml MBMC buffer (10 mM Mes-Tris, pH 6.1, 0.02% bovine serum albumin (BSA), 0.35 M mannitol, 0.1 mM CaCl₂) in a small blending jar. Blender speed-setting and blending time was adjusted according to the toughness of the leaves. The blended mixture was poured through a 200 µm nylon mesh. Broken mesophyll and epidermal cells passed through the mesh. The pale whitish-green epidermal pieces were put in 10 ml enzyme solution 1 (2% cellulase Onozuka R-10, 0.1% pectolyase Y-23, 2% BSA, 0.2 mM phenyl-methyl-sulfonyl-fluoride (PMSF) and 1 mM dithiothreitol (DTT) in MBMC buffer) in a 50 ml flask after rinsing thoroughly with cold distilled water. After shaking for at least 45 min at 78–85 rpm, the epidermal pieces were collected on 200 µm nylon mesh. Broken mesophyll and epidermal cells passed through the mesh. Then the followed mixture was poured through a 200 µm nylon mesh. Broken mesophyll and epidermal cells passed through the mesh. The pale whitish-green epidermal pieces were put in 10 ml enzyme solution 1 (2% cellulase Onozuka R-10, 0.1% pectolyase Y-23, 2% BSA, 0.2 mM phenyl-methyl-sulfonyl-fluoride (PMSF) and 1 mM dithiothreitol (DTT) in MBMC buffer) in a 50 ml flask after rinsing thoroughly with cold distilled water. After shaking for at least 45 min at 78–85 rpm, the epidermal pieces were collected on 200 µm nylon mesh, and rinsed thoroughly with MBMC buffer. Then the epidermal strips were transferred into 10 ml enzyme solution 2 (1% cellulase Onozuka R-10, 0.05% Pectolyase Y-23, 0.1 mM PMSF, 0.5 mM DTT in MBMC buffer) in a 50 ml flask with shaking at 35–45 rpm for 50–60 min. After guard cell protoplasts were separated from the guard cell walls, the sample was filtered through 30 µm nylon mesh into a 50 ml centrifuge tube. The protoplasts were centrifuged for 5 min at 200 × g in MBMC buffer for three times, the supernatant was carefully pipetted out leaving a minimum volume at the bottom of the tube, and the pellet was resuspended with MBMC buffer.

2.3. Experimental procedures involving pharmacological treatments

The lower epidermal strips were peeled from detached leaves in the morning, and the mesophyll cells washed away with distilled water. Then the strips were incubated in 10 mM Mes-Tris (pH 6.1) in the dark to induce stomatal closing. After stomata were closed in the dark, the epidermal strips were transferred into FC or IAA solution to induce stomatal opening, with plus-30 mM KCl and minus-K⁺ separately. A total of 20 µM Cytochalasin B (CB), an inhibitor of F-actin polymerization [2,3,15], or 50 µM oryzalin, an inhibitor of plant microtubule polymerization [16,17], were applied in this experiment to pre-treat closed stomata in the dark. Stomatal apertures did not change significantly within our applied concentrations for less than 60 min, which is consistent with the findings of Huang and Wang [3,5]. Then the epidermal strips were transferred into FC or IAA solution with or without cytoskeletal inhibitor.

For experiments with protoplasts, the freshly isolated protoplasts were suspended with MBMC buffer containing CB or oryzalin. Changes of volume were less than 3% after incubation in CB, oryzalin, or MBMC buffer for 30 min in the dark. Then the protoplasts were treated with FC and IAA plus cytoskeletal inhibitor separately. The volume of protoplasts was calculated by measuring their diameters with a ruler in the eyepiece under the microscope.

In parallel with each experiment, controls were conducted with dimethyl sulfoxide in which CB and oryzalin were dissolved separately (the stock concentrations are 52 mM of CB, 100 mM of oryzalin). The final concentration of DMSO was less than 0.05% (v/v). No significant effect on stomatal movement was found at these concentrations.

The viability of epidermal stomata and guard cell protoplasts in our experiments was tested by
the neutral red methods as described by Weyers and Meidner [18]. More than 90% stomata or protoplasts of guard cells had viability.

Measurements were made within 5 min of 50 stomata or protoplasts located at random, ten from each of the five strips or slides. Each experiment was conducted at least three times independently.

3. Results

3.1. Effect of FC, or IAA on stomatal opening

The effect of FC on stomatal opening was tested by treating epidermal strips for 90 min with different concentration of FC. FC induced stomatal opening in both plus- and minus-KCl buffer (Fig. 1A). The apertures were significantly increased until the concentration of FC added in plus-KCl solution and minus-KCl solution reached 0.73 and 2.19 μM, respectively, but not further increased at higher concentrations (Fig. 1A).

The time course of 1.46 μM FC on stomatal opening in plus- and minus-KCl buffers is shown in Fig. 1B. FC induced stomatal opening after 30 min. The maximum aperture was approached after 90 and 105 min after FC incubation in plus-KCl and minus-KCl, respectively (Fig. 1B).

IAA of different concentrations induced stomatal opening in plus- and minus-KCl for 90 min (Fig. 2A). Stomatal aperture increased with IAA concentration. The maximum aperture at concentration of 0.2 mM IAA was 8.6 μm in plus-KCl and 7.6 μm (Fig. 2A) in minus-KCl buffer. For the time course, the stomatal aperture was significantly increased in the first 90 min both in plus-KCl and minus-KCl as 0.2 mM IAA was added (Fig. 2B). Thus the results indicated that FC, or IAA induced stomatal opening in the dark without requiring the addition of KCl.

3.2. Effect of CB or oryzalin on stomatal opening-induced by FC or IAA

When stomata were treated with 20 μM CB for 50 min, the aperture induced by 1.46 μM FC was only 58.8% of the control’s (Fig. 3 Treatment 3). If FC were added in CB solution after CB pretreatment, the aperture was 42.2% of control’s (Fig. 3 Treatment 4).

Also when stomata were pretreated with 50 μM oryzalin for 60 min, the aperture induced by 1.46
Fig. 3. Effect of 20 μM CB or 50 μM oryzalin pretreatment on stomatal opening induced by 1.46 μM FC for 90 min in the dark. 1: control; Closed stomata were incubated in MAC buffer. 2: FC treatment, 3: FC treatment after 50 min pretreatment of CB. 4: FC treatment plus CB after pretreatment of CB. 5: FC treatment after 60 min pretreatment of oryzalin. 6: FC treatment plus oryzalin after pretreatment of oryzalin. Epidermal peels were in MAC buffer containing 10 mM Mes-Tris (pH 6.1) plus 1 mM AlCl₃ and 0.1 mM CaCl₂. The stomatal aperture was the average (±SE) of three separate experiments, counting at least 50 stomata for each experiment described as Section 2.

μM FC was 61.8% of control's (Fig. 3 Treatment 5). If FC was added to the oryzalin solution after oryzalin pretreatment, the aperture was 44.1% of control's (Fig. 3 Treatment 6).

Similarly, stomatal aperture induced by 0.2 mM IAA was suppressed when stomata were pretreated with either CB for 50 min or oryzalin for 60 min. Compared with the control the aperture was 59.3% for IAA treatment and 46.5% if IAA was added to the CB solution after CB pretreatment. Similar results were obtained with oryzalin (Fig. 4).

Thus our results demonstrate that pretreatment of CB or oryzalin suppressed stomatal opening induced by FC or by IAA in K⁺-free medium in the dark. These suggest that microtubules and microfilaments can affect stomatal opening independently of K⁺ fluxes.

3.3. Effect of CB, or oryzalin on the swelling of guard cell protoplasts induced by FC, or IAA

When freshly isolated guard cell protoplasts were incubated in MBMC, the volume of protoplasts did not change significantly. However, the protoplasts swelled if 0.73 μM FC or 100 μM IAA was added to the MBMC buffer. The relative volume of guard cell protoplasts was increased by 28–35% within 50–60 min following the addition of FC or IAA (Fig. 5 and Fig. 6). After this period, 78.1–85.7% protoplasts were broken.

The volume of guard cell protoplasts did not change significantly if protoplasts were treated with 20 μM CB, or 50 μM oryzalin for 30 min in minus-K⁺ buffer in the dark. The increase in volume of the protoplasts induced by FC and IAA after CB and oryzalin pretreatment respectively was less than 3% (Figs. 5 and 6). This result on protoplasts demonstrates that the induction is likely to reflect the inherent nature of the microtubule and microfilament system rather than some collateral effects of cell walls.

4. Discussion

Electrophysiological studies have demonstrated that there are two independent K⁺ channels in the

Fig. 4. Effect of 20 μM CB or 50 μM oryzalin on stomatal opening induced by 0.2 mM IAA in the dark for 90 min. 1: control: Closed stomata were incubated in MAC buffer. 2: IAA treatment. 3: IAA treatment after 50 min pretreatment of CB. 4: IAA treatment plus CB after pretreatment of CB. 5: IAA treatment after 60 min pretreatment of oryzalin. 6: IAA treatment plus oryzalin after pretreatment of oryzalin. Epidermal peels were in MAC buffer containing 10 mM Mes-Tris (pH 6.1) plus 1 mM AlCl₃ and 0.1 mM CaCl₂. The stomatal aperture was the average (±SE) of three separate experiments, counting at least 50 stomata for each experiment described as Section 2.
Fig. 5. Effect of 20 μM CB or 50 μM oryzalin on protoplast swelling-induced by 0.73 μM FC in minus-K⁺ in the dark. Protoplasts were treated with FC only (black circle). A: The protoplasts were incubated in FC plus CB after CB pretreatment for 30 min as indicated with arrow (black triangle). B: The protoplasts were incubated in FC plus oryzalin after oryzalin pretreatment for 30 min as indicated with arrow (black triangle). All the protoplasts were in MBMC buffer. The volume data were the average of three separate experiments, counting at least 50 protoplasts for each experiment. And the relative volume of guard cell protoplasts (GCPs) is the percentage of protoplast average volume (± SE) compared with control's (100%).

Fig. 6. Effect of 20 μM CB or 50 μM oryzalin on protoplast swelling-induced by 0.1 mM IAA in minus-K⁺ in the dark. Protoplasts were treated with IAA only (black circle). A: The protoplasts were incubated in IAA plus CB after CB pretreatment for 30 min as indicated with arrow (black triangle). B: The protoplasts were incubated in IAA plus oryzalin after oryzalin pretreatment for 30 min as indicated with arrow (black triangle). The protoplasts were in MBMC buffer. The volume data were the average of three separate experiments, counting at least 50 protoplasts for each experiment. And the relative volume of guard cell protoplasts (GCPs) is the percentage of protoplast average volume (± SE) compared with control’s (100%).

Plant growth regulators affect plant development and movement through the dynamics of microtubules or microfilaments. For example IAA changes the orientation of cortical microtubule [21,22], and regulates the elasticity of the actin network [23]. FC has many effects similar to IAA, such as promoting proton translocation [24–27], but it is not clear whether FC also regulates the cytoskeletal network. In stomatal movements, there is some evidence that microtubules and microfilaments are involved in stomatal opening and closing depending on K⁺ [1–5,8,9] and the activities of K⁺ channels [6]. In this paper our experi-

guard cell plasma membrane, e.g. inward rectified K⁺ channels and outward rectified K⁺ channels [1,19]. Al³⁺ was reported as an inhibitor of the inward rectified K⁺ channel [20], suggesting the central role played by K⁺ in stomatal movement. However, when KCl is absent from the incubation medium, stomatal aperture increases in response to blue light without any K⁺ accumulation [7]. Further, in K⁺-free medium in the dark, our results demonstrate that FC (Fig. 1) and IAA (Fig. 2) induce both stomatal opening and protoplast swelling (Figs. 5 and 6). The K⁺ concentration for minus-K⁺ buffer was less than 0.05 mM (measured with a Medal 6400-A Flame Photometer) before or after stomatal opening in our experimental system containing AlCl₃, in which the stomatal aperture did not change significantly (data not shown). These results suggest that stomatal opening induced by FC or IAA is independent of the K⁺ fluxes.

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Fig. 5. Effect of 20 μM CB or 50 μM oryzalin on protoplast swelling-induced by 0.73 μM FC in minus-K⁺ in the dark. Protoplasts were treated with FC only (black circle). A: The protoplasts were incubated in FC plus CB after CB pretreatment for 30 min as indicated with arrow (black triangle). B: The protoplasts were incubated in FC plus oryzalin after oryzalin pretreatment for 30 min as indicated with arrow (black triangle). All the protoplasts were in MBMC buffer. The volume data were the average of three separate experiments, counting at least 50 protoplasts for each experiment. And the relative volume of guard cell protoplasts (GCPs) is the percentage of protoplast average volume (± SE) compared with control’s (100%).

Fig. 6. Effect of 20 μM CB or 50 μM oryzalin on protoplast swelling-induced by 0.1 mM IAA in minus-K⁺ in the dark. Protoplasts were treated with IAA only (black circle). A: The protoplasts were incubated in IAA plus CB after CB pretreatment for 30 min as indicated with arrow (black triangle). B: The protoplasts were incubated in IAA plus oryzalin after oryzalin pretreatment for 30 min as indicated with arrow (black triangle). The protoplasts were in MBMC buffer. The volume data were the average of three separate experiments, counting at least 50 protoplasts for each experiment. And the relative volume of guard cell protoplasts (GCPs) is the percentage of protoplast average volume (± SE) compared with control’s (100%).
ments provided further evidence that stomatal opening and protoplast swelling induced by FC or IAA in the absence of KCl in the dark could be suppressed by CB or by oryzalin (Figs. 3 and 4). These results, taken together with those in the literature [2,8,9], suggest that microtubules and microfilaments might independently affect stomatal opening.

To further eliminate the effect of the cell wall in stomatal movement, we used guard cell protoplasts, thereby investigating a direct role for cytoskeletal components in stomatal opening. Our results show that protoplast swelling induced by FC or IAA was suppressed when protoplasts were pre-treated with CB or oryzalin (Figs. 5 and 6). This suggests that microtubules and microfilaments trigger stomatal opening directly rather than acting indirectly on guard cell walls.

In the light of this we hypothesize that the dynamics of both microtubules and microfilaments directly modulate the swelling of guard cell protoplasts at the beginning of stomatal opening. These changes of guard cell volume are, ultimately, the result of water movement. Aquaporins form water selective channels, allowing water to pass freely while excluding ions and metabolites [28–30]. It seems likely that water channels or aquaporins in the plasma membrane [31–33] control water movement in guard cells. The changes in the activity of microtubules and microfilaments could activate aquaporins which, depending on the existing osmotic potential, would drive water into guard cells, resulting in stomatal opening. Thus our results provide new clues for a fuller understanding of the mechanism of stomatal movement.

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