G Proteins Regulate Dihydropyridine Binding to Moss Plasma Membranes*

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The role of calcium as an activator and regulator of many biological processes is linked to the ability of the cell to rapidly change its cytoplasmic calcium levels. Calcium acts as an intracellular messenger in hormone-induced bud formation during the development of the moss Physcomitrella patens. Calcium transport and ligand binding studies have implicated plasma membrane-localized 1,4-dihydropyridine (DHP)-sensitive calcium channels in this increase in cellular calcium. To understand the regulation of the moss calcium channel, we investigated the involvement of GTP binding regulatory proteins (G proteins). Guanosine 5'-dithiodiphosphate (GTPγS), a nonhydrolyzable GTP analog that locks G proteins into their active state, stimulated DHP binding to high affinity receptors in the moss plasma membrane. DHP binding was measured as the ability of the DHP agonist Bay K8644 or the DHP antagonist nifedipine to compete with the DHP arylazide [3H]azidopine for binding to moss plasma membranes. G protein stimulation of binding was seen when competition was carried out with either nifedipine or Bay K8644. G proteins regulated the rates of association and dissociation of bound [3H]azidopine, and stimulation was dependent on GTPγS concentration. Guanosine 5'-dithiodiphosphate, a GDP analog that locks G proteins into their inactivated state, did not affect the dose dependence of either the agonist or the antagonist. These results suggest that G proteins may act via a membrane-delimited pathway to regulate calcium channels in the moss plasma membrane.

The growth and development of plants are strongly influenced by physiological and environmental signals. The pathway from these chemical or physical cues to a new developmental program involves biochemical and molecular changes in the cell. An important component in many of the signal transduction pathways in plants is a controlled change in cellular calcium concentrations through the coordination of passive fluxes and active transport across organelar and plasma membranes (1–3). One example of a dramatic developmental change that is modulated by calcium is the formation of buds during moss development (4–9). Early in moss development, a single cell spore germinates to form a thread-like protonema composed of a single cell type, the chloronema. Increased production of the hormone auxin induces divisions in the chloronema tip cell that give rise to a completely different cell type, the caulonema.

Accumulation of the hormone bryokinin, an adenine-type cytokinin, induces development of buds from single initial cells that form on most caulonema cells (10). Formation of buds is an integral part of the moss life cycle; it leads to the development of the mature gametophyte and subsequent sexual reproduction.

In vivo bud formation, calcium transport, and ligand binding studies implicated 1,4-dihydropyridine (DHP)3-sensitive calcium channels in the increase in cellular calcium during moss development. In moss protonemata, application of DHP calcium channel agonists blocked cytokinin-induced bud formation (8). Increases in calcium influx into moss protoplasts were seen within 15 s of addition of cytokinin, and calcium influx was inhibited by DHP antagonists and stimulated by DHP agonists (11). DHP-sensitive binding of the DHP arylazide [3H]azidopine to purified moss plasma membranes was found to be cytokinin-dependent (12). While physiological events implicating calcium in hormone-induced bud formation have been described, the biochemical pathways that link signal perception to channel regulation remain largely unknown.

In animal cells, G proteins have been implicated in the direct regulation of calcium channels. G proteins are αβγ-heterotrimers that share a common set of βγ-dimers and differ in the composition of their α-subunits. The α-subunits are similar yet distinct gene products that bind and hydrolyze GTP and act on specific effectors. These plasma membrane-localized regulatory G proteins transduce extracellular signals into intracellular events by coupling to membrane receptors (13); voltage-dependent calcium channels are common targets for direct G protein coupling independent of cytoplasmic factors (14–16). Studies of the effect of G proteins on DHP binding indicate a role for G proteins in the response of calcium channel ligand binding to the channel in its resting state (17, 18).

Evidence is accumulating for the presence and function of G proteins in plants. Plant G protein homologs have been identified in protein binding assays using radiolabeled GTP, antiserum to animal G proteins, or toxin-mediated ADP-ribosylation of G proteins using radioactive NAD (19). Genes encoding α-subunit homologs have also been isolated from a number of plants (20, 21). At the physiological level, G proteins have been implicated in phytochrome-induced chloroplast development and anthocyanin biosynthesis in tomato and in the regulation of stomatal aperture in Vicia faba guard cells (22–24). In guard cells, inward rectifying K+ currents were enhanced with G protein inhibitors and inhibited with G protein activators, cholera toxin, and pertussis toxin (22, 23).

To understand the regulation of calcium levels during moss

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1 The abbreviations used are: DHP, 1,4-dihydropyridine; bis-tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; GTPγS, guanosine 5'-dithiodiphosphate; GDPβS, guanosine 5'-(β-thio)diphosphate; GMP-PNP, 5'-guanylylimidodiphosphate.

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bud formation, we hypothesized that G proteins regulate calcium channels in the moss plasma membrane. In this study, we investigated whether the in vitro addition of stable GTP analogs activates a G protein in purified plasma membranes of the moss Physcomitrella patens. To do this, we determined if G proteins interact with DHP receptors, thereby altering the ability of DHP agonists and antagonists to bind to their recognition sites. We report evidence for G protein modification of DHP binding to calcium channel receptors in the moss plasma membrane. Our results suggest that G proteins may regulate calcium channels in moss and may do so via a membrane-delimited (cytoplasm-independent) pathway.

EXPERIMENTAL PROCEDURES

Plant Material—P. patens (Hedw.) Br. Eur. was cultured and grown aseptically using a modified Knop's medium solidified with 1.5% (w/v) agar (basal medium) (25). Plants were grown at 25°C under continuous white fluorescent light (45–50 microeinsteins/m² s⁻¹). Petri dishes containing appropriately supplemented basal medium overlaid with sterile cellophane were inoculated with spore suspensions. To prepare spore suspensions, capsules were washed by successive steps with sterile distilled water (10 ml), 2% (w/v) sodium hypochlorite, 0.1% Triton X-100 (10 ml) for 10 min with occasional swirling. The capsulizes were washed four times with sterile distilled water (10 ml each) and resuspended in 10 ml of sterile distilled water. Capsules were opened with sterile forceps, and plates were inoculated with 1 ml of sterile spore suspension (4 × 10¹¹ viable spores). After inoculation at room temperature for 2 weeks, protonemal tissue was harvested and ground in sterile water (1 g ml⁻¹) with a tissue homogenizer (Tissue Tearor, Biospec Products, Inc., Bartlesville, OK) for 2 min at 11,000 rpm. Ground tissue (0.25 g ml⁻¹) was transferred to sterile flasks containing 50 ml of Gottwald's medium (26). Flasks were incubated at room temperature under fluorescent lights (as described above) on a rotary shaker for 3 weeks at 160 rpm. The tissue was then subcultured by transferring 9 ml aliquots into flasks containing 50 ml of Gottwald's medium. Cultures were incubated for an additional 2 weeks, and the tissue was harvested, weighed, (yield of ~1 g of tissue/flask), and used for membrane isolation.

Plasma Membrane Isolation—All procedures were conducted at 4°C. Moss vegetative tissue (25–60 g) was homogenized by mortar and pestle in a medium containing 250 mM sorbitol, 3 mM EGTA, 25 mM Hepes-bis-tris propane (pH 7.4), 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, 0.01% (v/v) bovine serum albumin (fatty acid-free), and 0.2% bovine serum albumin (fatty acid-free), and 0.25 g/g of fresh weight polyvinylpyrrolidone. The homogenate was filtered through cheesecloth, the tissue was homogenized again in 1 ml of the homogenization medium, and the resulting homogenate was washed washed four times with sterile distilled water (10 ml) for 10 min with occasional swirling. The homogenate was centrifuged for 15 min at 13,000 × g, and the resulting supernatant was centrifuged for 90 min at 16,000 g (Beckman SW 28 rotor, rₕmax). The resulting pellet (crude microsomal fraction) was resuspended in 250 mM sorbitol, 2.5 mM Hepes-bis-tris propane (pH 7.2), and 1 mM dithiorthreitol (resuspension buffer). The suspension (1.1 ml) was layered over a two-step (6 and 12%, w/v) dextran gradient (5 ml each) prepared in resuspension buffer. After centrifugation for 2 h at 70,000 × g (Beckman SW 28.1 rotor, rₕmax), a turbid band at the 6–12% dextran interface was collected and is referred to as plasma membrane-enriched vesicles.

Protein Determination—Protein was determined by the method of Lowry et al. (27) with bovine serum albumin as the standard.

[^3H]Azidopine Binding Assays—A filtration assay was used to measure G protein regulation of DHP binding. Membranes were incubated at 20°C in a solution containing 20 mM Hapes-NaOH (pH 7.5), 0.1 mM phenylmethylsulfonyl fluoride, 5 mM KCl, 1 mM CaCl₂, 0.01% Triton X-100, and 10 nM[^3H]azidopine at a final protein concentration of 0.066 mg/ml. Incubations were stopped by rapid filtration of reaction aliquots (usually 450 μl) under reduced pressure through glass fiber filters (Whatman GF/C). The filters were immediately washed twice with 5 ml of an ice-cold solution containing 100 mM Tris-HCl (pH 7.5) and 0.1% bovine serum albumin. Duplicate experiments were systematically carried out using at least three different membrane preparations. Ten-μl samples of the incubation mixture were taken for measurement of the total amount of[^3H]azidopine present, and radioactivity was measured by scintillation counting. All experiments were carried out under dim light due to the light sensitivity of the DHPS. Solvent controls were included in all assays where necessary. The glass fiber filters used for filtration were presoaked for at least 1 h in 0.3% polyethyleneimine. Under these conditions,[^3H]azidopine bound to filters in the absence of membranes and could be displaced by nonradioactive ligand. Control experiments without vesicles were conducted, and the binding data were corrected for nonspecific filter interactions.

Nonspecific binding, measured by preincubation of membranes with a 100 μM concentration of the DHP antagonist nifedipine or with a 1 μM concentration of the DHP agonist Bay K8644 (methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine 5-carboxylate) on ice for 60 min, was <10% of total binding at a 10 nM concentration of ligand. Nonspecific binding was subtracted from total binding to yield specific binding. Variations of binding conditions for individual experiments are indicated in the appropriate figure and table legends. For determination of the effect of guanine nucleotide analogs on the association of DHP with its receptor, membranes were incubated with plasma membranes for 5 min at room temperature prior to the addition of[^3H]azidopine. For determination of the effect of guanine nucleotide analogs on the disassociation of DHP from its receptor, 10 nM[^3H]azidopine was first incubated with plasma membranes for 60 min on ice. Dissociation was initiated by adding excess unlabeled ligand (100 μM nifedipine or 1 μM Bay K8644) and filtering aliquots at the appropriate time.

Pseudo first-order plots of ln(Bt/B₀) versus time, where B₀ is the amount of specifically bound[^3H]azidopine at assay time and Bₜ is the equilibrium-specific binding of[^3H]azidopine (28). Dissociation rate constants (k₁) were determined by measuring the amount of labeled ligand-receptor complex remaining at various times after the addition of excess unlabeled ligand, and the data were analyzed using linear regression of first-order plots of ln(Bt/B₀) (28).

Materials—[^3H]Azidopine (70–90 Ci/mmol) was purchased from DuPont NEN. Unlabeled nifedipine and (+)-Bay K8644 were purchased from Calbiochem-Novabiochem Corp. Guanine nucleotide analogs were purchased from Sigma. All other chemicals were of the highest quality available from commercial sources.

RESULTS

Effects of Guanine Nucleotides on DHP Binding—G proteins have been implicated in the direct regulation of calcium channels in animal cells (13). Under these conditions, where regulation is independent of changes in intracellular second messengers, the binding of GTP may alter the interaction of ligands with their receptors (18, 29). To determine the role of G proteins in the regulation of the calcium channel in moss, we have investigated the effects of guanine nucleotide analogs on the properties of DHP binding to moss plasma membranes.

In the studies presented here, the binding of unlabeled DHPs to receptors in moss plasma membranes was measured by their ability to compete with the binding of the labeled DHP antagonist[^3H]azidopine. The amount of[^3H]azidopine specifically bound when 10 nM was incubated with 0.066 mg/ml membrane protein was increased by the presence of 1 μM BayK8644 or with a 1 μM concentration of the DHP antagonist nifedipine (Fig. 1A) or the DHP agonist Bay K8644 (Fig. 1C). The association reaction of[^3H]azidopine with moss DHP receptors reached a steady state within 30 min when either nifedipine or Bay K8644 was used. Kinetic data for association, plotted semilogarithmically (Fig. 1, B and D), showed the linear relationships that would be expected for pseudo first-order reactions. Using the first-order rate equation, this representation gives a k₂ of 1.1 × 10⁻³ M⁻¹ s⁻¹ for nifedipine (n = 4) and 8.4 × 10⁻⁴ M⁻¹ s⁻¹ (n = 3) for Bay K8644. The addition of GTPγS increased the rate constants by an average of 61% for nifedipine-sensitive binding (k₁ = 1.8 × 10⁻³ M⁻¹ s⁻¹) and 76% for Bay K8644-sensitive binding (k₁ = 1.1 × 10⁻³ M⁻¹ s⁻¹).

To determine the concentrations of GTPγS required to stimulate DHP-sensitive binding, we measured the effect of varying
the GTP·S concentration on the rates of association. Concentrations of GTP·S as low as 0.1 μM in the incubation medium stimulated DHP-sensitive binding (Fig. 2, A and C), and the effects were saturated by 1 μM. Two- and three-fold G protein stimulations of binding were seen, and half-maximal stimulation occurred at 4.1 and 3.9 μM GTP·S for nifedipine (Fig. 2B) and Bay K8644 (Fig. 2D), respectively. GDP·PS, a competitive inhibitor of G proteins, had no effect on either nifedipine- or Bay K8644-sensitive binding (Fig. 2, A and C).

As described previously, nifedipine increases the rate of dissociation of [3H]azidopine in moss membranes (12) (Fig. 3, A and C). Time courses of dissociation followed first-order kinetics, producing rate constants of dissociation of 1.6 × 10⁻³ s⁻¹ (nifedipine; n = 2) and 1.2 × 10⁻² s⁻¹ (Bay K8644; n = 3) (Fig. 3, A and C). GTP·PS enhances the displacement produced by both DHPs (Fig. 3, A and C); the addition of the stable GTP analog increased the dissociation rate constants by 76% for nifedipine-sensitive binding (k₉ = 2.1 × 10⁻³ s⁻¹) and by 60%

FIG. 2. Effect of nucleotides on the DHP-sensitive formation of the [3H]azidopine-moss plasma membrane DHP receptor complex. A, nucleotide regulation of nifedipine-sensitive [3H]azidopine binding to moss plasma membranes. Association rates were determined for nifedipine-sensitive binding in the presence of 0–10 μM GTP·S (○) or GDP·PS (▲). Points represent the means ± S.E. of three experiments. B, Eadie-Hofstee plot of the data (y = 0.032 – (4.412)x and R² = 0.931) resulting in a Kₐ for GTP·S of 4.4 μM. C, nucleotide regulation of Bay K8644-sensitive [3H]azidopine binding to moss plasma membranes. Association rates were determined for Bay K8644-sensitive binding in the presence of 0–10 μM GTP·S (○) or GDP·PS (▲). Points represent the means ± S.E. of three experiments. D, Eadie-Hofstee plot of the data (y = 0.032 – (3.90)x and R² = 0.883) resulting in a Kₐ for GTP·S of 3.9 μM.
calcium channel blocker \[^{3}H\]azidopine (12). In the absence of multiple classes of binding sites, other mechanisms must exist to provide a level of channel control during development. The ability of the stable GTP analog GTP\(_{S}\) to stimulate the interaction of DHPs with their receptors in moss plasma membranes suggests that coupling of a G protein to these high affinity DHP-binding sites may provide a mechanism of channel regulation. The specificity of G protein action is supported by stimulation of DHP binding by elevated concentrations of GTP and the active nucleotides GTP\(_{S}\) and GMP-PNP as well as the lack of effect of the inactive nucleotides GDP\(_{S}\), ATP, and cAMP. While the mechanism of G protein action is unknown, binding of the G protein may modify the conformation of the DHP-binding sites, enhancing the possibility of interaction with calcium channel antagonist and agonist ligands.

Low levels of Triton X-100 were required to see the effects of GTP and its analogs on DHP binding. Membranes used in this study were isolated using dextran gradients that enrich for sealed vesicles (30). While Triton X-100 at the concentration used had no effect on DHP binding, it prevented \[^{40}Ca\] uptake into moss protoplasts (data not shown), suggesting that membranes have been permeabilized. If, as expected, the membranes used represent a mixture of right-side-out and inside-out vesicles, the fact that no binding was observed in the absence of Triton X-100 suggests that GTP and the DHPs may bind to opposite faces of the plasma membrane.

While voltage-dependent calcium channels have been found in the plasma membrane of cells in carrot (31, 32), wheat (33, 34), and maize (35), no evidence currently exists for G protein modulation of these channels. G proteins have been shown to regulate inward rectifying K\(^+\) channels in the plasma membrane of V. faba guard cells. Using isolated membrane patches, G protein regulators were shown to affect the open state probability of single inward K\(^+\) channels and provided evidence for a membrane-delimited pathway of G protein regulation (23). Experiments presented here were done using isolated plasma

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**Table 1. Specificity of nucleotide effects on the DHP-sensitive formation of the \[^{3}H\]azidopine-moss plasma membrane DHP receptor complex**

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>[^{3}H]azidopine bound (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>100</td>
</tr>
<tr>
<td>GTP(_{S})</td>
<td>187 ± 6</td>
</tr>
<tr>
<td>GDP(_{S})</td>
<td>192 ± 4</td>
</tr>
<tr>
<td>GMP-PNP</td>
<td>186 ± 9</td>
</tr>
<tr>
<td>ATP</td>
<td>95 ± 7</td>
</tr>
<tr>
<td>cAMP</td>
<td>84 ± 12</td>
</tr>
<tr>
<td>Bay K8644</td>
<td>90 ± 21</td>
</tr>
</tbody>
</table>

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**Fig. 3. Kinetics of nucleotide effects on the DHP-sensitive dissociation of the \[^{3}H\]azidopine-moss plasma membrane DHP receptor complex.** A, after equilibrium was reached (60 min), the rate of dissociation of the \[^{3}H\]azidopine-receptor complex was monitored following the addition of 100 \(\mu M\) nifedipine in the absence of nucleotide (\(\square\)) or in the presence of 1 \(\mu M\) GTP\(_{S}\) (\(\bigcirc\)) or GDP\(_{S}\) (\(\bigcirc\)). Binding data are presented as percent \[^{3}H\]azidopine binding in the absence of nifedipine. Data are the means ± S.E. of three experiments. B, shown is the first-order representation of \[^{3}H\]azidopine binding: control (\(\square\)), \(y = 4.481 - (0.043)x\) and \(R^2 = 0.936\); GTP\(_{S}\) (\(\bigcirc\)), \(y = 4.231 - (0.055)x\) and \(R^2 = 0.825\); and GDP\(_{S}\) (\(\bigcirc\)), \(y = 4.469 - (0.039)x\) and \(R^2 = 0.900.\) B, represents the equilibrium-specific binding of \[^{3}H\]azidopine, and B, is specifically bound \[^{3}H\]azidopine at assay time \(t.\) The dissociation rate constants (k\(_{-1}\)) were as follows: control, 1.648 × 10\(^{-3}\) s\(^{-1}\); GTP\(_{S}\), 2.108 × 10\(^{-3}\) s\(^{-1}\); and GDP\(_{S}\), 1.495 × 10\(^{-3}\) s\(^{-1}\), C, after equilibrium was reached (60 min), the rate of dissociation of the \[^{3}H\]azidopine-receptor complex was monitored following the addition of 1 \(\mu M\) Bay K8644 in the absence of nucleotide (\(\square\)) or in the presence of 1 \(\mu M\) GTP\(_{S}\) (\(\bigcirc\)) or GDP\(_{S}\) (\(\bigcirc\)). Binding data are presented as percent \[^{3}H\]azidopine binding in the absence of Bay K8644. Data are the means ± S.E. of three experiments. D, shown is the first-order representation of \[^{3}H\]azidopine binding: control (\(\square\)), \(y = 4.577 - (0.031)x\) and \(R^2 = 0.998\); GTP\(_{S}\) (\(\bigcirc\)), \(y = 4.427 - (0.051)x\) and \(R^2 = 0.956\); and GDP\(_{S}\) (\(\bigcirc\)), \(y = 4.469 - (0.026)x\) and \(R^2 = 0.992.\) The dissociation rate constants (k\(_{-1}\)) were as follows: control, 1.188 × 10\(^{-3}\) s\(^{-1}\); GTP\(_{S}\), 1.955 × 10\(^{-3}\) s\(^{-1}\); and GDP\(_{S}\), 9.967 × 10\(^{-3}\) s\(^{-1}\).

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**Fig. 4. Effect of nucleotides on the DHP-sensitive dissociation of the \[^{3}H\]azidopine-moss plasma membrane DHP receptor complex.** A, nucleotide regulation of nifedipine-sensitive \[^{3}H\]azidopine binding to moss plasma membranes. Dissociation rates were determined for nifedipine-sensitive binding in the presence of GTP\(_{S}\) (\(\bigcirc\)) or GDP\(_{S}\) (\(\bigcirc\)). Points represent the means ± S.E. of three experiments. B, Eadie-Hofstee plot of the data (y = 0.065 - (4.297)x) and \(R^2 = 0.931\) resulting in a \(K_m\) for GTP\(_{S}\) of 4.3 \(\mu M\). C, nucleotide regulation of Bay K8644-sensitive \[^{3}H\]azidopine binding to moss plasma membranes. Dissociation rates were determined for Bay K8644-sensitive binding in the presence of GTP\(_{S}\) (\(\bigcirc\)) or GDP\(_{S}\) (\(\bigcirc\)). Points represent the means ± S.E. of three experiments. D, Eadie-Hofstee plot of the data (y = 0.045 - (4.319)x) and \(R^2 = 0.883\) resulting in a \(K_m\) for GTP\(_{S}\) of 4.3 \(\mu M\).
membranes and suggest that G protein regulation of DHP binding in moss may also take place via a membrane-associated or cytoplasm-independent pathway.

G protein regulation of DHP binding in moss indicates that the mechanism of binding differs from binding in animal cells in several ways. (i) While binding studies in animal cells suggest that DHP-binding sites may exist in distinct conformations with different affinity for antagonists and agonists (17, 18), the present study suggests that differences in affinity do not exist in plant cells. GTP and stable analogs showed similar affinities for antagonists and agonists (17, 18). GTP binding sites may exist in distinct conformations with different affinity for antagonists and agonists (17, 18). The data presented in this study support the hypothesis that G proteins regulate DHP binding to high affinity sites that are likely associated with voltage-dependent channels and suggest cytoplasm-independent regulation. Electrophysiological studies will allow us to link G proteins and the transport activity of the moss calcium channels directly. In addition, these studies will allow investigation into the requirement for cytoplasmic factors in G protein regulation and will allow us to determine if differential regulation of the channel is a determinant of bud formation during moss development.

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