Nodulin 26, a Nodule-specific Symbiosome Membrane Protein from Soybean, Is an Ion Channel*

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Nodulin 26 is an integral symbiosome membrane protein of nitrogen-fixing soybean nodules. Nodulin 26 is a member of a family of structurally homologous membrane proteins with diverse transport functions. Thus, it has been proposed to be involved in symbiosome membrane transport. Despite this claim, there has not been any evidence that nodulin 26 has a transport activity. In this study, nodulin 26 was purified from soybean nodules by a non-denaturing protocol and was reconstituted into liposomes for channel studies in planar lipid bilayers. Nodulin 26 readily incorporated into bilayers, forming single channels with a maximum unitary conductance of 3.1 nanosiemens (nS) in a recording buffer of 20 mM 3-(N-morpholino)propanesulfonate-NaOH, pH 7.4, 1 mM KCl. Nodulin 26 also exhibited multiple, discreet lower conductance states ranging from 0.5 to 2.5 nS. Nodulin 26 channels were voltage-sensitive. The maximal 3.1-nS state was preferentially occupied at lower applied voltages, whereas the lower conductance states were more frequently occupied at higher voltage potentials. Nodulin 26 channels transported both cations and anions, but showed a weak selectivity for anions. These results represent the first purification and functional characterization of the nodulin 26 channel and support a role for this protein in symbiosome membrane transport.

EXPERIMENTAL PROCEDURES

Isolated soybean nodule SM (1 mg of protein) (14) were resuspended in 10 ml of 7.5 mM sodium phosphate, pH 7.5, 1 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1 mM pepstatin. The sample was incubated for 30 min at room temperature and was centrifuged at 100,000 x g for 1 h at 4 °C. The supernatant fraction was discarded, and the pellet was rinsed twice with 5 ml of 10 mM sodium phosphate, pH 7.9, 2% (w/v) 1-O-n-octyl p-D-glucopyranoside (OG), 1 mM NaN3, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1 mM pepstatin. The sample was incubated overnight at 4 °C and was centrifuged at 100,000 x g for 1 h at 4 °C. The supernatant fraction was applied to a DEAE Fast Flow column (1.8 ml) attached to a Pharmacia fast protein liquid chromatography system. The column was washed with 20 ml of 10 mM Tris-HCl, pH 8.0, 1% (w/v) OG and then with 20 ml of 20 mM MES-NaOH, pH 6.2, 1% (w/v) OG. The column was eluted with a 30-ml linear gradient (0–0.4 mM NaCl in 20 mM MES-NaOH, pH 6.2, 1% (w/v) OG. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis (19) and immunoblotting with nodulin 26-specific antibodies (11). Fractions containing purified nodulin 26 were combined and concentrated to 0.2 mg/ml with a Centricon-10 ultrafiltration device (Amicon). Protein was determined by the bicinchoninic acid method (20).
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Fig. 1. Analysis of purified nodulin 26. 15% (w/v) SDS-polyacrylamide gels of A, purified SM (20 µg protein); B, purified nodulin 26 (5.0 µg protein); and C, purified nodulin 26 (0.5 µg protein) blotted onto nitrocellulose and probed with anti-nodulin 26 antibodies. The proteins in lanes A and B were stained with Coomassie blue.

(100 µg) was added to 3.6 µmol of soybean phosphatidylcholine (Avanti Polar Lipide) dissolved in 470 mM OG, 200 mM NaCl, 4 mM Tris base, 5 mM EDTA, 1 mM CaCl₂, 1.5 mM NaN₃. This ratio of nodulin 26 to lipid was chosen since it was likely to yield single channel incorporations based on previous work with MIP (4). The sample was dialyzed against 3 liters of 25 mM TES-NaOH, pH 7.0, 200 mM NaCl to remove residual detergent. The buffer was changed every 24 h over a 4-day period.

To address whether nodulin 26 possesses channel activity it was necessary to purify the protein from isolated SM (Fig. 1). SM were washed with 1 M KI to remove extrinsic membrane proteins and nodulin 26 was solubilized with OG. Solubilized nodulin 26 was isolated by fast protein liquid chromatography ion-exchange chromatography on DEAE Fast Flow. SDS-polyacrylamide gel electrophoresis of the final product showed only a single band with an apparent molecular weight of 27,000 (Fig. 1B). The identity of the purified protein as nodulin 26 was confirmed by immuno-blotted using nodulin 26-specific antibodies (Fig. 1C).

Purified nodulin 26 was reconstituted into liposomes and its ion channel activity was tested with a standard 1 M KCl recording solution (Fig. 2). Addition of nodulin 26 liposomes to planar lipid bilayers resulted in the appearance of channels, usually within 30 min (Fig. 2A). In contrast, in control experiments, addition of an equivalent amount of nodulin 26-free liposomes did not result in changes in bilayer conductance, even after 3 h of incubation. Addition of a large excess of control liposomes under conditions favoring vesicle fusion yielded nonspecific currents resulting from the disruption of the bilayer (data not shown). However, the control liposomes never produced channels with the characteristic conductance or gating properties observed with the nodulin 26 liposomes. Therefore, the channels we discuss below are due to the incorporation of nodulin 26 into the bilayer.

Ten out of 12 channel incorporations evaluated yielded a maximum conductance of 3.1 nS, and likely represent single channel incorporation (Fig. 2A). This conductance was derived from a current-voltage plot of the maximum current amplitude at each voltage (Fig. 2C). The other recordings showed much higher current amplitudes and probably resulted from the incorporation of multiple nodulin 26 channels.

Multiple conductance states are apparent in the single channel records of nodulin 26 (Fig. 2). Current-voltage relationships for the 3.1- and 1.6-nS conductance states were linear over the range of -100 to +100 mV, and the measured reversal potentials were near zero under standard conditions (Fig. 2C). Additional conductance states were identified from current-amplitude histograms determined at several voltages. Assuming a linear relationship (conductance = current/voltage, as determined for the 3.1- and 1.6-nS states), the most frequently observed conductance states were calculated to be 0.5, 1.1, 1.6, 1.9, 2.5, and 3.1 nS (Fig. 2).

The nodulin 26 channel exhibited variable open dwell times that ranged from 1 to 50 ms (Fig. 2A). Complete channel closures were rare, and were more often observed at higher voltages (60–100 mV) than at lower voltages (<40 mV). Channels typically exhibited transitions between the various open conductance states, and the occupancy of these states was voltage dependent (Fig. 3). At 30 mV, the channel occupied the 3.1-nS state 76% of the time, with infrequent transitions to the lower conductance states. In contrast, at 70 mV the channel occupied the 3.1-nS state only 7.2% of the time, and greater occupancy of the lower conductance states was observed. These data indicate that nodulin 26 forms a voltage-sensitive channel with lower conductance states preferentially occupied at higher voltage potentials.

To determine the specificity of nodulin 26 channels, the charge and size of the conducted ions were investigated. Changing the KCl concentration of the trans chamber solution from 1 to 0.1 M resulted in a shift in the reversal potential to -4 mV (data not shown). This shift corresponds to a permeability ratio \( P_{K^+}/P_{Cl^-} \) of 1:1.21, suggesting only a slight selectivity for anions under these conditions. After exchange of the trans chamber solution with 1 mM Tris-HEPES, pH 7.4, the reversal potential was shifted to -12 mV, indicating only a small (1.6-fold) selectivity for either K⁺ versus Tris, or for Cl⁻ versus...
HEPES (Fig. 4). In contrast to the small reversal potential shift, a large decrease in conductance was observed, from 3.1 to 0.5 nS at negative voltage potentials (i.e. trans side conductance) and from 3.1 to 1 nS at positive voltage potentials (i.e. cis side conductance) (Fig. 4). Thus, the larger Tris and HEPES ions appear to block the full opening of the nodulin 26 channel. Further, the observation that the trans side conductance (representing the current carried by the Tris ion from the trans to the cis side) is decreased more than the cis side conductance, suggests that Tris may be a more effective channel blocker than HEPES.

**FIG. 2.** Single channel recordings of purified, reconstituted nodulin 26. Channel recordings were done in symmetrical 1 mM KCl, 20 mM MOPS-NaOH, pH 7.4. A, representative channel traces. The holding potential was 70 mV. Channel openings are shown as upward deflections; -C on the right side of the traces indicates the closed (zero current) position. Arrows indicate conductance levels corresponding to the labeled conductance amplitude histogram peaks in Panel B. B, amplitude histogram for nodulin 26 channels showing principal conductance states. The ordinate represents the total number of events recorded. The events were not weighted according to the duration of the conductance state. C, current voltage relationships of the 3.1- and 1.6-nS conductance states. Single current amplitudes were determined from pulsed potentials to the indicated voltage.

**DISCUSSION**

In this report we have purified nodulin 26 from soybean symbiosome membranes and have shown that it forms ion-conducting channels with a maximum unitary conductance of 3.1 nS when incorporated into planar lipid bilayers. These channels exhibit voltage sensitivity with a tendency to occupy lower conductance states at high voltage potentials (>60 mV). In addition, reversal potential measurements suggest that nodulin 26 shows only a slight preference for Cl⁻ over K⁺. However, when Tris-HEPES was exchanged with KCl there was a reduced level of conductance compared to the symmetrical KCl conditions, suggesting a potential relationship between ion size and conductance state. Overall, the data provide the first evidence that nodulin 26 is a membrane channel, and suggest that nodulin 26 channels have a high conductance and low ion selectivity.

These findings are of interest with respect to the relationship of nodulin 26 to other MIP family members. The data suggest that nodulin 26 shares the greatest functional similarity with lens fiber MIP (4). Similar to the nodulin 26 channel, MIP forms single channels with high conductance (approximately 3 nS), exhibits lower conductance states that are preferentially occupied at higher voltage potentials, and shows a weak selectivity for anions (4). Studies with other family members suggest that several have functions that are distinct from nodulin 26 and MIP. For example, the channel-like intrinsic protein of M. 28,000 (CHIP281 from erythrocytes and the y isoform of the tonoplast intrinsic protein from plants form highly specific water channels (6–9). Ion conductances have not been observed for either of these proteins when assayed by voltage clamp after expression in Xenopus oocytes (6, 9) or after reconstitution of purified proteins into liposomes (7). The glycerol facilitator protein of Escherichia coli also appears to be distinct from nodulin 26 and MIP since it shows a transport preference for neutral straight chain polyols such as glycerol (5, 23).

An important physiological question that remains is the function of nodulin 26 in the SM. Three major ion transport activities have been described in soybean SM (reviewed in Ref. 13): 1) a C₂-dicarboxylate transporter which supplies organic acids, such as malate, to the rhizobia bacteroid to provide energy for nitrogen fixation (24–28); 2) an electrogenic H⁺-pumping ATPase (27, 29); and 3) a general anion transport system that is coupled to the H⁺-pumping ATPase (27). In preliminary experiments, we have found that the exchange of 0.1 M potassium malate for KCl in the trans chamber did not result in any significant change in nodulin 26 channel activity (data not shown). This suggests that malate may be transported as well as Cl⁻ by the nodulin 26 channel, supporting the previous suggestion that nodulin 26 may be responsible for dicarboxylate transport across the SM into the symbiosome space (13). However, in addition to dicarboxylate transport, the SM shows a wide range of ion transport activities that are coupled to the H⁺-pumping ATPase (27, 29). Thus, it is also possible that nodulin 26 has a broader function in symbiosome transport, and could supply other metabolites and nutrients to the bacteroid.
**Fig. 3. Voltage sensitivity of nodulin 26 channels.** Nodulin 26 channel conductance amplitude histograms corresponding to channels recorded in symmetric 1 mM KCl, 20 mM MOPS-NaOH, pH 7.4, at potentials of A, 30 mV and B, 70 mV are shown. Data was derived from 25 1-s sweeps similar to those shown to the right of each histogram. The ordinate represents the number of events recorded. The TRANSIT program (22) was used to weight the events by the duration of the occupancy of the indicated conductance states.

**Fig. 4. Current-voltage curves of nodulin 26 channel in Tris-HEPES.** Representative traces of a nodulin 26 channel recorded with the ramp protocol described under "Experimental Procedures" are shown. The recording solution was symmetrical 1 mM KCl, 20 mM MOPS-NaOH, pH 7.4 (trace 1), or cis 1 mM KCl, 20 mM MOPS-NaOH, pH 7.4, trans 1 mM Tris-HEPES, pH 7.4 (trace 2).

Another consideration with respect to the endogenous nodulin 26 channel activity is the effect of transmembrane voltages. The SM H^+-ATPase pump generates a significant ΔΨ in isolated symbiosomes (27, 29). Based on previous observations, transmembrane potentials across plant membranes can be extremely high, exceeding 100 mV (reviewed in Ref. 34). Because of the sensitivity of the nodulin 26 channel to high voltage potentials, the endogenous potential across the SM needs to be evaluated to determine whether this could control the permeability of the nodulin 26 channel.

Nodulin 26 is phosphorylated on serine 262 within the hydrophilic COOH-terminal domain in a calcium-dependent manner by a novel protein kinase (CDPK) found on the SM (11, 14). There are numerous precedents for the modulation of ion channel properties by phosphorylation (30). Of particular note is the finding that MIP is phosphorylated by cAMP-dependent protein kinase (31) and that phospho-MIP channels exhibit enhanced voltage sensitivity compared to unphosphorylated MIP (32). This may be particularly relevant considering the apparent similarities in the channel properties of MIP and nodulin 26, and the observation that the residue on MIP phosphorylated by cAMP-dependent protein kinase is in a location similar to the residue on nodulin 26 phosphorylated by CDPK (14, 33). A thorough examination of the specific functional effects of phosphorylation of nodulin 26 on serine 262 needs to be done. Hopefully, this will shed light on the function of nodulin 26 in the SM, and how its activity is modulated by calcium-dependent phosphorylation.

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**REFERENCES**

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