Effect of Fusicoccin on the Early Infection Process of Legume Roots by *Rhizobium* spp.

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Nod factors, the first detectable signals produced by *Rhizobium* spp., were reported to induce cytosolic pH changes and plasma membrane depolarization in root hairs as specific responses. In this study, it has been found that fusicoccin inhibits nodulation of alfalfa roots. This inhibition was only observed when fusicoccin was applied in the earlier steps of the bacteria-plant interaction. The observed effect is similar to that caused by the undissociated permeant acetic acid, which decreases the cytoplasmic pH and, like fusicoccin, significantly stimulates net H⁺ efflux. These results suggest that the fusicoccin-induced plasma membrane H⁺-ATPase activity and membrane hyperpolarization could be responsible for the nodulation inhibition observed. Moreover, it was found that nodulation was inhibited by removing external calcium with EGTA. When fusicoccin is present, a lower concentration of EGTA is necessary to inhibit nodulation. Furthermore, the addition of Ca²⁺ ionophore A23187 was found to inhibit H⁺ efflux by roots. These observations support the idea that the Nod factor-triggered calcium signal modulates the activity of the proton pump.

Members of the genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, and *Sinorhizobium* (hereafter, rhizobia) are bacteria that establish a mutualistic symbiosis with legume roots. They form nodules where dinitrogen is reduced to ammonia that is then transferred to the host. A signal exchange between the host and the microsymbiont is necessary for the establishment of the association. Apparently, almost all plants exhibit the ability to distinguish between pathogenic and mutualistic interactions (Quigley et al. 1996). There may be similarities between pathogenic and mutualistic modes of infection and at least some elements of the molecular recognition mechanisms may be common in both cases.

In higher plants, ion transport is largely organized around hydrogen ions, and the most solidly postulated function of the plasma membrane proton pump is to maintain a large, stable, electrochemical potential difference for protons, which serves

as the driving force for the concentration of metabolic substrates and the stabilization of intracellular pH (Serrano 1989). Changes in the host plasma membrane H⁺-ATPase activity (with the associated changes in the intracellular pH and membrane potential) are among the earliest events related to plant pathogen interactions (Vera-Estrella et al. 1994). It has been proposed that the differential effect of elicitors on the plasma membrane H⁺-ATPase and the resulting acidification or alkalization of the extracellular medium are produced in response to the difference between specific and nonspecific elicitors (De Witt 1995).

Nod factors are the first recognizable signal produced by rhizobia when *nod* genes are induced by specific flavonoids exuded by the roots of the corresponding legume. However, little is known about how these signals are specifically perceived by host plant cells. It has been demonstrated that a plasma membrane depolarization (Ehrhardt et al. 1992; Kurkdjian 1995; Felle et al. 1995) and intracellular alkalization (Felle et al. 1996) of alfalfa root hairs take place in response to Nod factors produced by *Sinorhizobium meliloti*. Recently, evidence has been provided for the potential role of plasma membrane H⁺-ATPase in the process of Nod factor-induced membrane depolarization and extracellular alkalization (Felle et al. 1998). It was concluded that the depolarization was caused by temporary short-circuiting of the proton pump through the rapid release of Cl⁻ ions over their steep electrochemical gradient. Moreover, the observation that the Ca²⁺ ionophore A23187 mimics the Nod factor response, and that the Ca²⁺ channel antagonist nifedipine inhibits this response, indicates a Nod signal-induced influx of calcium ions that is required for triggering the subsequent events of plasma membrane depolarization and pH changes. Previous work with a non-nodulating alfalfa mutant showed a periodic calcium spiking in root hairs in response to specific *S. meliloti* Nod factors (Ehrhardt et al. 1996).

In higher plants, fungal toxin fusicoccin and undissociated permeant weak acids stimulate net H⁺ efflux and hyperpolarize the membrane potential. It has also been suggested that fusicoccin and weak acids stimulate both ATP hydrolysis and ATP-dependent H⁺ pumping activities of plasma membrane H⁺-ATPase as a result of the cytoplasmic acidification that is followed by the acidification of the medium (Marré 1979; Brummer et al. 1984; Bertl and Felle 1985; Johansson et al. 1993; De Michelis et al. 1996). Likewise, it has now been

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demonstrated that fusicoccin binds to a single receptor in plants, and it has become clear that a functional FC-binding site is formed from a complex between the C-terminal regulatory domain of the H⁺-ATPase and 14-3-3 proteins (Baunsgaard et al. 1998). Because fusicoccin and weak acids activate the electrogenic proton pump, the aim of this work has been to determine whether the cytoplasmic acidification induced by these two factors can affect the nodulation of alfalfa roots by rhizobia, and whether the activity of the proton pump can also be related to the extracellular concentration of calcium.

RESULTS AND DISCUSSION

Results have shown that simultaneous addition of fusicoccin and bacteria causes a delay in root nodule emergence that is also detected when fusicoccin is applied 8 h after inoculation (Fig. 1A). A significant decrease in the number of nodules formed was also observed (Fig. 1B). The effect on nodulation is temporary since several days after nodule appearance the number of nodules per plant was almost the same. No significant effect was observed when fusicoccin was added 24 h after inoculation (data not shown). At that time, most of the bacteria have already entered the root hair cells. The effect on nodulation cannot be attributed to a bacteriostatic or bactericidal action of the fusicoccin since no effect on the bacterial growth rate has been observed at the concentration applied (data not shown). Previously, we also found that fusicoccin at a concentration of 125 nM did not affect shoot and root plant development, while higher concentrations clearly caused a chlorosis.

The expression of a *nodC-lacZ* fusion was analyzed at different pHs in order to determine whether or not the low extracellular pH induced by fusicoccin affected the expression of *nod* genes. No differences in expression were observed (data not shown) at any of the pHs studied, even at pH 5.2, at which the bacterial growth is hardly noticeable.

Table 1 shows that H⁺ extrusion from excised alfalfa roots was significantly stimulated by fusicoccin. These results are in line with the effects of this diterpene glucoside on the acidification of the external medium observed in practically all the higher plant species (Marré et al. 1974; Roldan et al. 1992). Thus, the inhibition of nodulation by the fusicoccin may be a consequence of plasma membrane hyperpolarization.

Since the hyperpolarization of plasma membrane by H⁺-ATPase activation is important for the inhibitory effect of fusicoccin on nodulation, a control experiment was carried out with alfalfa roots in which a weak permeant acid (acetic acid) was used to change the cytosolic pH and the polarization of plasma membrane. A delay in the nodulation rate (Fig. 2A) and a decrease in the number of nodules formed (Fig. 2B) were found when plants were maintained for 30 min in a medium with a pH lowered to 5.0 at both concentrations of acetic acid, 4.0 and 7.5 mM. The treatment of plants at a pH 5.0, which was obtained by adding succinic acid to the mineral solution, displayed no effect. This suggests that, rather than the external pH, it is the intracellular acidification that is an important factor in nodulation inhibition. In this respect, the decreased intracellular pH that is due to the addition of acetic acid does enhance proton pumping by root cells (Table 1). This finding is consistent with the notion that cytoplasmic H⁺ concentration provides additional substrate for the plasma membrane H⁺-ATPase. This increased H⁺ pumping, which is the result of cytoplasmic acidification, can be manifested as a membrane hyperpolarization. Moreover, the decrease of intracellular pH caused by acetic acid load may put limits on the increase of cytoplasmic pH in response to Nod factor, as was demonstrated in *Medicago sativa* root hairs after the addition of *S. meliloti* Nod factor (Felle et al. 1996).

Alternatively, the decrease in nodulation rate may be caused by a cytosolic pH change in response to the action of fusicoccin on the biochemical pH stat. Recently, Felle et al. (1998) have suggested that an intracellular alkalization of the root hair cells appears to be caused by a net metabolic consumption of protons. Since, when fusicoccin is added 24 h after inoculation, no inhibition of nodulation was observed, it is likely that fusicoccin acts at the early stages of the interaction.

Table 1. Effects of fusicoccin, acid load, and Ca²⁺ ionophore A23187 on the H⁺ extrusion of alfalfa root cells

Treatment	Maximum rate of H ⁺ extrusion ($\mu\text{mol H}^+ \cdot \text{g (FW)}^{-1} \cdot \text{h}^{-1}$)
Control	1.18 $\pm$ 0.05
125 nM fusicoccin	1.79 $\pm$ 0.10
5.0 μM A23187	0.85 $\pm$ 0.05
Succinate	1.20 $\pm$ 0.08
Succinate plus acetic acid	2.07 $\pm$ 0.20

Fig. 1. Effect of 125 nM fusicoccin on the nodulation rate of alfalfa roots. **A**, Percentage of nodulated plants. **B**, Number of nodules formed per plant. (▲) Fusicoccin added to the mineral solution at the time of inoculation. (■) Fusicoccin added 8 h later. (●) Control. Experiments were conducted three times with similar results.

Later, the signal derived from the recognition of Nod factors is transduced and the nodulation process remains unaffected by the presence of the fungal toxin. No effect of fusicoccin addition on the emergence of lateral roots was detected. The effect of fusicoccin on a gene-for-gene pathogen-resistance response (Roberts and Bowles 1999), and a possible mechanistic link between plasma membrane H^+ -ATPase activity and the induction of plant defense genes (Schaller and Oecking 1999) have been recently reported. According to Martínez-Abarca et al. (1998), the gene-for-gene system does not seem to operate when legumes are inoculated with their specific rhizobia. The data presented here are therefore not a consequence of a defense response.

On the other hand, since cytoplasmic calcium spiking is associated with the infection of root hairs of plants treated with specific *S. meliloti* Nod factors (Ehrhardt et al. 1996), the restriction of the passage of Ca^{2+} from the medium into the cell with agents that bind Ca^{2+} , as EGTA, could either arrest or greatly inhibit nodulation. It was found that when plants were grown in a medium containing 1.0 mM EGTA, nodulation was delayed and, moreover, only a very few nodules appeared. Furthermore, the effect of EGTA on nodulation in the presence of fusicoccin was investigated. The results (Fig. 3) indicated that, in the presence of fusicoccin (125 nM), a lower concentration of EGTA was necessary to inhibit nodulation (Fig. 3C,D). Additional evidence in support of a relationship between the activity of the proton pump and the Nod factor-triggered calcium signal was provided by the calcium ionophore A23187, which mimics the responses of the root to Nod factors with striking similarity (Felle et al. 1998). As shown in Table 1, roots of alfalfa responded to 5 μ M A23187 with a clear inhibition of H^+ extrusion. As reported by other authors (Munns 1970; Allen et al. 1994; Eckhardt et al. 1996; Felle et al. 1998), these results also confirm that calcium plays an important role in nodulation signal response. A plausible mechanism to explain the results could be activation of plasma membrane receptors by Nod factors that could lead to Ca^{2+} signaling events that regulate proton pump and ion channel activity. The rapid activation of ion channels could cause membrane depolarization, allowing Ca^{2+} influx from the extracellular space and Ca^{2+} release from intracellular stores (Felle et al. 1998). The increase of cytosolic Ca^{2+} , membrane depolarization, and other signaling events (phosphorylation/de-

phosphorylation of proteins) would mediate the inhibition of the proton pump and a long-term membrane depolarization by which nodulation could be regulated. Because of their role in converting ATP into electrical and pH gradient across the membrane, the activation of H^+ -ATPase and plasma membrane hyperpolarization induced by FC could interfere with the symbiotic signals implicated in nodulation.

MATERIALS AND METHODS

Fusicoccin and EGTA assays.

Ten-day-old alfalfa plants (cv. Aragón), axenically grown in test tubes as described by Olivares et al. (1980), were inoculated up to 10^6 cells $\cdot$ ml $^{-1}$ of the *S. meliloti* strain GR4 (Casadesús and Olivares 1979), from a 24-h culture grown in tryptone yeast extract medium. Fusicoccin (Sigma, St. Louis, MO) at a final concentration of 125 nM was added to the plant growth medium at the same time as bacteria, 8 or 24 h later. Previously, we found that fusicoccin at that concentration did not affect shoot and root plant development. Higher concentrations caused a clear chlorosis. Nodule appearance and number of nodules formed were recorded each 24 h. *S. meliloti* GR4 containing a *nodC-lacZ* fusion (pRmM57) (Mulligan and Long 1985) was grown in minimal medium up to a concentration of 10^9 cells $\cdot$ ml $^{-1}$. Two sets of tubes containing 3 ml of buffered medium at pH 7.2, 6.8, 6.4, 6.0, 5.6, and 5.2 were prepared. The tubes with the three highest pH were inoculated with 10^7 cells $\cdot$ ml $^{-1}$, whereas those with the lowest pHs were inoculated with 10^8 cells $\cdot$ ml $^{-1}$. Luteolin was added to one set of tubes up to 5 μ M and all tubes were incubated for 18 h at 30°C. β -Galactosidase assays were performed as described by Miller (1972) with the same number of cells for each pH. For the assays with EGTA, this compound was added to the mineral solution at the time of inoculation with the bacteria acid at final concentrations of 0.1, 0.5, or 1.0 mM. This assay was performed in both the presence and absence of fusicoccin.

Acid load treatments.

Before the inoculation of the plants prepared as above, the mineral growth medium of one set of tubes (usually 20) was discharged under sterile conditions and pooled for its later use. The solution was substituted by a new one supplemented with

Fig. 2. A, Percentage of nodulated alfalfa plants. B, Number of nodules formed. Roots treated with the following: (■) 3.75 mM acetic acid; (◆) 7.5 mM acetic acid; (▲) 20 mM succinic acid; (●) control; as described in Material and Methods. Experiments were conducted three times with similar results.

20 mM succinic acid (impermeable weak acid) adjusted to pH 5.0 with Tris (extracellular acidification) or by the same acid plus 3.75 or 7.5 mM acetic acid (permeable weak acid) also adjusted to pH 5.0 with Tris. After 30 min, the solutions were thrown out and the roots were washed three times with fresh mineral solution. A third set of tubes was used as the control and treated in the same way as the other two sets except that no acids were added to the mineral medium. The pooled original medium was inoculated with GR4 up to 10^6 cells $\cdot$ ml $^{-1}$, as above, and distributed equally in all the tubes. Nodule appearance and number of nodules formed were recorded every 24 h.

H⁺ extrusion.

For the measurement of H⁺ extrusion by roots a Metrohm pH Stat System (Metrohm, Herisau, Switzerland) was used.

Excised alfalfa roots were kept in a cool 0.2 mM CaSO₄ solution bubbled with air for 30 min and, afterwards, incubated in a thermo-regulated (27°C) Büchner glass. The funnel was mounted on a filter flask and CO₂-free air was pumped through the roots, which were placed in a standard assay medium (40 ml per g fresh weight) containing 50 μ M CaSO₄ and 2 mM K₂SO₄. The apparent H⁺ extrusion was estimated over a 4-h period by the amount of KOH needed in order to maintain the pH at the selected value (6.50 ± 0.02 U) according to Davidian et al. (1984). Fusicoccin or calcium ionophore A23187 (Sigma) was added at a final concentration of 125 nM

or 5.0 μ M, respectively, to alfalfa roots incubated for 1 h in the standard assay medium.

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Fig. 3. Nodulation rate of alfalfa roots when calcium is removed from the mineral solution by the addition of EGTA in the (A, B) absence or (C, D) presence of 125 nM fusicoccin. (●) Control. (◆) 0.1 mM EGTA. (■) 0.5 mM EGTA. (▲) 1.0 mM EGTA. Experiments were conducted three times with similar results.

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