Depolarization of Alfalfa Root Hair Membrane Potential by *Rhizobium meliloti* Nod Factors

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Although much is known about the bacterial genetics of early nodulation, little is known about the plant cell response. Alfalfa root hair cells were impaled with intracellular microelectrodes to measure a membrane potential depolarizing activity in *Rhizobium meliloti* cell-free filtrates, a plant response dependent on the bacterial nodulation genes. The depolarization was desensitized by repeated exposure to factors and was not observed in a representative nonlegume. A purified extracellular Nod factor, NodRm-IV(S), caused membrane potential depolarization at nanomolar concentrations. This rapid single-cell assay provides a tool for dissecting the mechanisms of host cell response in early nodulation.

The bacterium *Rhizobium meliloti* causes changes in the growth and development of cells in its host, alfalfa, that give rise to a new organ, the root nodule. At least two distinct cell types in the alfalfa root respond to this bacterium: inner cortical cells and epidermal root hair cells. In their response, cortical cells de-differentiate, undergo a series of mitotic divisions, and organize into a meristem that forms a mature nodule; root hair cells grow into curled forms before their infection by bacteria. Both of these cell types fail to undergo these responses, however, if the infecting bacteria are mutated in the common bacterial nodulation genes *nodABC* (*1*), which suggests that *nodABC* may be required for the production of a signal or signals (*2*, *3*) that cause the early events of nodulation. A family of molecules has been identified that causes both root hair distortion (*4*, *5*) and cortical cell division (*6*–*9*) and that meets several biochemical and genetic criteria for having essential early nodulation signals (*4*–*6*, *9*, *10*). These Nod factors are oligomers of *N*-acylated, *N*-acetylglucosamine (*R. meliloti* Nod factors are also sulfated) (*4*, *5*, *9*). Although the initial interaction of host and microsymbiont has been studied at the histological and ultrastructural levels, little is known of individual plant cell function at this stage. We measured transmembrane potential change in single infectible root hair cells in response to *Rhizobium* Nod factors. The host responded rapidly to Nod factors with a depolarization of transmembrane potential.

We impaled untreated young root hair cells of whole seedlings with glass microelectrodes and consistently measured large transmembrane potentials in untreated root hair cells (-134 ± 11 mV) (*11*). Preparations were stable up to 90 min. We reasoned that we were measuring potentials across the plasma membrane, rather than across both the tonoplast and plasma membranes for several reasons. For one, young root hair cells are much richer in cytoplasm than many plant cell types (*12*) and are therefore less likely to be impaled through the vacuole. In addition, large negative potentials have been interpreted as cytoplasmic potentials (*13*). Furthermore, when the electrode was advanced farther into the cell, less negative potential was measured. When the electrode was withdrawn to its original position, a large negative potential was again observed, consistent with observations that the vacuole potential is positive relative to that of the cytoplasm in some plant cells (*14*).

Genetically tailored *Rhizobium* was used as a control for the nodulation specificity of membrane potential changes. After a large and stable membrane potential was measured for several minutes in a young root hair cell, the butanol-extractable components (*15*) of a 1021/pRmJT5 cell-free culture filtrate (Nod^+ extract) were applied by means of perfusion. This application caused a slow and often large depolarization of the membrane potential in most cells (Fig. 1A, panels 2 to 4), with a delay of 30 to 60 s between the time extracts first reached root hair cells [estimated by comparison to depolarization onset times to a final concentration of 30 mM KCl (*9*)] and the onset of depolarization. Once initiated, depolarization was slow, taking a mean time of 540 s (range = 330 to 980 s) to reach a maximum response. As with root hair distortion, depolarization activity was absent in extracts prepared from the Nod^- strain TJ1A3/pRmJT5 (Nod^- extract) (Fig. 1A, panel 1). In three experiments, we applied a Nod^+ extract several minutes after application of the Nod^- extract; in all three cases, addition of the Nod^+ extract was followed by a large depolarization that was not caused by the Nod^- extract (Fig. 1A, panel 4). Depolarization responses varied among cells. Mean magnitude was -20 mV, and maximum response was -45 mV (Fig. 1B). As a result of baseline variation over the time scale of these recordings, changes in potential of fewer than 10 mV could not be reliably distinguished from the background.

Membrane potential depolarization induced by Nod factors displayed a characteristic often seen in animal cell signal trans-

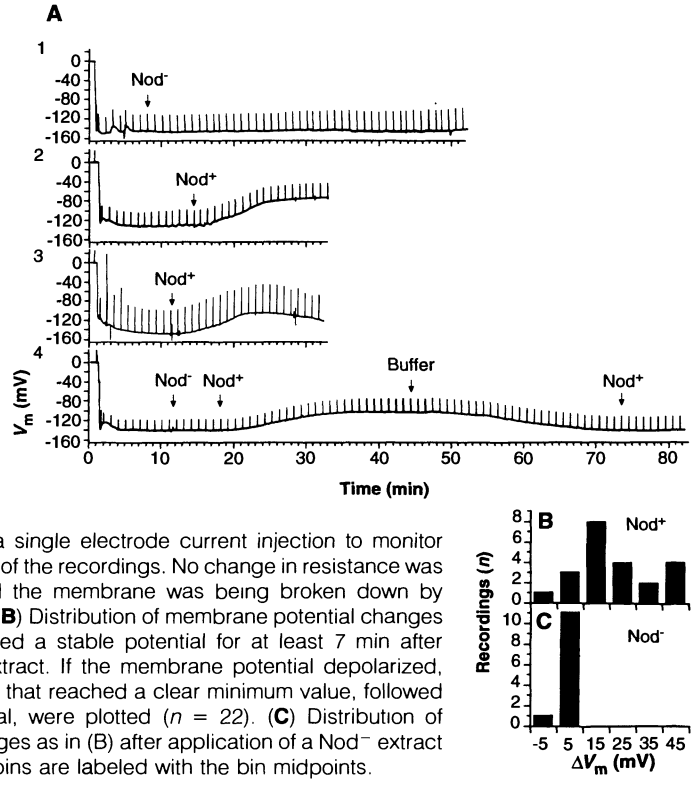

Fig. 1. Membrane potential depolarization of alfalfa root hair cells in response to butanol extracts of cell-free filtrates of *R. meliloti* strains 1021/pRmJT5 (Nod^+) and TJ1A3/pRmJT5 (Nod^-) (*15*). (**A**) Membrane potential of cells under perfusion with Nod^- extract (panel 1), Nod^+ extract (panels 2 and 3), and Nod^- extract followed by Nod^+ extract (panel 4). Extracts were applied at a 10^{-3} dilution of the original culture volume in ARB (*26*). The regularly spaced spikes are the result of a single electrode current injection to monitor changes in the resistance of the recordings. No change in resistance was observed that suggested the membrane was being broken down by exposure to Nod factors. (**B**) Distribution of membrane potential changes for all cells that maintained a stable potential for at least 7 min after application of a Nod^+ extract. If the membrane potential depolarized, only those measurements that reached a clear minimum value, followed by a recovery in potential, were plotted ($n = 22$). (**C**) Distribution of membrane potential changes as in (B) after application of a Nod^- extract ($n = 12$). The histogram bins are labeled with the bin midpoints.

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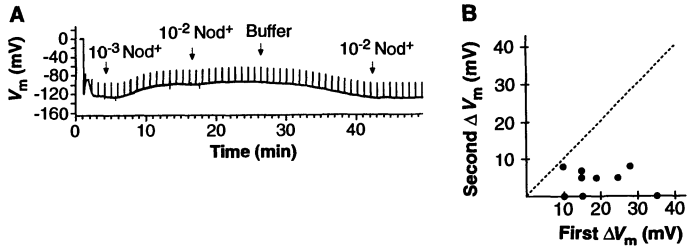

Fig. 2. Desensitization of response to Nod+ culture filtrate extract. (**A**) Membrane potential of a cell challenged with tenfold higher doses of Nod+ extract after an initial 10^{-3} challenge. Nod+ dilution is relative to original culture. (**B**) Change in membrane potential caused by the first application of Nod+ extract plotted against the change in membrane potential caused by a second application. Data are presented for all experiments in which a depolarizing first dose was followed by a recovery of membrane potential and then a second dose of extract was followed by at least 6 min of uninterrupted measurement (n = 9).

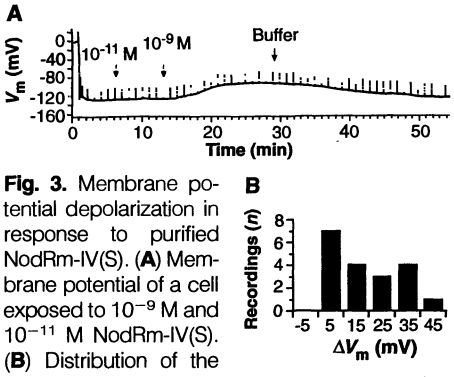

Fig. 3. Membrane potential depolarization in response to purified NodRm-IV(S). (**A**) Membrane potential of a cell exposed to 10^{-9} M and 10^{-11} M NodRm-IV(S). (**B**) Distribution of the changes in membrane potential after perfusion with 10^{-9} M NodRm-IV(S) (n = 19).

duction: root hair cells became desensitized to depolarization with repeated doses of factors. After observing depolarization, we allowed the membrane potential to recover to its initial value, which would occur spontaneously over 25 to 30 min but was hastened by removal of the Nod+ extract (*9*). When cells were exposed to a second dose of Nod+ extract, tenfold larger than the first, they no longer responded with large depolarizations (Fig. 2). This experiment was varied in several ways: factor was presented by perfusion or spot inoculation; varying doses of factor for first and second treatment were tried; and both supernatants and purified factors were presented to the root hair. All trials showed the same lack of response to factor by cells after an initial dose (*9*). Cells impaled this long before a first exposure to Nod+ extract still responded with large depolarizations, so this desensitization is not a result of long impalement times (*9*). As a result of the difficulty in maintaining a stable cell impalement for longer than 60 to 90 min, the refractory time for desensitization was not measured.

Although depolarization activity of filtrate extracts was dependent on the *nod* gene, the extracts were a complex mixture of molecules. To discover whether depolarization was caused by the same extract components that cause root hair distortion, we applied both 10^{-11} M and 10^{-9} M pure NodRm-IV(S) (*4*) to alfalfa roots by perfusion. Only 1 cell in 15 responded to 10^{-11} M NodRm-IV(S) with significant (>9 mV) depolarization, although 12 of 19 cells depolarized upon exposure to 10^{-9} M NodRm-IV(S) (Fig. 3). This latter concentration is in the range required for root hair branching activity (*4*). The pattern of depolarization magnitudes at this concentration (Fig. 3), and the kinetic profiles of the response, were similar to those obtained with the raw filtrate extract. We have found molecules similar in structure to NodRm-IV(S) in this filtrate extract that cause both root hair distortion and cortical cell division (*9*).

Membrane potential depolarization could be caused by a number of mechanisms that may not function in early nodulation. An important criterion for specificity of any host response to bacteria or bacterial factors is host selectivity, as only particular bacteria can nodulate particular hosts. One of the broadest limitations is the confinement of *Rhizobium* nodulation to members of the legume family (*16*). If the response we measured was specifically relevant to nodulation, it should not occur in a plant that does not nodulate. Such a plant, however, should still be responsive to common plant growth regulators and transported solutes. We therefore chose tomato, a well-characterized plant with accessible root hairs, as a representative nonhost control. We applied Nod factors prepared by high-performance liquid chromatography (HPLC) fractionation of Nod+ extracts (*17*) to young alfalfa and tomato root hairs. Because we had limited quantities of fractionated factors, we used a spot application technique to conserve material (*18*). Alfalfa root hairs showed a pattern of depolarization responses similar to that seen under perfusion, whereas the tomato root hairs failed to exhibit significant changes in membrane potential in all 17 cells measured (Fig. 4). Because the depolarization activity in Nod+ extracts did not extend to a representative nonhost, it is therefore not likely to be a result of a mechanism that is shared by all plant species.

Other characteristics of the depolarization response also support its specificity to

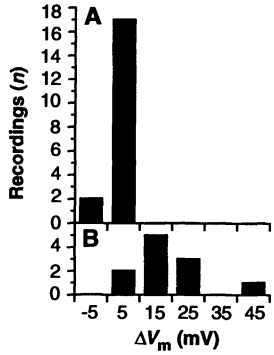

Fig. 4. Membrane potential response of (**A**) tomato (n = 19) and (**B**) alfalfa (n = 11) root hair cells (*17*) to HPLC-fractionated nodulation factors (*18*). Extract fractions (×2 culture concentration) were applied to the space immediately surrounding the hair cell by means of a micropipette.

nodulation. Because root hair cells exhibit sensitivity in nanomolar concentrations to purified NodRm-IV(S), it is unlikely that this depolarization could be a result of simple cotransport mechanisms, as hypothesized for depolarization with solutes such as sucrose. For example, it takes 10 μM sucrose to cause measurable depolarization in soybean cotyledon cells (*19*). The desensitization to Nod factors exhibited by root hair cells is also inconsistent with depolarization patterns described for cotransported solutes (*19*). These two features also argue against the possibility that the depolarization is caused by nonspecific disruption of the membrane structure, an initial concern because of the amphipathic nature of NodRm-IV(S).

The ionic redistributions we have observed, which result in membrane potential depolarization, may merely accompany a cellular signaling event or may play a mechanistic role in nodulation. One possible role for an ionic redistribution might be alteration of cell-wall growth in root hair cells. Changes in pH and extracellular Ca^{2+}, for example, alter cell wall extensibility (*20*). Another possibility is that ion changes may occur as part of secondary signaling mechanisms. Ionic fluxes mediate or accompany many plant cell responses to hormonal and environmental signals (*21*). In root hair cells, a secondary signal may couple Nod factor perception to cell growth mechanisms or to other functions needed for the root hair cell to interact productively with *Rhizobium*. It is also possible that a secondary signal is needed to communicate with the inner cortical cells.

Depolarization of plant cell membrane potential is also a response to bacterial pathogens (*22*) and to oligosaccharide elicitors prepared by enzymatic digestion of fungal cell walls (*23*); these oligosaccharides may serve as plant defense signals

(*24*). Given that *Rhizobium*-plant interactions may be modified pathogen-host relationships (*25*), it is interesting to note that NodRm-IV(S) is essentially a modified chitin tetramer. The optical and mechanical accessibility of root hairs, together with the possible use of bacterial genetics to modify signal molecules, will provide new opportunities to study the mechanism of action of these oligosaccharide signals.

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11. Alfalfa seeds (AS13, Ferry-Morse Seed, Modesto CA) were surface-sterilized and germinated as described (*26*). Seedlings (24 hours old) were transferred to the surface of square petri plates that contained buffered nodulation medium (BNM) (*27*) and 12% Bacto agar (Difco Laboratories, Detroit, MI). The plates were sealed with Parafilm, and the plants were grown for 2 days at 29°C under a cycle of 16 hours of light and 8 hours of dark. Whole seedlings were mounted in a custom perfusion chamber, and the zone of emerging root hairs was suspended in a 250-μl chamber. The chamber was perfused with alfalfa recording buffer (ARB) (1 ml/min). The root tips were excised halfway between the root cap and the point of root hair emergence to prevent movement of the root as a result of gravitropic bending. Because this procedure might interfere with nodule initiation, we spot-inoculated *R. meliloti* onto the root hair elongation zone at the same time. Nodules developed at the same frequency and emerged at the same time as they did on unexcised controls (*9*). After a 20-min recovery period under perfusion with ARB, emergent hair cells were impaled with a Prior (Stoelting, Wood Dale, IL) micromanipulator and a Wild (Technical Instrument Co., San Francisco) dissecting microscope at ×50 magnification. The equipment was set up on a vibration-resistant concrete table inside a Faraday cage. Glass microelectrodes were pulled from filamented glass capillaries (World Precision Instruments) on a Flaming/Brown micropipette puller (Sutter, model P-87). The final outer diameter of the tips was approximately 0.2 mm as measured by scanning electron microscopy. Pipettes were back-filled with 3 M KCl and placed in holders with a sintered Ag-AgCl pellet (World Precision Instruments). The reference electrode was connected to the recording bath with an agar bridge. Electrical potential was measured with a Getting model 5 amplifier, and the output was filtered through a six-pole Bessel filter at a 10-Hz corner frequency (Frequency Devices, Model 902LPF) and sent to an oscilloscope (Tektronix Instruments, Santa Clara, CA, model 5111A) and a chart recorder (Gould Electronics, Haywood, CA, model 2400S). When filled with 3 M KCl and placed in ARB, the electrodes had a resistance of approximately 30 megohms.
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17. Tomato and alfalfa seeds were surface-sterilized and germinated on 12% water agar in inverted petri plates. After 2 days of growth, they were placed on the surface of a thin layer of solidified 0.6% agarose-BNM (2 ml) that was spread over the surface of a glass microscope slide. The radicles were covered with Spectra/Por6 dialysis membrane, and the slides were placed in 50-ml conical screw-cap tubes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) with 5 ml of BNM. After 2 days of growth, the dialysis membrane was removed, and the slides were placed in chambers under 1 mm of ARB.
18. Extracts were fractionated by HPLC on a Hewlett-Packard 1090M with a modified protocol (*28*). Samples were injected on a C_{18} reverse-phase column with a 2-mm inside diameter and eluted at 0.2 ml/min with a 20-min isocratic elution with 20% acetonitrile, followed by a 10-min gradient to 50% acetonitrile, and finally with a 15-min isocratic elution with 50% acetonitrile. Eluted species were detected by ultraviolet (UV) absorption at 215 nm. Fractions that contained the unique UV-absorbing peaks in 1021/pMH36 extracts were collected and pooled. The corresponding fractions were also collected from TJ1A3/pMH36 extracts.
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27. The BNM was composed of 2 mM Mes, 2 mM $CaSO_4 \cdot 2H_2O$, 0.5 mM KH_2PO_4, and 0.5 mM $MgSO_4 \cdot 7H_2O$, with the minor salts 50 nM Na_2EDTA, 50 nM H_3BO_3, 50 nM $FeSO_4 \cdot 7H_2O$, 50 nM $MnSO_4 \cdot H_2O$, 16 nM $ZnSO_4 \cdot 7H_2O$, 1 nM $Na_2MoO_4 \cdot 2H_2O$, 0.1 nM $CoCl_2 \cdot 6H_2O$, and 0.1 nM $CuSO_4 \cdot 5H_2O$. KOH (approximately 1.2 mM final concentration) was added to adjust the pH to 6.0. The ARB is the same composition but does not have the minor salts.
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29. We thank R. Scheller for equipment; J. Schroeder, E. Serrano, and B. Lucas for advice; K. Faull for assistance with HPLC and mass spectroscopy; and J. Dénarié for purified NodRm-IV(S). Supported by Department of Energy contract grant DE-A503-82ER12084 (S.R.L.), a National Science Foundation Predoctoral Fellowship (D.W.E.), and an NIH training grant in molecular biology awarded to Stanford University (E.M.A.).

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Paleotemperatures in the Southwestern United States Derived from Noble Gases in Ground Water

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A paleotemperature record based on measurements of atmospheric noble gases dissolved in ground water of the Carrizo aquifer (Texas) shows that the annual mean temperature in the southwestern United States during the last glacial maximum was about 5°C lower than the present-day value. In combination with evidence for fluctuations in mountain snow lines, this cooling indicates that the glacial lapse rate was approximately the same as it is today. In contrast, measurements on deep-sea sediments indicate that surface temperatures in the ocean basins adjacent to our study area decreased by only about 2°C. This difference between continental and oceanic records poses questions concerning our current understanding of paleoclimate and climate-controlling processes.

Paleoclimate records for the oceans and the continents during the last glacial maximum are inconsistent. The observation that the 0°C isotherm on mountains from almost all geographic settings and latitudes in both the Northern Hemisphere and the Southern Hemisphere dropped by about 950 m during the peak of the last glacial suggests that the temperature was lowered by 4.2° to 6.5°C at elevations between 3 and 5 km (*1*). On the other hand, paleoclimatic information derived from oxygen isotope measurements (on foraminifera) and faunal abundances indicate that most of the low-latitude ocean surface cooled by less than 2°C. No process has yet been identified that could cause marked high-

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